THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 12, Issue of March 23, pp. 8681–8694, 2001

Printed in U.S.A.

An Oxysterol-derived Positive Signal for 3-Hydroxy-3-methylglutaryl-CoA Reductase Degradation in Yeast*

Received for publication, August 29, 2000, and in revised form, December 13, 2000
Published, JBC Papers in Press, December 27, 2000, DOI 10.1074/jbc.M007888200

Richard G. Gardner‡, Hui Shan§, Seiichi P. T. Matsuda§¶, and Randolph Y. Hampton‡‖

From the ‡Section of Cell and Developmental Biology, Division of Biology, University of California at San Diego, La Jolla, California 92093 the Departments of §Biochemistry and Cell Biology and ¶Chemistry, Rice University, Houston, Texas 77005

Sterol synthesis by the mevalonate pathway is modulated, in part, through feedback-regulated degradation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). In mammals, both a non-sterol isoprenoid signal derived from farnesyl diphosphate (FPP) and a sterol-derived signal appear to act together to positively regulate the rate of HMGR degradation. Although the nature and number of sterol-derived signals are not clear, there is growing evidence that oxysterols can serve in this capacity. In yeast, a similar non-sterol isoprenoid signal generated from FPP acts to positively regulate HMGR degradation, but the existence of any sterol-derived signal has thus far not been revealed. We now demonstrate, through the use of genetic and pharmacological manipulation of oxidosqualene-lanosterol cyclase, that an oxysterol-derived signal positively regulated HMGR degradation in yeast. The oxysterol-derived signal acted by specifically modulating HMGR stability, not endoplasmic reticulum-associated degradation in general. Direct biochemical labeling of mevalonate pathway products confirmed that oxysterols were produced endogenously in yeast and that their levels varied appropriately in response to genetic or pharmacological manipulations that altered HMGR stability. Genetic manipulation of oxidosqualene-lanosterol cyclase did result in the buildup of detectable levels of 24,25-oxidolanosterol by gas chromatography, gas chromatography-mass spectroscopy, and NMR analyses, whereas no detectable amounts were observed in wild-type cells or cells with squalene epoxidase down-regulated. In contrast to mammalian cells, the yeast oxysterol-derived signal was not required for HMGR degradation in yeast. Rather, the function of this second signal was to enhance the ability of the FPP-derived signal to promote HMGR degradation. Thus, although differences do exist, both yeast and mammalian cells employ a similar strategy of multi-input regulation of HMGR degradation.

Many essential cellular molecules are derived from products of the mevalonate pathway, including sterols, coenzyme Q, prenyl groups, and dolichol (1). As one tactic for regulating pathway production, the cell exerts tight transcriptional and post-transcriptional control over the steady-state levels of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR),[1] which catalyzes a rate-limiting, irreversible step in sterol synthesis (1). In fact, a significant component of this control is effected through regulation of HMGR stability (2–4). When the cellular amounts of mevalonate pathway products are abundant, HMGR degradation is rapid, and the protein's steady-state levels are correspondingly low. Conversely, when the cellular amounts of pathway products are insufficient for the cell's needs, HMGR degradation is slow, and the steady-state levels of HMGR are relatively high.

Analyses of HMGR stability in both mammalian cells and yeast have revealed many aspects of HMGR-regulated degradation that are conserved among eukaryotes (4–7). To gain a better understanding of this aspect of cellular sterol regulation, we have been studying the genetics and biochemistry of the regulated degradation of the HMGR isozyme Hmg2p in yeast (4, 8–11). Through use of the powerful genetic manipulations possible in this organism, we have identified genes required for the degradation of Hmg2p, termed *HRD* genes (8), and genes required for normal regulation of Hmg2p degradation, termed *COD* genes (11). These studies have led to a clearer understanding of the degradation pathway, as they have demonstrated that the key components, Hrd1p and Hrd3p, form the core of an endoplasmic reticulum (ER)-localized ubiquitin ligase complex required for ubiquitination and degradation of HMGR (12, 13) and numerous other quality control substrates (14). Regulation of HMGR degradation appears to occur through pathway signals controlling entry of the protein into the constitutively active *HRD* degradation pathway (11, 13), perhaps by allosteric conversion of HMGR from a stable protein into a quality control substrate. Recently, it has been confirmed that mammalian HMGR similarly undergoes regulated ubiquitination (15), most likely mediated by an ER-localized ubiquitin ligase complex similar to the *HRD* complex in yeast.

In parallel studies, we have been examining the identities of the mevalonate-derived signals that control Hmg2p stability. Through use of both pharmacological and genetic manipulations of mevalonate pathway enzymes, we have identified the pathway intermediate farnesyl pyrophosphate (FPP) as the source of a positive signal for Hmg2p degradation (9, 10). FPP is the substrate for squalene synthase (see Fig. 1) and occupies a key branch point in the mevalonate pathway for which its conversion is the committed step for each separate pathway (1). The identification of FPP as the primary signal for regulation of HMGR degradation in yeast is especially intriguing, as farnesol, a derivative of FPP, has also been implicated as the source

* This work was supported by National Institutes of Health Grants HL49122 (to S. P. T. M.) and DK5199601 (to R. Y. H.) and by 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.

‖ To whom correspondence should be addressed. Tel.: 858-822-0511; Fax: 858-534-0555; E-mail: rhampton@biomail.ucsd.edu.

[1] The abbreviations used are: HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; ER, endoplasmic reticulum; FPP, farnesyl diphosphate; HA, hemagglutinin; HPLC, high pressure liquid chromatography; GFP, green fluorescence protein.

of the signal for regulation of mammalian HMGR stability (16–20).

In addition to farnesol, mammalian HMGR stability is also regulated by the levels of cellular sterols. Mammalian HMGR degradation is rapid when abundant sterols are present; HMGR degradation is slow when cellular sterol levels are insufficient (2, 21–25). Although the exact identity of the sterol-derived signal is unknown, 25-hydroxycholesterol and 24,25-oxidolanosterol have been shown to be potent enhancers of HMGR degradation when added to mammalian cells (2, 3, 21–25), suggesting that an oxysterol-derived molecule may be a source of the sterol signal that positively regulates mammalian HMGR degradation. These and related oxysterols can be generated *in vivo* either by an "alternate" pathway involving cyclization of 2,(3*S*),(22*S*),23-dioxidosqualene (see Fig. 1) or through the action of a family of enzymes that directly oxidize cholesterol (26). Identification of an oxysterol-derived signal for HMGR degradation has added significance due to a growing body of evidence that oxysterols also function in numerous other aspects of cellular regulation and physiology (26), allowing for the possibility that oxysterols may serve to integrate cholesterol homeostasis with other distinct biological processes. Until now, it was unknown whether yeast also employed an oxysterol-derived positive signal for HMGR degradation or if this was only a characteristic of HMGR degradation in mammalian cells.

Through pharmacological and genetic manipulations of mevalonate pathway enzymes downstream of squalene synthase, we have explored the possibility that yeast also utilizes an oxysterol-derived signal for HMGR degradation. In the following results, we have demonstrated the existence of a secondary positive signal for yeast HMGR degradation that is derived from the alternate oxysterol pathway. This secondary oxysterol-derived signal enhances Hmg2p degradation in addition to, but not in the absence of, the primary FPP-derived signal. Our results demonstrate that there is further striking conservation for mevalonate pathway regulation of HMGR degradation among eukaryotes.

EXPERIMENTAL PROCEDURES

Materials and Reagents—All enzymes were obtained from New England Biolabs Inc. (Beverly, MA). Bis(trimethylsilyl)trifluoroacetamide, pyridine, pyrogallol, and potassium hydroxide were purchased from Aldrich. Methanol, methyl *t*-butyl ether, dichloromethane, and hexane were purchased from EM Science (Gibbstown, NJ). 24,25-Oxidolanosterol and 26-hydroxycholesterol were synthesized according to published methods (27, 28). Lovastatin, L659,699, and zaragozic acid were generously donated by Merck. Ro48-8071 was kindly provided by Dr. Olivier Morand (F. Hoffman-La Roche Ltd., Basel, Switzerland). Terbinafine was obtained as a 1% Lamisil® solution from a local drugstore. ECL™ chemiluminescence immunodetection reagents and sodium [^{14}C]acetate were from Amersham Pharmacia Biotech. All other chemical reagents were obtained from Sigma. Silica Gel 60 F_{254} TLC plates were obtained from Fisher and EM Science. The anti-Myc antibody 9E10 was used as a cell culture supernatant obtained by growing the 9E10 hybridoma (American Type Culture Collection CRL 1729) in RPMI 1640 medium (Life Technologies, Inc.) with 10% fetal calf serum. The anti-HA antibody was an ascites fluid obtained from BabCo (Berkeley, CA). Affinity-purified horseradish peroxidase-conjugated goat anti-mouse antiserum was obtained from Sigma.

Recombinant DNA and Molecular Cloning—The polymerase chain reaction was performed as previously described (29). The gene encoding oxidosqualene-lanosterol cyclase (*ERG7*) was polymerase chain reaction-amplified from yeast strain RHY623 genomic DNA (10) using separate primers containing *Pst*I and *Bgl*II sites in the upstream primers and *Nhe*I and *Sal*I sites in the downstream primers. The amplified *ERG7* gene was digested with *Bgl*II and *Sal*I, and the 2.22-kilobase fragment was then cloned between the *Bam*HI and *Sal*I sites in pRH440 to yield P_{TDH3}-*ERG7* (pRH1205). The amplified *ERG7* gene was digested with *Bgl*II and *Hpa*I, and the 1.04-kilobase fragment was then cloned between the *Bam*HI and *Hpa*I sites in pRH973 to yield P_{MET3}-*ERG7* (pRH1206). The gene encoding squalene epoxidase (*ERG1*) was previously polymerase chain reaction-amplified from yeast strain RHY623 genomic DNA using separate primers containing *Pst*I and *Bgl*II sites in the upstream primers and *Nhe*I and *Sal*I sites in the downstream primers (10). The amplified *ERG1* gene was digested with *Bam*HI and *Sal*I, and the 1.5-kilobase fragment was then cloned between the *Bam*HI and *Sal*I sites in pRH440 to yield P_{TDH3}-*ERG1* (pRH1203).

Strains and Media—Escherichia coli DH5α strains were grown at 37 °C in LB medium + ampicillin (100 μg/ml). Yeast strains were grown at 30 °C in minimal medium supplemented with glucose and the appropriate amino acids as described (4). The lithium acetate method was used to transform yeast with plasmid DNA (30).

Yeast strains RHY1326 (*α his3Δ200 lys2-801 ade2-101 leu2Δ ura3-52::LEU2::* P_{TDH3}*-hmg2-GFP MET2 hmg1::LYS2 hmg2::HIS3::* P_{TDH3}*-1MYC-HMG2*) and RHY1462 (*α his3Δ200 lys2-801 ade2-101 leu2Δ::* P_{TDH3}*-6myc-hmg2-GFP::LEU2 ura3-52::* P_{TDH3}*-6MYC-HMG2 MET2 hmg1::LYS2 hmg2Δ::HIS3*) (10) were the parent strains for transformation of the plasmid containing the P_{MET3}-expressed *ERG7* gene and the P_{TDH3}-expressed *ERG1*, *ERG7*, and *ERG9* genes. Plasmid pRH1206 (P_{MET3}-*ERG7*) was introduced into RHY1326 or RHY1462 cells by targeted integration at the *Bam*HI site of *ERG7*. Plasmid pRH440 (P_{TDH3}-*ERG9*), pRH1203 (P_{TDH3}-*ERG1*), and pRH1205 (P_{TDH3}-*ERG7*) were introduced into RHY1326 cells by targeted integration at the *Stu*I site of *ura3-52*. All yeast transformants were selected for Ura$^+$ prototrophy.

Biochemical Assays—Cycloheximide-chase assays, log-phase steady-state assays, and methionine-chase assays were performed as previously described (10, 29). Hmg2p ubiquitination assays were performed as previously described (31). Strains were transformed with pRH1100 (31), which expresses a triple HA epitope-tagged ubiquitin from the constitutive *TDH3* promoter (alias the glyceraldehyde-3-phosphate dehydrogenase promoter) (32). Transformants were selected for Ade$^+$ prototrophy. Hmg2p ubiquitination was assayed by immunoprecipitation of 1Myc-Hmg2p and then immunoblotting the precipitate for covalently linked HA-tagged ubiquitin.

Flow Cytometric Analysis—Flow cytometry was performed as previously described (29). Living cells were analyzed by flow microfluorometry using a FACSCalibur flow microfluorometer (Becton Dickinson, Palo Alto, CA) with settings for fluorescein-labeled antibody analysis. Histograms were produced from 10,000 individual cells and plotted with log fluorescence (arbitrary units) on the horizontal axis and with cell number on the vertical axis.

