Regulation of 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase Degradation by the Nonsterol Mevalonate Metabolite Farnesol in Vivo*

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We have previously reported that degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme in the isoprenoid pathway leading to cholesterol production, can be accelerated in cultured cells by the addition of farnesyl compounds, which are thought to mimic a natural, nonsterol mevalonate metabolite(s). In this paper we report accelerated reductase degradation by the addition of farnesol, a natural product of mevalonate metabolism, to intact cells. We demonstrate that this regulation is physiologically meaningful, shown by its blockage by several inhibitory conditions that are known to block the degradation induced by mevalonate addition. We further show that intracellular farnesol levels increase significantly after mevalonate addition. Based on these results, we conclude that farnesol is a nonsterol, mevalonate-derived product that plays a role in accelerated reductase degradation. Our conclusion is in agreement with a previous report (Correll, C. C., Ng, L., and Edwards, P. A. (1994) J. Biol. Chem. 269, 17390–17393), in which an in vitro system was used to study the effect of farnesol on reductase degradation. However, the apparent stimulation of degradation in vitro appears to be due to nonphysiological processes. Our findings demonstrate that in vitro, farnesol causes reductase to become detergent insoluble and thus lost from immunoprecipitation experiments, yielding apparent degradation. We further show that another resident endoplasmic reticulum protein, calnexin, similarly gives the appearance of protein degradation after farnesol addition in vitro. However, after the addition of farnesol to cells in vivo, calnexin remains stable, whereas reductase is degraded, providing further evidence that the in vivo effects of farnesol are physiologically meaningful and specific for reductase, whereas the in vitro effects are not.

The isoprenoid metabolic pathway, which leads to the production of cholesterol among other essential cellular products, is tightly regulated at the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate, catalyzed by the enzyme HMG-CoA reductase, a 97-kDa glycoprotein of the endoplasmic reticulum membrane (1, 2). The levels of this enzyme are governed by regulation of transcription (3–5), mRNA translation (6–9), and enzyme degradation (10–13).

We have studied the regulation of the degradation rate of reductase. Although production of sterols has a role in triggering accelerated reductase degradation, it has become clear that an unidentified nonsterol product is necessary for this acceleration to occur. This has been shown by treating cells with a potent competitive inhibitor of reductase, compactin, or by using a cell line, UT2, that lacks HMG-CoA reductase. Under these conditions exogenous cholesterol does not trigger accelerated degradation of reductase, unless the metabolic block is bypassed with exogenous mevalonate (14, 15). The reciprocal relationship also appears to exist that the nonsterol component requires the presence of the sterol regulatory component to cause accelerated degradation. This has been demonstrated using inhibitors of enzymes in the squalene branch of the isoprenoid pathway (16), and in our current study using a cell line deficient in squalene synthase. In both of these instances endogenous production of sterols, but not nonsterol products, was effectively blocked, and in both instances accelerated reductase degradation did not occur. The identity of the nonsterol mevalonate-derived regulatory component, however, has remained unknown. Recently Bradfute and Simoni (17) and others (18) reported that reductase degradation can be accelerated in intact cultured cells by the addition of farnesyl derivatives, which appear to act by mimicking the elusive mevalonate-derived metabolite.

Here we report acceleration of reductase turnover in intact cells by the addition of farnesol. We demonstrate that this regulation is physiologically meaningful by showing that the effect of farnesol is sensitive to inhibitory agents and a mutational condition that are known to stunt the regulatory effect of exogenously added mevalonate. Furthermore we report that intracellular levels of farnesol increase after mevalonate addition, a treatment known to accelerate reductase degradation, and decrease after compactin addition, a treatment that blocks mevalonate production and is known to increase reductase stability.