Metabolic Labeling of Mevalonate Pathway Products and Detection by Thin Layer Chromatography—TLC was performed similarly as previously described (33). Cells were grown at 30 °C to log phase (A_{600} = 0.3) in 25 ml of minimal medium. In some cases, 50 μg/ml lovastatin, 10 μg/ml zaragozic acid, 40 μg/ml Ro48-8071, or 50 μg/ml terbinafine was added to the cells. Sodium [^{14}C]acetate was added at the same time as the drugs to a final concentration of 2 μCi/ml, and the cells were incubated for an additional 6 h at 30 °C. In the case of gene down-regulation, cells were incubated in the presence of 2 mM methionine for 19 h at 30 °C. Sodium [^{14}C]acetate was added to a final concentration of 2 μCi/ml either at the same time as methionine addition or after 13 h of incubation with methionine. After incubation with [^{14}C]acetate, cells were washed once with 0.5 volume of sterile water. ^{14}C-Labeled lipids were extracted from the cells by addition of 9 ml of methanol. Extracts were saponified by addition of 6 ml of 10% aqueous potassium hydroxide such that the methanol/water ratio was 3:2. The mixture was incubated overnight at room temperature. ^{14}C-Labeled lipids were extracted twice from the saponification mixture by addition of 4 ml of petroleum ether, which was subsequently concentrated to 150 μl. The entire 150-μl fraction was applied to Silica Gel 60 F_{254} plates (Fisher). The ^{14}C-labeled lipids were resolved by developing the plates twice with 5:1 benzene/ethyl acetate to 10 cm and once with 95:5:1 petroleum ether/diethyl ether/glacial acetic acid to 15 cm. The resolved ^{14}C-labeled lipids were detected by autoradiography. Reference compounds were visualized by brief exposure of the plates to I_2 vapor.

High Pressure Liquid Chromatography (HPLC), Gas Chromatography, Gas Chromatography-Mass Spectroscopy, and NMR Analyses—Cells containing the appropriate P_{MET3}-expressed construct were grown in 2 liters of minimal medium containing 2 mM methionine for 19 h at 30 °C. Cells were harvested by centrifugation and stored at −80 °C prior to the analyses. For processing, the samples were thawed, weighed, and combined with 1 mg of butylated hydroxytoluene, 15 mg of pyrogallol, 7.5 ml of methanol, and 3 ml of 60% aqueous KOH. The resulting mixtures were deoxygenated for 10 min with nitrogen and heated at 70 °C (1.5 h). After cooling to ambient temperature, the

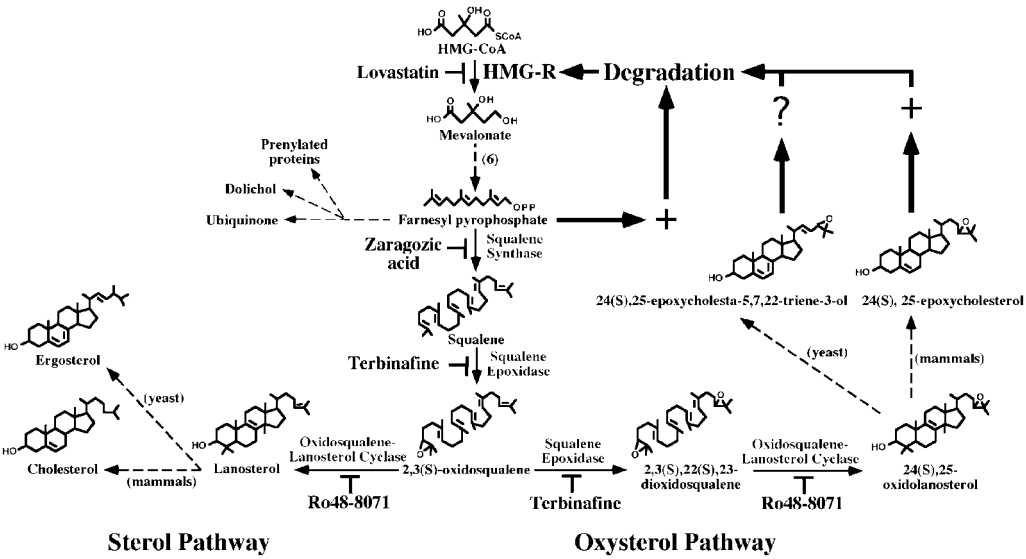

FIG. 1. **The alternate pathway.** Shown is a schematic representation of the mevalonate pathway and the alternate oxysterol pathway. Enzymes responsible for each step and their yeast inhibitors are marked accordingly. This figure was adapted from Goldstein and Brown (1) and Dolis and Schuber (35).

samples were extracted with methyl *t*-butyl ether (4 × 12 ml). For each sample, the combined extracts were washed with 12 ml of water and 12 ml of brine, dried over anhydrous sodium sulfate, and evaporated to dryness under nitrogen. A sample (10%) of each crude extract was reserved for analysis. The resulting residue (90% of the crude extract containing the non-saponifiable lipids) was subjected to normal-phase separation on an SPE cartridge. The residues were dissolved in 2 ml of 1:1 CH_2Cl_2/hexane and placed onto the SPE cartridge (6 ml, 1 g of silica; J & W Scientific, Folsom, CA) pretreated with the same solvent. Elution was conducted with 1:1 CH_2Cl_2/hexane (32 ml), then undiluted CH_2Cl_2 (36 ml), and finally 10% isopropyl alcohol/hexane (36 ml). TLC (Silica Gel 60 plates, EM Science) was used to identify the appropriate fractions, which were combined. Sterols were visualized with 5% ammonium molybdate in 10% sulfuric acid. Reversed-phase HPLC was used to purify material with a retention time corresponding to that 24,25-oxidolanosterol. HPLC was carried out with a Sphereclone 5μ ODS(2) reversed-phase column (4.6 mm (inner diameter) × 250 mm; Phenomenex Inc., Torrance, CA) with methanol isocratically at 1 ml/min and UV detection at 210 nm. Gas chromatography analyses were performed with derivatives (80 μl of pyridine and 20 μl of bis(trimethylsilyl)trifluoroacetamide at 25 °C) and DB-5 capillary column (0.25 mm (inner diameter) × 30 cm, 5% phenyl and 95% methyl polysiloxane, 0.1-μm film thickness; J & W Scientific) with helium carrier gas (1.1 kg/cm^2) on a Shimadzu GC-9A instrument (35:1 split ratio, 200 °C for 2 min, 10 °C/min to 280 °C, and injector and FID detector at 280 °C). Gas chromatography-mass spectroscopy analyses employed a VG ZAB-HF spectrometer interfaced to a Hewlett-Packard 5890A chromatograph (splitless injection, helium carrier gas at 1.4 kg/cm^2, and the same column and program as for gas chromatography). NMR experiments were carried out with Bruker AMX500 and Avance 500 spectrometers (500.1 MHz for ^{1}H) equipped with a 5-mm inverse geometry broadband or 5-mm normal geometry probe. ^{1}H NMR spectra were measured at 25 °C in $CDCl_3$ solution and referenced to internal bis(trimethylsilyl) trifluoro acetamide.

RESULTS

In mammalian cells, oxysterols have been implicated as positive signals for HMGR degradation. The existence of such oxysterol signals has been proposed from the observations that HMGR degradation is enhanced as a result of either 24,25-oxidolanosterol or 25-hydroxycholesterol addition to cells (2, 3, 21–25) or when endogenous oxysterols are increased by inhibition of oxidosqualene-lanosterol cyclase (34). It appears that oxysterols do not alter HMGR degradation alone, but require the presence of a non-sterol positive signal derived from FPP to enhance HMGR degradation (20). In our following examination of oxysterol-derived signals for yeast HMGR degradation, we utilized genetic and biochemical approaches to address this issue.

Oxysterols can be produced in the cell from the alternate oxysterol pathway (Fig. 1) (35), which branches from the sterol biosynthetic pathway at 2,(3*S*)-oxidosqualene. These oxysterols are initially generated from 2,(3*S*)-oxidosqualene by an additional epoxidation at positions 22 and 23 through the action of squalene epoxidase (36, 37). Oxidosqualene-lanosterol cyclase plays a critical role in determining availability of the dioxidosqualene required for production of oxysterols in the alternate pathway. By catalyzing the cyclization of 2,(3*S*)-oxidosqualene to lanosterol (35), the activity of oxidosqualene-lanosterol cyclase determines steady-state cellular levels of 2,(3*S*)-oxidosqualene available for further epoxidation to 2,(3*S*),(22*S*),23-dioxidosqualene. If conversion of 2,(3*S*)-oxidosqualene to lanosterol by oxidosqualene-lanosterol cyclase is relatively slow, some 2,(3*S*)-oxidosqualene may be further epoxidized to 2,(3*S*),(22*S*),23-dioxidosqualene by squalene epoxidase (36, 37). The 2,(3*S*),(22*S*),23-dioxidosqualene can be further converted to 24,25-oxidolanosterol by oxidosqualene-lanosterol cyclase (36, 38). Oxidosqualene-lanosterol cyclase has a higher affinity for the dioxidosqualene than for the monooxidosqualene (39); and thus, the cyclization of the dioxidosqualene is likely the favored reaction when both substrates are present. Through this competition, partial inhibition of oxidosqualene-lanosterol cyclase causes increased production of 24,25-oxidolanosterol (38), and later oxysterols may be derived from this product. Thus, pharmacological or genetic manipulations of oxidosqualene-lanosterol cyclase served as a useful starting point to examine the effect of oxysterol production on yeast HMGR degradation.

Oxidosqualene-lanosterol Cyclase Inhibition or Down-regulation Enhances Hmg2p Degradation—To determine whether HMGR degradation in yeast could be hastened by an oxysterol-derived product, we examined the effect of altering cellular oxidosqualene-lanosterol cyclase activity on the degradation of the yeast HMGR isozyme Hmg2p. We used yeast strains that coexpress two versions of Hmg2p, the single Myc epitope-tagged 1Myc-Hmg2p and the autofluorescent reporter protein Hmg2p-GFP (9, 40), to detect changes in Hmg2p levels by immunological or optical means, respectively. Alterations in Hmg2p stability brought about by physiological or genetic means are correctly reported by changes in 1Myc-Hmg2p im-

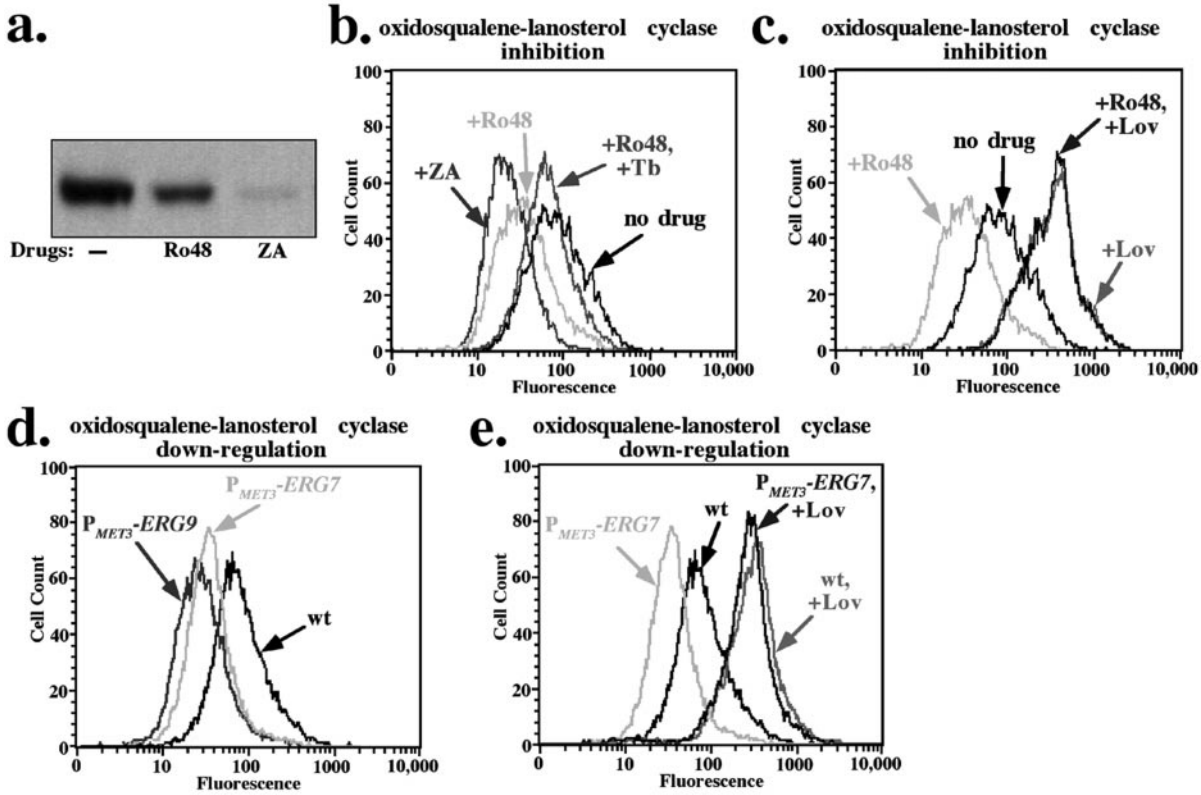

FIG. 2. **Inhibition or down-regulation of oxidosqualene-lanosterol cyclase enhances Hmg2p degradation.** *a*, effect of Ro48-8071 on 1Myc-Hmg2p steady-state levels. Log-phase cultures were treated for 4 h with no drug (−), 40 μg/ml Ro48-8071 (*Ro48*), or 10 μg/ml zaragozic acid (*ZA*) and then subjected to immunoblotting with anti-Myc antibody 9E10 to determine 1Myc-Hmg2p steady-state levels. *b*, effect of Ro48-8071 on Hmg2p-GFP steady-state levels. Log-phase cultures were treated for 4 h with no drug, 40 μg/ml Ro48-8071 (*+Ro48*), or 10 μg/ml zaragozic acid (*+ZA*) and then analyzed by flow cytometry to determine Hmg2p-GFP steady-state fluorescence. 80 μg/ml terbinafine was added at the same time as Ro48-8071 addition for the indicated sample (*+Ro48, +Tb*). *c*, upstream inhibition blocks the effect of Ro48-8071 on Hmg2p-GFP steady-state levels. The same experiment as described for *b* was carried out, except that 25 μg/ml lovastatin was added alone (*+Lov*) or at the same time as Ro48-8071 addition (*+Ro48, +Lov*) in the indicated samples. *d*, effect of oxidosqualene-lanosterol cyclase down-regulation on Hmg2p-GFP steady-state levels. Otherwise identical strains expressing oxidosqualene-lanosterol cyclase from either the wild-type promoter (*wt*) or the *MET3* promoter (P_{MET3}-*ERG7*) or squalene synthase from the *MET3* promoter (P_{MET3}-*ERG9*) were grown for 16 h at 30 °C in the presence of 2 mM methionine. Hmg2p-GFP steady-state fluorescence was analyzed by flow cytometry. *e*, upstream inhibition blocks the effect of oxidosqualene-lanosterol cyclase down-regulation on Hmg2p-GFP steady-state levels. The same experiment as described for *d* was carried out, except that 25 μg/ml lovastatin (*+Lov*) was added to the indicated samples after 12 h of incubation.

munoreactivity and Hmg2p-GFP steady-state fluorescence (9, 29, 41).

We first examined the effect of decreased oxidosqualene-lanosterol cyclase activity on Hmg2p degradation by addition of the drug Ro48-8071, which is a specific inhibitor of oxidosqualene-lanosterol cyclase (42), to cells. Addition of Ro48-8071 to cells coexpressing both versions of Hmg2p caused an increase in Hmg2p degradation in a dose-dependent manner, as seen by the reduced steady-state levels of both 1Myc-Hmg2p and Hmg2p-GFP (Fig. 2, *a* and *b*, *+Ro48*, respectively). However, the maximal degradation-enhancing effect of Ro48-8071 was not as great as that caused by addition of zaragozic acid (Fig. 2*a*, *+Ro48 versus +ZA*). Zaragozic acid is an inhibitor of squalene synthase that, when added to growing cells, results in the rapid degradation of Hmg2p through buildup of its substrate FPP (9, 10). In contrast, addition of the HMGR inhibitor lovastatin to growing cells results in stabilization of Hmg2p by blockade of mevalonate pathway product synthesis (Fig. 2*c*, *+Lov*) (4). Ro48-8071 addition to cells treated with lovastatin had no effect on the stabilized Hmg2p-GFP steady-state levels (Fig. 2*c*, *+Ro48, +Lov versus +Lov*). Furthermore, the degradation-enhancing effect of Ro48-8071 addition was suppressed by addition of terbinafine, a potent antifungal squalene epoxidase inhibitor (43–45), to the cells (Fig. 2*b*, *+Ro48, +Tb versus +Ro48*). This implicated the substrate of oxidosqualene-lanosterol cyclase, 2,(3*S*)-oxidosqualene, as the source of the signal for enhanced Hmg2p degradation by Ro48-8071 addition.

It was possible that the Hmg2p degradation-enhancing effect of Ro48-8071 addition to cells was through an action other than oxidosqualene-lanosterol cyclase inhibition, so we next tested the effect of reducing cellular oxidosqualene-lanosterol cyclase activity on Hmg2p degradation by genetic down-regulation of oxidosqualene-lanosterol cyclase. To do so, we constructed a "promoter-switch" plasmid containing a truncated version of the oxidosqualene-lanosterol cyclase gene, *ERG7*, placed behind the *MET3* promoter, which is repressed by the presence of high extracellular concentrations (>0.5 mM) of methionine (46, 47). This was similar to our previously described conditional alleles of other mevalonate pathway enzymes, including squalene synthase (*ERG9*), farnesyl-diphosphate synthase (*ERG20*), and squalene epoxidase (*ERG1*) (10). Targeted integration of this plasmid into the *ERG7* locus resulted in the creation of a single functional copy of the oxidosqualene-lanosterol cyclase gene under the control of the regulated *MET3* promoter (P_{MET3}). The plasmid was used to transform the 1Myc-Hmg2p- and Hmg2p-GFP-coexpressing, methionine prototroph yeast strain described above. When grown in the absence of methionine, normal regulated Hmg2p degradation was observed in the P_{MET3}-*ERG7* yeast strain.[2]

[2] R. G. Gardner and R. Y. Hampton, unpublished observations.

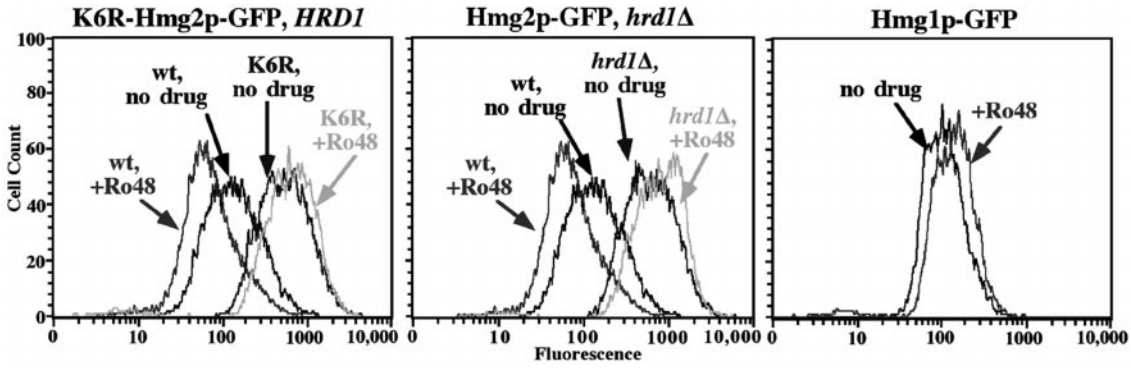

FIG. 3. **Oxidosqualene-lanosterol cyclase inhibition does not lower steady-state levels of degradation-deficient Hmg2p-GFP.** Otherwise identical strains expressing K6R-Hmg2p-GFP (*left panel*), Hmg2p-GFP in the presence of the *hrd1*Δ allele (*middle panel*), or Hmg1p-GFP (*right panel*) were grown to log phase and then treated with 40 μg/ml Ro48-8071 (+*Ro48*) or no drug for 4 h at 30 °C. Cultures were analyzed by flow cytometry to determine cellular Hmg2p-GFP or Hmg1p-GFP steady-state fluorescence. *wt*, wild-type cells.

Down-regulation of oxidosqualene-lanosterol cyclase, by growth of the P_{MET3}-*ERG7* strain in 2 mM methionine, resulted in increased degradation of Hmg2p, as seen by reduced Hmg2p-GFP steady-state levels (Fig. 2*d*, P_{MET3}-*ERG7 versus wt*). The effect was similar to that previously reported for squalene synthase down-regulation (10), but was not as drastic (Fig. 2*d*, P_{MET3}-*ERG7 versus* P_{MET3}-*ERG9*). These observations were identical to the effects seen upon inhibition of each respective enzyme with Ro48-8071 or zaragozic acid shown above (compare with Fig. 2*b*). Furthermore, Hmg2p-GFP was still stabilized by addition of lovastatin even after genetic down-regulation of the oxidosqualene-lanosterol cyclase gene (Fig. 2*e*, P_{MET3}-*ERG7, +Lov*). The degree of stabilization by lovastatin addition was similar to the degree of Hmg2p-GFP stabilization by lovastatin addition to the wild-type strain (Fig. 2*e*, P_{MET3}-*ERG7, +Lov* compared with *wt, +Lov*), indicating that enhanced Hmg2p degradation caused by oxidosqualene-lanosterol cyclase down-regulation was still regulated by mevalonate pathway flux. Thus, the effect of oxidosqualene-lanosterol cyclase down-regulation on Hmg2p degradation was identical to that of Ro48-8071 addition.

Effect of Oxidosqualene-lanosterol Cyclase Inhibition Is Not through Altered Transcription or Translation of Hmg2p—The possibility existed that the effect of decreased oxidosqualene-lanosterol cyclase activity on Hmg2p steady-state levels was not due to enhanced degradation, but occurred through decreased Hmg2p synthesis as a result of diminished transcription and/or translation. We eliminated transcriptional regulation in the cells used for the above analyses by expression of 1Myc-Hmg2p and Hmg2p-GFP from the constitutive *TDH3* promoter (alias the glyceraldehyde-3-phosphate dehydrogenase promoter) (32). However, it was still possible that the effect of Ro48-8071 on the Hmg2p steady-state levels was through alteration of Hmg2p synthesis due to changes in Hmg2p translation.

To rule out the possibility that oxidosqualene-lanosterol cyclase inhibition resulted in altered translation and decreased Hmg2p synthesis, we examined the effect of Ro48-8071 addition on Hmg2p-GFP steady-state levels in cells that were deficient for Hmg2p degradation through either in *cis* or in *trans* stabilization. The effect of Ro48-8071 addition was first examined with the in *cis*-stabilized Hmg2p reporter protein K6R-Hmg2p-GFP. Introduction of the K6R mutation in both 1Myc-Hmg2p and Hmg2p-GFP results in complete stabilization (31). When Ro48-8071 was added to cells that expressed stable K6R-Hmg2p-GFP, no decrease in the steady-state levels of K6R-Hmg2p-GFP was observed (Fig. 3, *left panel*). Next, the effect of Ro48-8071 addition was examined with normal Hmg2p-GFP expressed in cells deficient for ER degradation by the presence of an *hrd1* null allele, which results in the in *trans* stabilization of Hmg2p (8). Hrd1p is an ER-associated ubiquitin ligase essential for ubiquitin-dependent Hmg2p degradation (8, 12). Similar to the in *cis* stabilization by introduction of the K6R mutation, no decrease in the steady-state levels of normal Hmg2p-GFP in cells carrying the *hrd1*Δ allele was observed after Ro48-8071 addition (Fig. 3, *middle panel*). In fact, for each stabilized version of Hmg2p-GFP, whether in *cis* or in *trans*, there appeared to be a slight increase in the steady-state levels after Ro48-8071 addition, which was also observed with the steady-state levels of the normally stable Hmg1p-GFP protein (Fig. 3, *right panel*). Thus, the decrease in 1Myc-Hmg2p and Hmg2p-GFP steady-state levels as a result of Ro48-8071 addition to cells was even more striking and was the result of enhanced degradation, not decreased Hmg2p synthesis.

Oxidosqualene-lanosterol Cyclase Inhibition Does Not Affect General ER Degradation—An alternative model for the degradation-stimulating effect of reduced oxidosqualene-lanosterol cyclase activity on Hmg2p degradation was that the effect occurred through enhancement of ER degradation in general, rather than through the specific action of mevalonate pathway regulation on Hmg2p degradation. To test this, we examined if Ro48-8071 addition to cells altered the degradation of 6Myc-Hmg2p, a previously described mutant of Hmg2p that is degraded in a similar *HRD*-dependent, ER-associated manner as normal Hmg2p (8), but its degradation is not regulated by signals from the mevalonate pathway (8, 10). When Ro48-8071 was added to cells coexpressing 6Myc-Hmg2p and 6Myc-Hmg2p-GFP in place of 1Myc-Hmg2p and Hmg2p-GFP, no decrease in the steady-state levels of 6Myc-Hmg2p-GFP (Fig. 4) or 6Myc-Hmg2p [2] was observed. Thus, reduced oxidosqualene-lanosterol cyclase activity enhanced only the mevalonate pathway-regulated degradation of Hmg2p, not general *HRD*-dependent, ER-associated degradation.

Oxidosqualene-lanosterol Cyclase Inhibition Enhances Hmg2p Ubiquitination—Previously, we demonstrated that the upstream FPP-derived signal enhanced Hmg2p degradation by acting to increase Hmg2p ubiquitination (10). Because inhibition or down-regulation of oxidosqualene-lanosterol cyclase resulted in increased degradation of Hmg2p, we examined if this effect was through enhanced Hmg2p ubiquitination. To assay Hmg2p ubiquitination, we utilized strains that coexpress 1Myc-Hmg2p and a triple HA epitope-tagged version of ubiquitin (3HA-ubiquitin) (31). Immunoprecipitation of 1Myc-Hmg2p and subsequent immunoblotting of the immunoprecipitate with anti-HA antisera allow for the detection of higher molecular mass Hmg2p-ubiquitin conjugates (9, 29).

When the cells coexpressing 1Myc-Hmg2p and 3HA-ubiquitin were treated with Ro48-8071, Hmg2p ubiquitination was

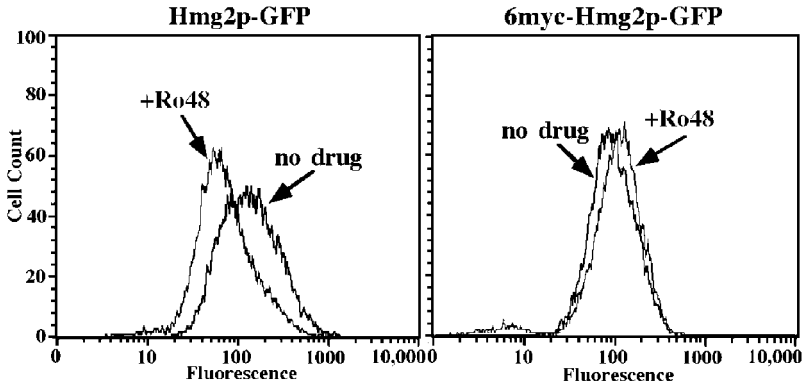

FIG. 4. **Inhibition of oxidosqualene-lanosterol cyclase does not affect general ER degradation.** Otherwise identical strains expressing either the mevalonate pathway-regulated, degradation substrate Hmg2p-GFP or the constitutive degradation substrate 6Myc-Hmg2p-GFP were grown to log phase and then treated with 40 μg/ml Ro48-8071 (*+Ro48*) or no drug for 4 h at 30 °C. Cultures were analyzed by flow cytometry to determine cellular Hmg2p-GFP or 6Myc-Hmg2p-GFP steady-state fluorescence.

increased (Fig. 5, *R*), but the degree of enhanced ubiquitination was slightly lower than that observed when cells were incubated with zaragozic acid (compare *R* with *ZA*). The effect of Ro48-8071 on Hmg2p ubiquitination was blocked by addition of lovastatin at the same time as Ro48-8071 and was identical to the effect of only lovastatin addition (Fig. 5, compare *R+L* with *L*), indicating that the Ro48-8071 effect on Hmg2p ubiquitination was regulated by the mevalonate pathway. Thus, increased regulated Hmg2p ubiquitination by addition of Ro48-8071 strongly supported the observation that decreased Hmg2p steady-state levels by reduced oxidosqualene-lanosterol cyclase activity were the result of enhanced Hmg2p degradation.

Partial Inhibition of Oxidosqualene-lanosterol Cyclase Enhances Hmg2p Degradation, whereas Complete Inhibition Has No Effect—Oxysterols have been implicated as positive signals for mammalian HMGR degradation (2, 3, 21–25). In mammalian cells, endogenous oxysterol and sterol levels can be oppositely manipulated through varying degrees of oxidosqualene-lanosterol cyclase inhibition, *i.e.* partial inhibition of oxidosqualene-lanosterol cyclase increases production of molecules in the alternate oxysterol pathway, but decreases production of molecules in the sterol pathway (38, 48). Complete inhibition of oxidosqualene-lanosterol cyclase blocks production of molecules in both the sterol and oxysterol pathways (38, 48). Thus, the effects of partial oxidosqualene-lanosterol cyclase inhibition *versus* complete inhibition provide strict criteria to test whether HMGR degradation is regulated by products of either the oxysterol or sterol pathway. If HMGR degradation is positively regulated by a sterol pathway product, then partial or complete inhibition of oxidosqualene-lanosterol cyclase should result in reduced HMGR degradation. In contrast, if HMGR degradation is positively regulated by an oxysterol pathway product, then partial inhibition of oxidosqualene-lanosterol cyclase should result in enhanced degradation, whereas complete inhibition should have the opposite effect. Indeed, partial inhibition of oxidosqualene-lanosterol cyclase in mammalian cells results in decreased HMGR activity, whereas complete inhibition results in increased HMGR activity (48). Whether this effect was due to alteration of only HMGR degradation or all forms of HMGR regulation was not examined. In an earlier study, inhibition of oxidosqualene-lanosterol cyclase in mammalian cells did result in enhanced HMGR degradation (34); however, it was not reported if the degree of oxidosqualene-lanosterol cyclase inhibition was partial or complete.

In yeast, it has been observed that partial inhibition of oxidosqualene-lanosterol cyclase, *i.e.* inhibition that does not compromise the growth of the cells and that still allows production of ergosterol, results in an increase in the cellular

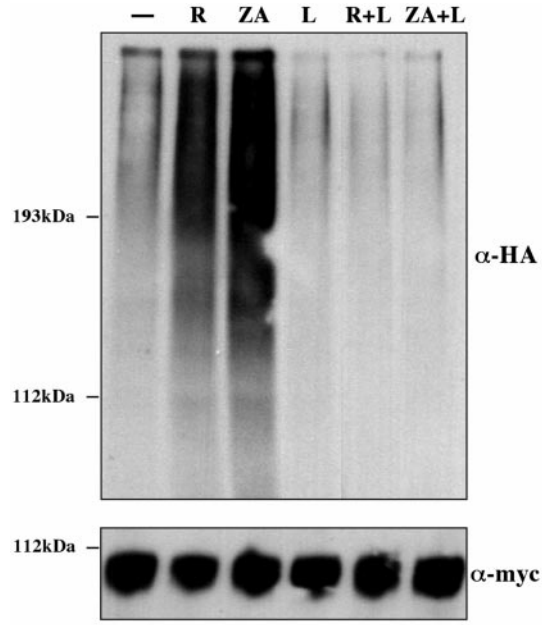

FIG. 5. **Oxidosqualene-lanosterol cyclase inhibition increases Hmg2p ubiquitination.** Cells expressing 1Myc-Hmg2p and 3HA-ubiquitin were grown to log phase. Ubiquitination assays were performed with no drug (−) or the presence of 40 μg/ml Ro48-8071 (*R*), 10 μg/ml zaragozic acid (*ZA*), of 25 μg/ml lovastatin (*L*). Lovastatin and Ro48-8071 were added 3 h prior to cell lysis, and zaragozic acid was added 10 min prior to cell lysis. The *upper panel* is the result of anti-HA (*α-HA*) immunoblotting for covalently linked HA-tagged ubiquitin. The *lower panel* is the result of parallel immunoblotting of an aliquot (0.125 total volume) of the same immunoprecipitates with anti-Myc antibody 9E10 (*α-myc*) to assess total immunoprecipitated Hmg2p.

levels of 2,(3*S*)-oxidosqualene, 2,(3*S*),(22*S*),23-dioxidosqualene, and an unidentified molecule with greater polarity than ergosterol, likely an oxysterol molecule, with a concomitant decrease in ergosterol and ergosterol esters (33, 36, 49). Enzymatic conversion of 2,(3*S*),(22*S*),23-dioxidosqualene to 24,25-oxidolanosterol by oxidosqualene-lanosterol cyclase could be observed by incubation of the cells under anaerobic conditions (49). Otherwise, incubation of cells under aerobic conditions results in the enzymatic conversion of 2,(3*S*),(22*S*),23-dioxidosqualene to an unidentified, putative oxysterol molecule (49), consistent with the requirement of oxygen for enzymatic actions directly downstream of oxidosqualene-lanosterol cyclase in the sterol/oxysterol biosynthetic pathways. These previous observations suggest that the down-regulation or inhibition of oxidosqualene-lanosterol cyclase we employed above to examine Hmg2p degradation likely resulted in the production of 2,(3*S*),(22*S*),23-

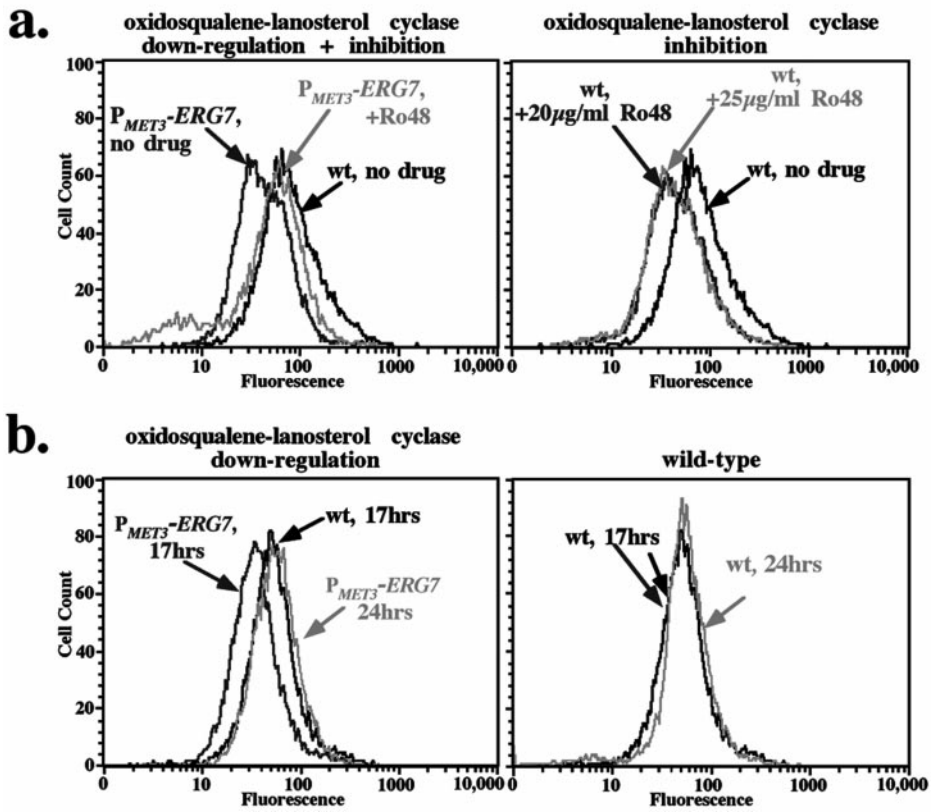

FIG. 6. **Manipulation of the oxysterol pathway alters Hmg2p degradation accordingly.** *a*, partial inhibition of oxidosqualene-lanosterol cyclase enhanced Hmg2p degradation, whereas complete inhibition had no effect. Otherwise identical strains expressing oxidosqualene-lanosterol cyclase from either the wild-type promoter (*wt*) or the *MET3* promoter (P_{MET3}-*ERG7*) were grown for 15 h at 30 °C in the presence of 2 mM methionine. Ro48-8071 (+*Ro48*; *left panel*, 5 μg/ml; *right panel*, 20 and 25 μg/ml) was added to the appropriate cultures, and the cells were incubated for an additional 4 h at 30 °C. Hmg2p-GFP fluorescence was analyzed by flow cytometry. *b*, an experiment similar to that described for *a* was carried out, except that the strains were allowed to grow for 17 or 24 h in 2 mM methionine, and no drug was added.

dioxidosqualene and possibly a downstream oxysterol derivative of this molecule.

Because downstream products of the alternate pathway are produced in yeast (33, 36, 49), and partial inhibition or down-regulation of oxidosqualene-lanosterol cyclase resulted in enhanced Hmg2p degradation (see above), we tested if complete inhibition of oxidosqualene-lanosterol cyclase had an additional enhancing effect on Hmg2p degradation, consistent with the substrate of oxidosqualene-lanosterol cyclase acting as a positive signal for HMGR degradation, or if complete inhibition was without effect on Hmg2p degradation, consistent with an oxysterol-derived molecule acting as a positive signal for HMGR degradation. To effect complete oxidosqualene-lanosterol cyclase inhibition, we first added increasing concentrations of Ro48-8071 to cells, but high concentrations (>150 μg/ml) rapidly destroyed the cells, and Hmg2p degradation could not be assessed.[2] Therefore, we employed a more sophisticated way to acutely inhibit all of the available oxidosqualene-lanosterol cyclase in the cell while avoiding the extremely destructive, high concentrations of Ro48-8071. Specifically, production of oxidosqualene-lanosterol cyclase was first down-regulated by addition of 2 mM methionine to the P_{MET3}-*ERG7* cells. This resulted in the expected decrease in Hmg2p-GFP steady-state levels (Fig. 6*a*, *left panel*, P_{MET3}-*ERG7*, *no drug versus wt, no drug*). The decrease in these cells was similar to that observed in wild-type cells after incubation with 20 μg/ml Ro48-8071 (Fig. 6*a*, compare *left panel*, P_{MET3}-*ERG7, no drug*, with *right panel*, *wt, +20μg/ml Ro48*). The remaining oxidosqualene-lanosterol cyclase activity in the P_{MET3}-*ERG7* cells under these down-regulated conditions should be completely inhibited by addition of relatively low concentrations of Ro48-8071. In fact, when these down-regulated cells were additionally incubated with just 5 μg/ml Ro48-8071, Hmg2p-GFP steady-state fluorescence was identical to Hmg2p-GFP steady-state levels in wild-type cells without down-regulation or drug treatment (Fig. 6*a*, *left panel*, P_{MET3}-*ERG7, +Ro48* compared with *wt, no drug*). In contrast, the same incremental addition of 5 μg/ml Ro48-8071 to wild-type cells did not alter the Hmg2p degradation-enhancing effect observed after incubation of cells with 20 μg/ml Ro48-8071 (Fig. 6*a*, *right panel*, *wt, +25μg/ml Ro48* compared with *wt, +20μg/ml Ro48*).

In addition to complete inhibition of oxidosqualene-lanosterol cyclase by the combination of pharmacological manipulation and genetic down-regulation, we tested if oxidosqualene-lanosterol cyclase down-regulation for further extended periods of time, which should result in almost complete loss of oxidosqualene-lanosterol cyclase activity in the cell, would have the same effect. Indeed, continued incubation of the P_{MET3}-*ERG7* strain in 2 mM methionine after the maximal Hmg2p-GFP degradation-enhancing effect, which was observed at 17 h, resulted in a complete reversal by 24 h (Fig. 6*b*, *left panel*), similar to the effect of Ro48-8071 addition. This effect was not seen for the wild-type strain (Fig. 6*b*, *right panel*). Thus, complete inhibition or down-regulation of oxidosqualene-lanosterol cyclase had no effect on Hmg2p degradation, whereas partial inhibition or down-regulation had an enhancing effect.

Moderate Reduction in Oxidosqualene-lanosterol Cyclase Activity Increases Production of Oxysterol Pathway Products—To verify that the partial inhibition employed in our studies did indeed result in increased production of oxysterol pathway products in the cell, we examined the relative abundance of these molecules in the radiolabeled non-saponifiable lipid frac-

tions from cells treated with or without the oxidosqualene-lanosterol cyclase inhibitor Ro48-8071. TLC analysis of the lipid fractions indicated that incubation of cells with 40 μg/ml Ro48-8071 resulted in a slight reduction in the free ergosterol pool and a drastic reduction in ergosterol esters (Fig. 7*a*, *R versus nd*). More importantly, incubation of cells with Ro48-8071 resulted in the detectable accumulation of 2,(3*S*)-oxidosqualene, 2,(3*S*),(22*S*),23-dioxidosqualene, and an unidentified molecule with a greater polarity than ergosterol (Fig. 7*a*, *R versus nd*). Accumulation of this putative oxysterol product and 2,(3*S*),(22*S*),23-dioxidosqualene in the cells treated with Ro48-8071 was blocked by simultaneous inhibition of the upstream enzyme squalene epoxidase by addition of terbinafine (Fig. 7*a*, *R+T*), indicating that these molecules were derived from squalene by the action of squalene epoxidase and later downstream enzymes in the sterol/oxysterol biosynthetic pathways. Although the identity of the putative oxysterol molecule remains unknown, we believe it may be 24,25-epoxycholesta-5,7,22-triene-3-ol based on its production from 2,(3*S*),(22*S*),23-dioxidosqualene by the enzymatic actions downstream of squalene epoxidase, its greater polarity that that of ergosterol, and its almost identical polarity to that of 25-hydroxycholesterol. We are initiating studies to characterize the identity of this putative oxysterol molecule.

It was possible that the Hmg2p degradation-enhancing effect of oxidosqualene-lanosterol cyclase inhibition was mediated by similar molecules as the degradation-enhancing effect of squalene synthase inhibition. Therefore, we examined the radiolabeled non-saponifiable lipid fractions from cells treated with the squalene synthase inhibitor zaragozic acid, which strongly enhances Hmg2p degradation (Refs. 9 and 10 and see above). Addition of zaragozic acid to cells resulted in a decrease in all downstream products of the mevalonate pathway and a simultaneous increase in two unidentified molecules (Fig. 7*a*, *ZA*), neither of which had the same mobility as farnesol nor geraniol, the dephosphorylated products of the immediate upstream enzyme farnesyl-diphosphate synthase. We are also currently attempting to characterize the identity of these molecules to determine whether they are directly responsible for enhanced Hmg2p degradation as a result of squalene synthase inhibition. However, buildup of different molecules after oxidosqualene-lanosterol cyclase inhibition and squalene synthase inhibition indicated that the Hmg2p degradation-enhancing effects as a result of these separate manipulations were mediated through different molecules. Inhibition of HMGR with lovastatin resulted in a decrease in all downstream products of the mevalonate pathway (Fig. 7*a*, *L*), consistent with the complete stabilization of Hmg2p.

We next examined the radiolabeled non-saponifiable lipid fractions from cells in which oxidosqualene-lanosterol cyclase was down-regulated. In these cells, an accumulation of 2,(3*S*)-oxidosqualene, 2,(3*S*),(22*S*),23-dioxidosqualene, and the putative oxysterol molecule with a concomitant decrease in lanosterol, free ergosterol, and ergosterol esters was observed by TLC (Fig. 7*b*, *OSC erg7 versus wt*). Down-regulation of squalene epoxidase reduced the endogenous levels of the putative oxysterol molecule (Fig. 7*b*, *SE erg1*), confirming that this molecule was indeed formed by enzymatic conversion of squalene by the action of squalene epoxidase and later downstream enzymes in the sterol/oxysterol biosynthetic pathways. Additionally, squalene synthase down-regulation resulted in an increase in the same two molecules observed after zaragozic acid addition (Fig. 7*b*, *SS erg9*), indicating that the Hmg2p degradation-enhancing effects of oxidosqualene-lanosterol cyclase down-regulation and squalene synthase down-regulation were also mediated by different molecules, identical to those observed after inhibition of the respective enzymes. More importantly, partial inhibition and down-regulation of oxidosqualene-lanosterol cyclase both resulted in decreased levels of sterol pathway molecules with a concomitant increase in oxysterol pathway molecules.

Because we are as yet unable to characterize the actual identity of this putative oxysterol molecule, we required a separate confirmation that flux through the oxysterol pathway was indeed increased due to our manipulations of oxidosqualene-lanosterol cyclase activity. The only product of the yeast oxysterol pathway that has been isolated and completely characterized is 24,25-oxidolanosterol (36). Although the TLC analyses performed here and in earlier studies did not have sufficient sensitivity to assess the levels of 24,25-oxidolanosterol (33, 36), previous studies with HPLC analysis did have sufficient resolution to detect increases in 24,25-oxidolanosterol upon partial inhibition of oxidosqualene-lanosterol cyclase (36). Due to the large volume of culture (>1 liter) and mass of cells required to isolate sufficient quantities of compounds for HPLC and the corresponding large amount of Ro48-8071 required to partially inhibit oxidosqualene-lanosterol cyclase in the cells from these large cultures, we opted to conduct HPLC analyses on the lipid fractions from cells in which oxidosqualene-lanosterol cyclase was down-regulated. As controls, we also examined the lipid fractions from wild-type cells and cells in which squalene epoxidase was down-regulated. Reversed-phase HPLC was performed on these samples and used to purify the appropriate fraction from each sample with the retention time corresponding to that of 24,25-oxidolanosterol. All three crude samples and their respective HPLC-purified fractions corresponding to 24,25-oxidolanosterol were analyzed by NMR. Only in the HPLC-purified fraction obtained from cells with oxidosqualene-lanosterol cyclase down-regulated were trace amounts (<1 μg) of 24,25-oxidolanosterol detected. Further analysis by gas chromatography-mass spectroscopy confirmed this result. The HPLC-purified fractions corresponding to 24,25-oxidolanosterol from all three samples were evaporated to dryness, converted to the corresponding tetramethylsilane ethers, and analyzed by gas chromatography-mass spectroscopy. Again, only samples from the cells with oxidosqualene-lanosterol cyclase down-regulated had a peak with a retention time (14.4 min) and mass spectrum (m/z 514) identical to those of authentic 24,25-oxidolanosterol (Fig. 7, *d* and *e*). There was insufficient 24,25-oxidolanosterol to be detected in wild-type cells (Fig. 7*d*) or in cells with squalene epoxidase down-regulated.[2]

A second analysis using a greater quantity of cells was performed to quantitate the amount of 24,25-oxidolanosterol in the cells with oxidosqualene-lanosterol down-regulated. To facilitate quantitation, the internal standard 26-hydroxycholesterol (100 ng/g of wet cells) was added before processing. The three samples were analyzed with selected-ion monitoring of the molecular ions of 26-hydroxycholesterol (m/z 546) and 24,25-oxidolanosterol (m/z 514). The amount of 24,25-oxidolanosterol was estimated from the ratio of the two peaks (m/z 546 and 514). The cells with oxidosqualene-lanosterol cyclase down-regulated contained ~150 ng of 24,25-oxidolanosterol/g of wet cells. In contrast, wild-type cells or cells with squalene epoxidase down-regulated contained <15 ng of 24,25-oxidolanosterol/g of wet cells.

Thus, moderate reduction in the activity of oxidosqualene-lanosterol cyclase, either by down-regulation or pharmacological inhibition, resulted in hastened Hmg2p degradation that correlated with the buildup of products in the oxysterol pathway, not the sterol pathway. In contrast, complete inhibition of oxidosqualene-lanosterol cyclase did not result in hastened Hmg2p degradation. This biphasic nature of Hmg2p degrada-

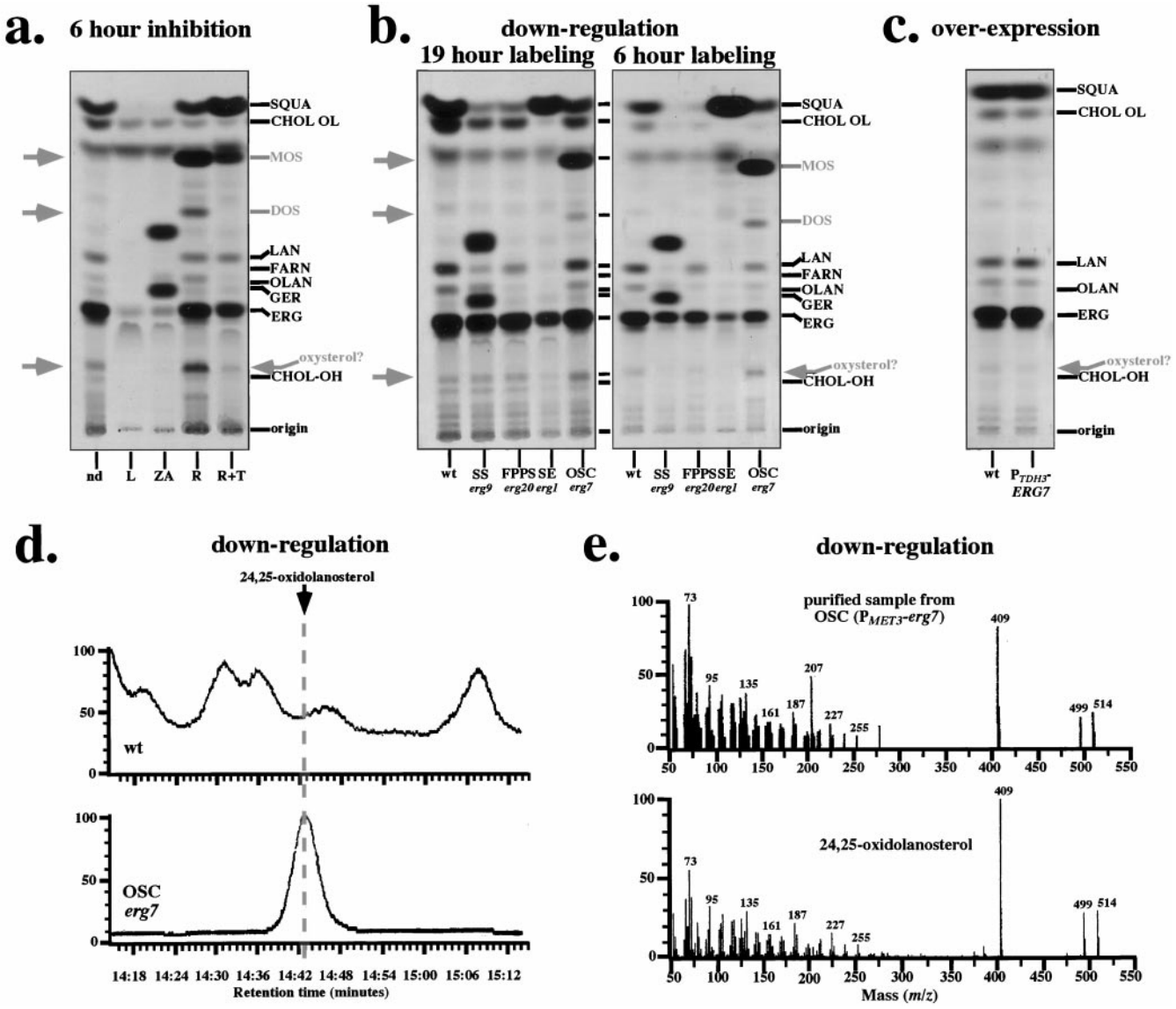

FIG. 7. **Altered activity levels of oxidosqualene-lanosterol cyclase alter the levels of oxysterol pathway products accordingly.** *a*, inhibition of oxidosqualene-lanosterol cyclase resulted in an increase in oxysterol pathway products. Shown are the results from TLC analysis of the radiolabeled non-saponifiable lipid fraction from yeast cells grown in the absence (no drug (*nd*)) or presence of 50 μg/ml lovastatin (*L*), 10 μg/ml zaragozic acid (*ZA*), 40 μg/ml Ro48-8071 (*R*), or 40 μg/ml Ro48-8071 plus 80 μg/ml terbinafine (*R*+*T*) for 6 h at 30 °C. [^{14}C]Acetate was added at the same time as the drugs. ^{14}C-Labeled lipids were extracted and gently saponified to preserve the ergosterol esters, and the radiolabeled non-saponifiable fraction was extracted, concentrated, and resolved by TLC. The migration positions of the known reference compounds are indicated on the right: squalene (*SQUA*), cholesterol oleate (*CHOL OL*; a standard for sterol esters), 2,(3*S*)-oxidosqualene (*MOS*), 2,(3*S*),(22*S*),23-dioxidosqualene (*DOS*), lanosterol (*LAN*), farnesol (*FARN*), 24,25-oxidolanosterol (*OLAN*), geraniol (*GER*), ergosterol (*ERG*), and 25-hydroxycholesterol (*CHOL-OH*). *Arrows* on the left indicate the buildup of the identifiable products. *b*, shown are the results from TLC analysis of the radiolabeled non-saponifiable lipid fraction from yeast cells without (wild-type cells (*wt*)) or with down-regulation of squalene synthase (*SS*), farnesyl-diphosphate synthase (*FPPS*), squalene epoxidase (*SE*), or oxidosqualene-lanosterol cyclase (*OSC*). *Left panel*, analysis of total changes in non-saponifiable lipids over the entire course of down-regulation. Cells were grown in minimal medium with 2 mM methionine and [^{14}C]acetate for 19 h at 30 °C. *Right panel*, analysis of changes in non-saponifiable lipids after 13 h of down-regulation, when Hmg2p degradation was maximally enhanced. Cells were grown in minimal medium with 2 mM methionine for 13 h at 30 °C. [^{14}C]Acetate was then added, and the cells were incubated for a further 6 h at 30 °C. ^{14}C-Labeled lipids were extracted and gently saponified to preserve the ergosterol esters, and the non-saponifiable fraction was extracted, concentrated, and resolved by TLC. The migration positions of the known reference compounds are marked on the right. *Arrows* on the left indicate the buildup of the identifiable products. *c*, overexpression of oxidosqualene-lanosterol cyclase resulted in decreased levels of the putative oxysterol. Shown are the results from TLC analysis of the radiolabeled non-saponifiable lipid fraction from yeast cells without (*wt*) or with overexpression of oxidosqualene-lanosterol cyclase (P_{TDH3}-*ERG7*). [^{14}C]Acetate was added to the cells in early log phase (A_{600} = 0.3), and the cells were incubated at 30 °C for 6 h. ^{14}C-Labeled lipids were extracted and gently saponified to preserve the ergosterol esters, and the non-saponifiable fraction was extracted, concentrated, and resolved by TLC. The migration positions of the known reference compounds are marked on the right. *d*, down-regulation of oxidosqualene-lanosterol cyclase resulted in an increase in 24,25-oxidolanosterol. Shown are the results from gas chromatography analysis of HPLC-purified fractions from wild-type cells (*wt*; *upper panel*) or cells with oxidosqualene-lanosterol cyclase down-regulated (*OSC erg7*; *lower panel*). The retention time of purified 24,25-oxidolanosterol is indicated by the *dashed line*. The quantity of 24,25-oxidolanosterol was determined by calculating the area under the appropriate peak and comparing it with that of an internal 26-hydroxycholesterol standard (not shown). *e*, the material in the HPLC-purified fraction from cells with oxidosqualene-lanosterol cyclase down-regulated was 24,25-oxidolanosterol. Mass spectroscopy was carried out on the sample from *d*. The mass/charge (*m*/*z*) ratios for some of the peaks are noted.

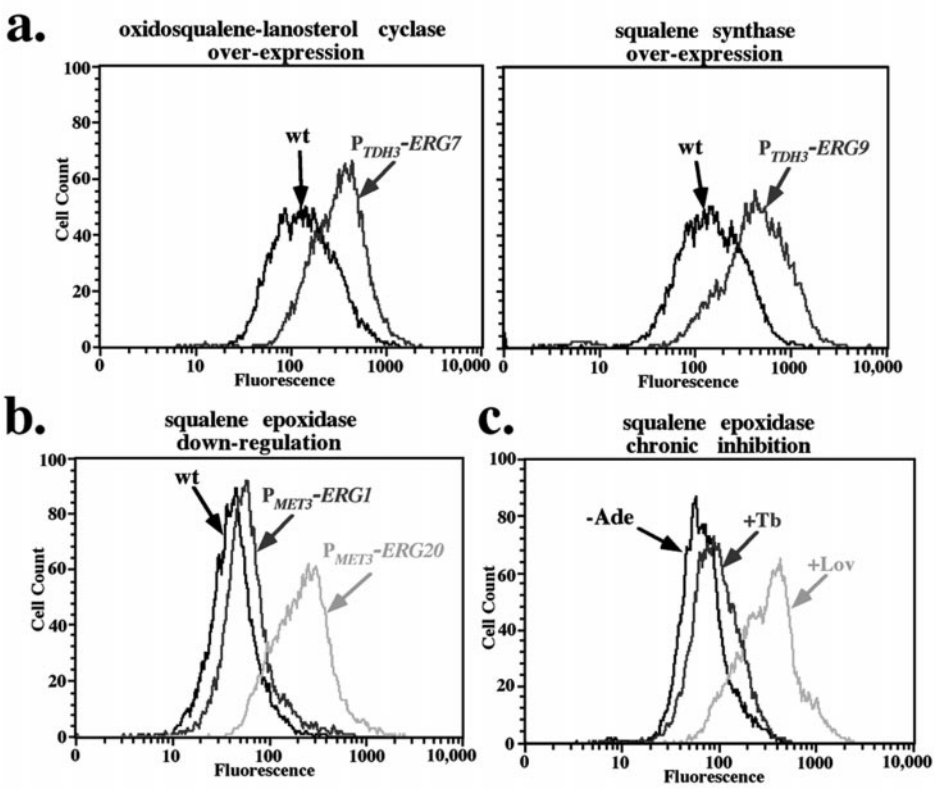

FIG. 8. **Endogenous levels of an oxysterol-derived signal control Hmg2p degradation.** *a*, overexpression of oxidosqualene-lanosterol cyclase stabilized Hmg2p. Otherwise identical strains expressing oxidosqualene-lanosterol cyclase from the *TDH3* promoter (P_{TDH3}-*ERG7*), squalene synthase from the *TDH3* promoter (P_{TDH3}-*ERG9*), or both from the native promoters (*wt*) were grown to log phase and analyzed by flow cytometry to determine Hmg2p-GFP steady-state fluorescence. *b*, squalene epoxidase down-regulation had a modest stabilizing effect on Hmg2p degradation. Otherwise identical strains expressing squalene epoxidase from either the wild-type promoter (*wt*) or the *MET3* promoter (P_{MET3}-*ERG1*) or farnesyl-diphosphate 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 8 mM methionine and then analyzed by flow cytometry to determine Hmg2p-GFP steady-state fluorescence. *c*, chronic treatment of cells with a squalene epoxidase inhibitor modestly stabilized Hmg2p. Log-phase cells expressing Hmg2p-GFP were treated first for 24 h with 10 μg/ml terbinafine, an antifungal squalene epoxidase inhibitor, followed by 18 h with 80 μg/ml terbinafine (+*Tb*). To compare cells inhibited for growth, log-phase cells not treated with terbinafine were incubated for 18 h either in the absence of adenine (−*Ade*), an essential nutrient, or in the presence of a lethal amount of lovastatin (300 μg/ml; +*Lov*).

tion was reminiscent of HMGR regulation in mammalian cells by oxysterol pathway products (37) and suggested that a downstream product of the oxysterol pathway acted as a positive signal for HMGR degradation in yeast.

Yeast Contains Endogenous Levels of Oxysterols That Enhance Hmg2p Degradation—In the above experiments, we observed a small amount of the putative oxysterol molecule in the non-saponifiable lipid fractions under normal growth conditions (Fig. 7*b*, *wt*). Thus, it is likely that under normal growth conditions, the enzymatic conversion of 2,(3*S*)-oxidosqualene to lanosterol by oxidosqualene-lanosterol cyclase is sufficiently rate-limiting to allow a small amount of 2,(3*S*)-oxidosqualene to become a substrate for squalene epoxidase. This would result in the formation of 2,(3*S*),(22*S*),23-dioxidosqualene (37), with the subsequent production of oxysterol pathway products (Fig. 1). Accordingly, increased expression of oxidosqualene-lanosterol cyclase may hasten 2,(3*S*)-oxidosqualene conversion to lanosterol, thereby reducing the probability that 2,(3*S*)-oxidosqualene would be available for further epoxidation and subsequent enzymatic conversion to an oxysterol. If an oxysterol pathway molecule were acting as a positive signal for Hmg2p degradation, then such overexpression of oxidosqualene-lanosterol cyclase might result in reduced Hmg2p degradation.

To examine the effect of increased oxidosqualene-lanosterol cyclase expression, we placed the *ERG7* gene behind the strong constitutive *TDH3* promoter and transformed the P_{TDH3}-*ERG7* gene into yeast cells expressing 1Myc-Hmg2p and Hmg2p-GFP. Increased expression of oxidosqualene-lanosterol cyclase resulted in a decrease (~50%) in the cellular levels of the putative oxysterol, as measured by TLC analysis of the radiolabeled non-saponifiable lipid fractions (Fig. 7*c*, P_{TDH3}-*ERG7 versus wt*). Increased expression of oxidosqualene-lanosterol cyclase also had a stabilizing effect on Hmg2p degradation, as seen by the rightward shift in the Hmg2p-GFP fluorescence histogram of the P_{TDH3}-*ERG7* cells compared with wild-type *ERG7* cells (Fig. 8*a*, *left panel*, P_{TDH3}-*ERG7 versus wt*). This result was similar to that previously reported for overexpression of squalene synthase (Fig. 8*b*, *left panel*), which stabilizes Hmg2p through reduced FPP levels by enhanced conversion to squalene (10). These results suggested that endogenous levels of oxysterols were acting to program the normal rate of Hmg2p degradation under typical growth conditions.

If endogenous levels of oxysterols were acting to program a specific rate of Hmg2p degradation, then it might be expected that inhibition of squalene epoxidase would have a stabilizing effect on Hmg2p degradation. Previously, we had shown that acute treatment of cells with lethal doses of terbinafine, an antifungal squalene epoxidase inhibitor (43–45), had no effect on Hmg2p-GFP steady-state levels (10). However, it was possible that the limited acute treatment had little effect on the preexisting pool of oxysterols present as a result of oxysterol pathway production prior to inhibition of squalene epoxidase. We had also previously shown that down-regulation of squalene epoxidase had little effect on Hmg2p degradation under the conditions tested (10). However, TLC analysis revealed that under these conditions, there were still detectable levels of

the putative oxysterol molecule (Fig. 7*b*, *left panel*, *SE erg1*). To reduce flux through the oxysterol pathway more stringently and to reduce the pool of potential accumulated oxysterols, we examined if either greater down-regulation or chronic inhibition of squalene epoxidase resulted in stabilization of Hmg2p degradation.

To induce greater down-regulation of squalene epoxidase, we explored whether incubation of cells expressing squalene epoxidase from the *MET3* promoter (P_{MET3}-*ERG1*) in different concentrations of methionine and/or with different incubation periods after methionine addition would reveal if squalene epoxidase down-regulation had any stabilizing effect on Hmg2p degradation. In fact, we were able to observe a modest stabilizing effect on Hmg2p-GFP degradation due to squalene epoxidase down-regulation, as cells carrying the P_{MET3}-*ERG1* gene had higher steady-state levels of Hmg2p-GFP after incubation with 8 mM methionine, rather than 2 mM, for 24 h compared with wild-type cells with the same treatment (Fig. 8*b*, P_{MET3}-*ERG1 versus wt*). However, down-regulation of squalene epoxidase did not have nearly the same stabilizing effect as down-regulation of the upstream mevalonate pathway enzyme farnesyl-diphosphate synthase (Fig. 8*b*, P_{MET3}-*ERG1 versus* P_{MET3}-*ERG20*), which results in complete stabilization of Hmg2p by reduced FPP production within the cell (10).

We also tested if chronic treatment of cells with terbinafine would have a similar stabilizing effect on Hmg2p degradation as down-regulation of squalene epoxidase. When cells were incubated with lethal doses of terbinafine for increasing periods of time past the inhibition of cell growth, modest stabilization of Hmg2p degradation was observed, as seen by the increased Hmg2p-GFP steady-state levels compared with wild-type cells that were similarly growth-compromised by removal of adenine from the growth medium (Fig. 8*c*, +*Tb versus* −*Ade*). However, chronic treatment of cells with lethal doses of lovastatin resulted in much greater stabilization and greatly increased steady-state levels of Hmg2p (Fig. 8*c*, +*Lov*). Thus, the Hmg2p-stabilizing effect of reduced oxysterol production was observable, but limited in its ability to alter Hmg2p degradation.

Together, the genetic and pharmacological manipulations of both oxidosqualene-lanosterol cyclase and squalene epoxidase activities revealed that a product of the oxysterol pathway acted to positively regulate Hmg2p degradation. Furthermore, it appeared that endogenous levels of the oxysterol-derived molecule were acting to program, in part, the specific rate of Hmg2p degradation under normal growth conditions.

The Sterol-derived Signal Functions Only when Sufficient Levels of the Primary FPP-derived Signal Are Present—The above results suggested that the oxysterol-derived molecule was an additional signal for Hmg2p degradation and not the primary signal. In mammalian cells, the secondary oxysterol-derived signal cannot work independently of the primary FPP-derived signal (2, 20, 25). To test if this was similar for Hmg2p degradation, we required a means to reduce FPP levels that still allowed production of downstream mevalonate pathway molecules. Previously, we constructed strains that overexpress squalene synthase from the *TDH3* (glyceraldehyde-3-phosphate dehydrogenase) promoter (10), which resulted in stabilization of Hmg2p through reduced FPP levels. In these strains, mevalonate pathway production was still fully operational because FPP levels were reduced by increased conversion to squalene, not by inhibition of FPP production. When cells that overexpressed squalene synthase were incubated with Ro48-8071, no effect on Hmg2p degradation was observed (Fig. 9*a*, *left panel*, +*Ro48* compared with *no drug*), even though we used up to 4-fold higher concentrations of Ro48-8071 than were sufficient to enhance degradation in a wild-type squalene synthase strain (*right panel*, +*Ro48* compared with *no drug*). Addition of zaragozic acid, which inhibits squalene synthase, did result in enhanced Hmg2p-GFP degradation in the strain overexpressing squalene synthase as well as the wild-type strain (Fig. 9*a*, *both panels*, +*ZA*). Thus, the action of the oxysterol-derived positive signal for HMGR degradation in yeast appeared to require a sufficient level of the primary FPP-derived positive signal.

The Secondary Oxysterol-derived Signal Acts to Enhance Hmg2p Degradation, but Is Not an Essential Requirement—Previous studies delineating the regulatory signals required for mammalian HMGR degradation demonstrated that the primary FPP-derived signal does not function to enhance HMGR degradation in the absence of sufficient levels of the oxysterol-derived signal (17, 18, 20). In one study, the obligatory nature of the oxysterol-derived signal was examined by observing HMGR degradation after inhibition of squalene epoxidase (17). The other two studies examined HMGR degradation in cell lines that are deficient for squalene synthase activity (18, 20). In both cases, sterol synthesis was severely reduced, and the evidence supported a codependent nature for the HMGR degradation-enhancing effect of the FPP-derived signal and the oxysterol-derived signal.

To examine further the requirement for the oxysterol-derived signal in yeast, we tested if overexpression of oxidosqualene-lanosterol cyclase prevented the ability of FPP accumulation to enhance Hmg2p degradation. We expected that if the oxysterol-derived signal were obligatory, then the hastened conversion of 2,(3*S*)-oxidosqualene to lanosterol would result in a blunted effect of zaragozic acid addition to cells overexpressing oxidosqualene-lanosterol cyclase. However, addition of zaragozic acid to the cells overexpressing oxidosqualene-lanosterol cyclase had an equivalent effect on Hmg2p-GFP steady-state fluorescence as zaragozic acid addition to wild-type cells (Fig. 9*b*, *both panels*, +*ZA*) at all concentrations tested.[2] Addition of Ro48-8071 to the cells overexpressing oxidosqualene-lanosterol cyclase did result in enhanced Hmg2p-GFP degradation (Fig. 9*b*, *left panel*, +*Ro48*), but it required 4-fold higher concentrations than those needed to enhance degradation in the wild-type cells,[2] as would be expected for increased expression of the drug's target enzyme oxidosqualene-lanosterol cyclase. Thus, it appeared that the upstream FPP-derived signal could work in the absence of the downstream oxysterol-derived signal.

These results implied that the function of the oxysterol-derived signal was to enhance FPP-dependent degradation of Hmg2p at lower levels of cellular FPP, rather than to serve as an essential signal. If this were the case, then it would be expected that the oxysterol-derived signal would act synergistically with the FPP-derived signal. To examine this scenario, we treated cells expressing Hmg2p-GFP with concentrations of zaragozic acid that normally did not enhance Hmg2p degradation combined with increasing concentrations of Ro48-8071 that also normally did not enhance Hmg2p degradation. When we treated the cells in this manner, we observed that the oxysterol-derived signal did indeed work synergistically with the FPP-derived signal, as a greater than additive decrease in Hmg2p-GFP steady-state levels was observed (Fig. 9*c*, *left panel*, +*ZA, +Ro48 versus* +*ZA* and +*Ro48*), with the maximal synergistic effect produced by addition of 5 μg/ml Ro48-8071 (*right panel*). However, sufficiently high levels of the FPP-derived signal in the cell, accomplished by inhibition or down-regulation of squalene synthase, did allow the fastest rate of Hmg2p degradation even with genetic or pharmacological manipulated decreases in the oxysterol-derived signal (Fig. 9*b*),

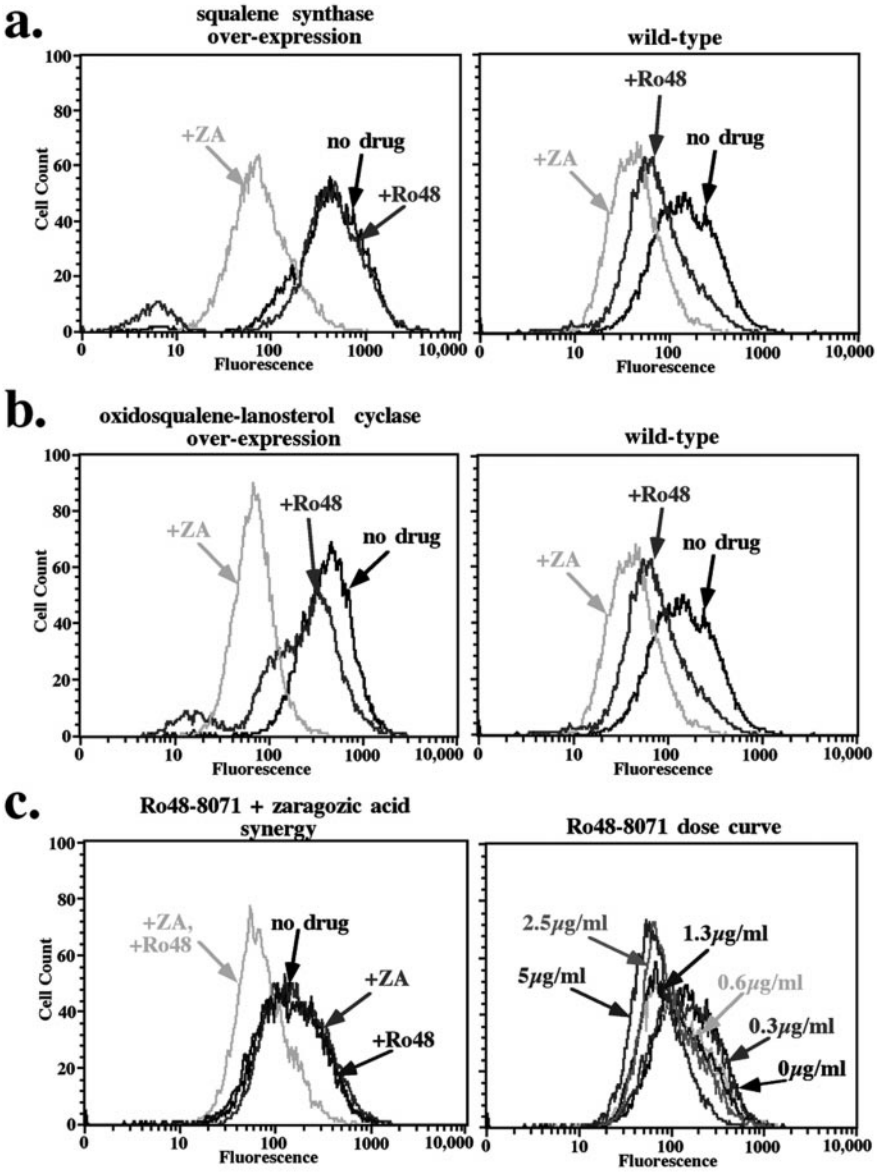

FIG. 9. **The oxysterol-derived signal is not required, but works synergistically with the FPP-derived signal to promote Hmg2p degradation.** *a*, squalene synthase overexpression blocked the degradation-enhancing effect of oxidosqualene-lanosterol cyclase inhibition. Otherwise identical strains expressing squalene synthase from a single integrated allele with the *TDH3* promoter (P_{TDH3}-*ERG9*) or from the wild-type promoter were grown to log phase and treated with no drug, 40 μg/ml Ro48-8071 (+*Ro48*), or 10 μg/ml zaragozic acid (+*ZA*) for 4 h at 30 °C. Cells were analyzed by flow cytometry to determine Hmg2p-GFP levels. *b*, oxidosqualene-lanosterol cyclase overexpression did not block the degradation-enhancing effect of squalene synthase inhibition. Otherwise identical strains expressing oxidosqualene-lanosterol cyclase from a single integrated allele with the *TDH3* promoter (P_{TDH3}-*ERG7*) or from the wild-type promoter were grown to log phase and treated with no drug, 40 μg/ml Ro48-8071, or 10 μg/ml zaragozic acid for 4 h at 30 °C. Cells were analyzed by flow cytometry to determine Hmg2p-GFP levels. *c*, the oxysterol-derived signal worked synergistically with the FPP-derived signal to promote Hmg2p degradation. Cells were grown to log phase and treated with a dose of zaragozic acid that did not elicit a degradation-enhancing effect (2.5 μg/ml; *left panel*, +*ZA*). Increasing concentrations of Ro48-8071, which did not promote Hmg2p degradation alone, were added to these zaragozic acid-treated cells. All cultures were incubated at 30 °C for 4 h after drug addition. Cultures were analyzed by flow cytometry to determine Hmg2p-GFP steady-state fluorescence. The maximal effect of Ro48-8071 was observed at a final concentration of 5 μg/ml (*right panel*; and *left panel*, +*ZA, +Ro48 versus +Ro48* and +*ZA*).

suggesting the independence of the FPP-derived signal. Thus, the function of the oxysterol-derived signal in yeast was to enhance Hmg2p degradation beyond the limited capacity of low levels of the FPP-derived signal.

DISCUSSION

In both mammalian cells and yeast, the rate of HMGR degradation is regulated by signals from the mevalonate pathway. Mammalian cells appear to utilize two separate signals from the mevalonate pathway to enhance HMGR degradation: a non-sterol signal derived from FPP (16–20) and a sterol signal derived from the oxysterol pathway (2, 3, 21–26). In yeast, HMGR degradation is enhanced by a signal generated from FPP (10), but no oxysterol-derived signal had yet been identified. Through use of pharmacological inhibition and genetic manipulation of the mevalonate pathway, we have explored if the degradation of the yeast HMGR isozyme Hmg2p is regulated by an oxysterol-derived signal.

The results detailed in this work strongly implicated a product of the alternate oxysterol pathway as a source of a positive signal for Hmg2p degradation in yeast. Moderate inhibition or genetic down-regulation of oxidosqualene-lanosterol cyclase increased Hmg2p degradation, whereas complete inhibition had no effect. These manipulations were consistent with increased oxysterol production by moderate oxidosqualene-lanosterol cy-

clase inhibition previously demonstrated in mammalian cells (38, 48). In fact, moderate inhibition or down-regulation of oxidosqualene-lanosterol cyclase did result in the buildup of 24,25-oxidolanosterol and a putative oxysterol molecule, which had greater polarity than ergosterol as expected. Furthermore, Hmg2p was stabilized by increased expression of oxidosqualene-lanosterol cyclase, which decreased production of the putative oxysterol through possibly hastened conversion of 2,(3*S*)-oxidosqualene to lanosterol, thereby reducing the further epoxidation of 2,(3*S*)-oxidosqualene. Hmg2p was partially stabilized by inhibition or down-regulation of squalene epoxidase, which decreased production of oxysterols by blocking epoxidation of squalene. Thus, all manipulations predicted to increase oxysterols in the cell resulted in increased Hmg2p degradation, whereas all manipulations predicted to decrease oxysterols in the cell resulted in stabilization of Hmg2p.

Although we observed an increase in a distinct putative oxysterol molecule upon manipulation of oxidosqualene-lanosterol cyclase in our TLC analysis of the radiolabeled non-saponifiable lipid fractions, the actual identity of this molecule remains unknown. We believe this putative oxysterol molecule may be 24,25-epoxycholesta-5,7,22-triene-3-ol based on its polarity and the available chemistry of the remaining downstream enzymes in the sterol/oxysterol biosynthetic pathways. Because 24,25-epoxycholesta-5,7,22-triene-3-ol or any of the other yeast oxysterol pathway intermediates are not readily available, we will have to synthesize the appropriate standards to completely characterize this putative oxysterol molecule. We are initiating experiments to purify sufficient amounts of this molecule from Ro48-8071-treated cells to characterize its identity, to synthesize the appropriate standards, and to determine whether this molecule is actually responsible for enhanced Hmg2p degradation by exogenous addition to cells. Despite this remaining work, the evidence presented in this report strongly indicates that increased production of the oxysterol pathway does enhance HMGR degradation in yeast.

Hmg2p Degradation Requires the FPP-derived Signal, but Not the Oxysterol-derived Signal—It appears that in mammalian cells, the FPP-derived signal and the oxysterol-derived signal are both required for HMGR degradation (17, 18, 20). The evidence for this dual requirement was obtained by pharmacological manipulation of squalene epoxidase (17) and by genetic manipulation of squalene synthase (18, 20). We examined if the oxysterol-derived signal in yeast was similarly required for Hmg2p degradation by pharmacological inhibition of squalene epoxidase and by increased expression of oxidosqualene-lanosterol cyclase. In all cases, we could not establish an essential requirement of the oxysterol-derived signal for Hmg2p degradation. However, it is possible that these manipulations did not effectively remove all the oxysterols present within the cell and that the residual oxysterols acted to allow Hmg2p degradation.

We did demonstrate that the oxysterol-derived signal required a sufficient level of the FPP-derived signal to promote Hmg2p degradation, as similar manipulations of the oxysterol pathway that enhanced Hmg2p degradation in normal cells no longer promoted Hmg2p degradation in cells with decreased levels of FPP by overexpression of squalene synthase. In these squalene synthase-overexpressing cells, Hmg2p is stabilized due to the low cellular levels of FPP by the hastened conversion of FPP to squalene (10). Although Hmg2p degradation was stimulated by sufficient squalene synthase inhibition in these cells, Hmg2p degradation could not be stimulated by inhibition of oxidosqualene-lanosterol cyclase. Consistent with this, we have clearly demonstrated that the Hmg2p degradation-enhancing function of the oxysterol-derived signal was through a synergistic effect with the FPP-derived signal. Pharmacological inhibition of squalene synthase and oxidosqualene-lanosterol cyclase, at levels that did not enhance Hmg2p degradation when inhibited separately, did result in a greater than additive enhancement of Hmg2p degradation when inhibited together. Thus, the levels of the FPP-derived signal programmed a particular rate of Hmg2p degradation, which could be accordingly hastened by increased oxysterol production.

Possible Mechanism for the Oxysterol-derived Signal—The transmembrane domain of the yeast HMGR isozyme Hmg2p shares homology with the transmembrane domains of both mammalian HMGR and the mammalian protein SCAP (SREBP cleavage-activating protein), both of which are postulated to act as sterol-sensing domains (50, 51). It was previously not clear if the Hmg2p transmembrane domain had any sterol-sensing ability, as it was unknown if any sterol signal acted to enhance Hmg2p degradation. As we have provided evidence for the existence of an oxysterol-derived signal for Hmg2p degradation, it may be likely that the Hmg2p transmembrane domain does possess a sterol-sensing capability similar to its mammalian counterpart. Based upon this function, it is possible that the oxysterol-derived signal in yeast acts to alter the structure of the Hmg2p transmembrane domain to achieve a greater degradation-promoting sensitivity to lower cellular levels of the FPP-derived signal. In the absence of the oxysterol-derived signal, the Hmg2p transmembrane domain would have a reduced sensitivity to the action of the FPP-derived signal, which could be overcome by sufficient accumulation of the FPP-derived signal. This action of the oxysterol-derived signal may be similar in mammalian HMGR degradation. However, because mammalian HMGR requires the oxysterol-derived signal for degradation, it is likely that the transmembrane sterol-sensing domain of mammalian HMGR cannot effect HMGR degradation by action of the FPP-derived signal at any cellular concentration in the absence of the oxysterol-derived signal. Further molecular studies will be required for both yeast and mammalian HMGR degradation to elucidate the actual mechanism of both the oxysterol-derived signal and the FPP-derived signal.

Alternatively, it is possible that oxysterols affect some global biophysical property of the ER membrane, which subsequently affects the regulated stability of HMGR. Properties of the ER membrane subject to possible alteration by oxysterols may include the width, fluidity, packing density, or protein density of the ER membrane. The observation that oxysterols, under certain genetic conditions, affect the stability and cellular localization of several membrane proteins not related to HMGR provides modest evidence for such a possibility (52).

Eukaryotic Conservation of an Oxysterol-derived Signal for HMGR Degradation—The fact that we have demonstrated the existence of an oxysterol-derived signal for yeast HMGR degradation further strengthens the similarities between mammalian and yeast HMGR degradation already established. Although yeast and mammals may regulate sterol production for different purposes, it is becoming increasingly clear that the mechanism of HMGR degradation as a means to control sterol production is highly conserved between the two organisms. These similarities include degradation without exit from the ER (4–6), degradation dependent upon both the proteasome and ubiquitination (8, 9, 15), a well defined N-terminal transmembrane domain that is both necessary and sufficient for regulated degradation (4–7), and the use of both an FPP-derived positive signal and an oxysterol-derived positive signal to effect degradation (Refs. 2, 3, 10, and 20 and this work).

In mammalian cells and yeast, FPP serves as a source for a primary positive signal for HMGR degradation (9, 10, 16–20).

FPP occupies a key branch point in the mevalonate pathway (1), from which numerous other essential molecules and cellular modifications are derived. The cellular levels of FPP would be contingent on the demands from each branch pathway. If the demands of each pathway are minimal in relation to FPP production, FPP would most likely accumulate. Conversely, if the demands of each pathway are maximal in relation to FPP production, FPP would most likely be scarce. Thus, it is not surprising that both yeast and mammalian cells utilize this key branch point as a sensor to optimally control mevalonate pathway production.

Oxysterols also serve as a source for a positive signal for HMGR degradation in both mammalian cells (2, 3, 21–25) and yeast (this work). The alternate pathway for oxysterol production branches from the mevalonate pathway at 2,(3*S*)-oxidosqualene (Fig. 1). Formation of oxysterols would be an effective measure of sterol content within the cell. For instance, insufficient levels of sterols in the cell may bias the conversion of 2,(3*S*)-oxidosqualene to lanosterol and thus decrease the availability of 2,(3*S*)-oxidosqualene for further epoxidation and subsequent oxysterol production. Conversely, abundant levels of sterols may allow increased levels of 2,(3*S*)-oxidosqualene that would be readily available for further epoxidation by squalene epoxidase and greater production of oxysterols. Such regulation of 2,(3*S*)-oxidosqualene levels could be effected by regulation of oxidosqualene-lanosterol cyclase activity by sterol abundance in the cell. Whatever the cellular mechanism may be to allow increased or decreased oxysterol production, it is clear that both yeast and mammals utilize this sensor, most likely as a means to prevent the detrimental buildup of sterols. However, both organisms also have vital interest in sufficient FPP production and appear to engage a feedback regulation process for HMGR degradation that is critically dependent upon FPP production within the cell. The use of an FPP-derived signal and an oxysterol-derived signal for HMGR degradation bolsters the idea that yeast and mammalian cells effect regulated degradation of HMGR by a highly conserved mechanism, thus allowing for rigorous experimentation and comparison between two highly divergent species.

Acknowledgments—We gratefully thank Merck for the generous gifts of zaragozic acid and lovastatin, Dr. Olivier Morand for Ro48-8071, Dr. Harry Mountain for the *MET3* plasmids, Dr. Robert Rickert for the use of the FACSCalibur™ flow microfluorometer and software, and Jihai Pang for measuring mass spectra. R. G. G. thanks Dustin Byrnes for greater stability due to increased expression.

REFERENCES

1. Goldstein, J. L., and Brown, M. S. (1990) *Nature* **343,** 425–430
2. Nakanishi, M., Goldstein, J. L., and Brown, M. S. (1988) *J. Biol. Chem.* **263,** 8929–8937
3. Chun, K. T., Bar-Nun, S., and Simoni, R. D. (1990) *J. Biol. Chem.* **265,** 22004–22010
4. Hampton, R. Y., and Rine, J. (1994) *J. Cell Biol.* **125,** 299–312
5. Gil, G., Faust, J. R., Chin, D. J., Goldstein, J. L., and Brown, M. S. (1985) *Cell* **41,** 249–258
6. Jingami, H., Brown, M. S., Goldstein, J. L., Anderson, R. G., and Luskey, K. L. (1987) *J. Cell Biol.* **104,** 1693–1704
7. Skalnik, D. G., Narita, H., Kent, C., and Simoni, R. D. (1988) *J. Biol. Chem.* **263,** 6836–6841
8. Hampton, R. Y., Gardner, R. G., and Rine, J. (1996) *Mol. Biol. Cell* **7,** 2029–2044
9. Hampton, R. Y., and Bhakta, H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 12944–12948
10. Gardner, R. G., and Hampton, R. Y. (1999) *J. Biol. Chem.* **274,** 31671–31678
11. Cronin, S. R., Khoury, A., Ferry, D. K., and Hampton, R. Y. (2000) *J. Cell Biol.* **148,** 915–924
12. Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A., and Hampton, R. Y. (2001) *Nat. Cell Biol.* **3,** 24–29
13. Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S., Wilhovsky, S., Seelig, L., Kim, C., and Hampton, R. Y. (2000) *J. Cell Biol.* **151,** 69–82
14. Brodsky, J. L., and McCracken, A. A. (1999) *Semin. Cell Dev. Biol.* **10,** 507–513
15. Ravid, T., Doolman, R., Avner, R., Harats, D., and Roitelman, J. (2000) *J. Biol. Chem.* **275,** 35840–35847
16. Bradfute, D. L., and Simoni, R. D. (1994) *J. Biol. Chem.* **269,** 6645–6650
17. Correll, C. C., Ng, L., and Edwards, P. A. (1994) *J. Biol. Chem.* **269,** 17390–17393
18. Meigs, T. E., Roseman, D. S., and Simoni, R. D. (1996) *J. Biol. Chem.* **271,** 7916–7922
19. Lopez, D., Chambers, C. M., and Ness, G. C. (1997) *Arch. Biochem. Biophys.* **343,** 118–122
20. Meigs, T. E., and Simoni, R. D. (1997) *Arch. Biochem. Biophys.* **345,** 1–9
21. Faust, J. R., Luskey, K. L., Chin, D. J., Goldstein, J. L., and Brown, M. S. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 5205–5209
22. Chin, D. J., Gil, G., Faust, J. R., Goldstein, J. L., Brown, M. S., and Luskey, K. L. (1985) *Mol. Cell. Biol.* **5,** 634–641
23. Meigs, T. E., and Simoni, R. D. (1992) *J. Biol. Chem.* **267,** 13547–13552
24. Panini, S. R., Delate, T. A., and Sinensky, M. (1992) *J. Biol. Chem.* **267,** 12647–12654
25. Roitelman, J., and Simoni, R. D. (1992) *J. Biol. Chem.* **267,** 25264–25273
26. Schroepfer, G. J., Jr. (2000) *Physiol. Rev.* **80,** 361–554
27. Panini, S. R., Sexton, R. C., Gupta, A. K., Parish, E. J., Chitrakorn, S., and Rudne, H. (1986) *J. Lipid Res.* **27,** 1190–1204
28. Ni, Y., Kim, H.-S., Wilson, W. K., Kisic, A., and Schroepfer, G. J., Jr. (1993) *Tetrahedron Lett.* **34,** 3687–3690
29. Gardner, R., Cronin, S., Leader, B., Rine, J., and Hampton, R. (1998) *Mol. Biol. Cell* **9,** 2611–2626
30. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
31. Gardner, R. G., and Hampton, R. Y. (1999) *EMBO J.* **18,** 5994–6004
32. Bitter, G. A., and Egan, K. M. (1984) *Gene* (*Amst.*) **32,** 263–274
33. Fung, B., and Holmlund, C. E. (1976) *Biochem. Pharmacol.* **25,** 1249–1254
34. Peffley, D. M., and Gayen, A. K. (1997) *Arch. Biochem. Biophys.* **337,** 251–260
35. Dolis, D., and Schuber, F. (1994) *Biochem. Pharmacol.* **48,** 49–57
36. Field, R. B., and Holmlund, C. E. (1977) *Arch. Biochem. Biophys.* **180,** 465–471
37. Peffley, D. M., Gayen, A. K., and Morand, O. H. (1998) *Biochem. Pharmacol.* **56,** 439–449
38. Bai, M., Xiao, X. Y., and Prestwich, G. D. (1992) *Biochem. Biophys. Res. Commun.* **185,** 323–329
39. Boutaud, O., Dolis, D., and Schuber, F. (1992) *Biochem. Biophys. Res. Commun.* **188,** 898–904
40. Hampton, R. Y., Koning, A., Wright, R., and Rine, J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 828–833
41. Cronin, S. R., and Hampton, R. Y. (1999) *Methods Enzymol.* **302,** 58–73
42. Morand, O. H., Aebi, J. D., Dehmlow, H., Gains, Y. H. J., Lengsfeld, H., and Himber, J. (1997) *J. Lipid Res.* **38,** 373–390
43. Ryder, N. S. (1989) *Clin. Exp. Dermatol.* **14,** 98–100
44. Jandrositz, A., Turnowsky, F., and Högenauer, G. (1991) *Gene* (*Amst.*) **107,** 155–160
45. Ryder, N. S. (1992) *Br. J. Dermatol.* **126,** Suppl. 39, 2–7
46. Cherest, H., Nguyen, N. T., and Surdin-Kerjan, Y. (1985) *Gene* (*Amst.*) **34,** 269–281
47. Mountain, H. A., Byström, A. S., Larsen, J. T., and Korch, C. (1991) *Yeast* **7,** 781–803
48. Mark, M., Muller, P., Maier, R., and Eisele, B. (1996) *J. Lipid Res.* **37,** 148–158
49. Field, R. B., and Holmlund, C. E. (1979) *Lipids* **14,** 741–747
50. Kumagai, H., Chun, K. T., and Simoni, R. D. (1995) *J. Biol. Chem.* **270,** 19107–19113
51. Hua, X., Nohturfft, A., Goldstein, J. L., and Brown, M. S. (1996) *Cell* **87,** 415–426
52. Du, E. Z., Fleming, J. F., Wang, S. L., Spitsen, G. M., and Davis, R. A. (1999) *J. Biol. Chem.* **274,** 1856–1862