The possible role of farnesol in regulation of reductase degradation has been recently suggested (19) based on findings in an in vitro system. However, several findings of ours suggest that reductase protein loss in this system is largely due to nonphysiological causes. We show that a significant fraction of reductase protein becomes detergent-insoluble during incubation with farnesol in vitro, causing a depletion of immunoprecipitable reductase and thus the appearance of degradation. Also, our studies of another endoplasmic reticulum resident

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§ The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; CHO, Chinese hamster ovary; SSD, squalene synthase-deficient; HMGal, fusion protein between the membrane domain of HMG-CoA reductase and β-galactosidase; MEM, minimum essential medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ALLN, N-acetyl-Leu-Leu-norleucinal.

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protein, calnexin, reveal that this protein likewise appears to be rapidly lost in permeabilized cells in response to farnesol but actually is largely rendered detergent-insoluble. Neither of these apparently nonphysiological effects occur in intact cells treated with farnesol. Based on our findings, we conclude that farnesol is a likely physiological, nonsteroid regulatory molecule with a critical role in accelerated reductase degradation.

EXPERIMENTAL PROCEDURES

Materials—Minimum essential medium (MEM), fetal calf serum, and trypsin were from Life Technologies, Inc. [3H]trans-[trans-Farnesol (20 Ci/mmol) and [1-35S]farnesyl pyrophosphate (15 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO), and trans-[trans-farnesol was obtained from Aldrich. Tran[s]Label metabolic labeling reagent (>1000 Ci/mmol) was obtained from ICN (Costa Mesa, CA). Castin was the generous gift of Dr. Akira Endo (Tokyo Noko University, Tokyo). Anti-calnexin polyclonal antibodies were kindly provided by Dr. John Bergeron (McGill University, Montreal) and Dr. Ron Kopito (Stanford University). ALLN and thapsigargin were obtained from Calbiochem (San Diego, CA). All other reagents were obtained from Sigma.

Cell Culture—Chinese hamster ovary (CHO) cells were maintained as monolayers in MEM supplemented with 5% fetal calf serum. Squale- lene synthase-deficient CHO (SSD) cells were maintained in the same medium but with 20% fetal calf serum. Unless otherwise indicated, medium was replaced 24 h before experiments with MEM containing 5% lipid poor serum, prepared by the method of Rothblat et al. (20, supplemented with 10 μM compstatin and 100 μM mevalonate.

Measurement of Protein Degradation in Vivo by Pulse-Chase—Cells grown in 60-mm dishes were washed with 4 ml of phosphate-buffered saline. Cells were then incubated for 1 h in 1 ml of methionine-free MEM/1% Triton-poor serum following by 30-min labeling period in 0.3 M of starvation medium containing 333 μCi/mmol Tran[s]Label. This medium was removed, and chase medium (MEM/1% lipid-poor serum containing 2 mM cold methionine) was applied to cells. At various times during the chase period, cells were washed three times with ice-cold PBS and scraped from the dish in 0.7 ml of ice-cold solubilization buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 2 μg/ml calpain inhibitor I). Lysates were centrifuged at 16,000 × g for 30 min at 4 °C. Supernatants were removed, and aliquots were taken for total labeled protein quantitation (see below). Supernatants were incubated overnight with 2 μl of anti-reductase antibody, which was prepared against synthetic peptides corresponding to the membrane domain of reductase (21). Next 20 μl of protein A-Sepharose was added to adsorb immunoprecipitated protein, and then samples were centrifuged 2 min at 16,000 × g, and pellets were rinsed with solubilization buffer followed by 10 μl Tris-Cl, pH 7.5, 0.1% Nonidet P-40. Pellets were dissolved in loading buffer (62.5 mM Tris, pH 6.8, 8% urea, 0.25% sodium dodecyl sulfate, 20% glycerol, 5% [3H]farnesol), incubated 30 min at 37 °C, and subjected to 5–15% gradient polyacrylamide gel electrophoresis (PAGE) for 16 h. The gel was fixed in a 45% methanol, 10% acetic acid solution, impregnated with Enlightning, and then appraised in x-ray film for 5–30 min. Bands were quantified using a phosphorimager system.

Immunoblotting—Proteins were separated by 5–15% gradient PAGE for 1 h and then transferred to a nitrocellulose membrane using a Hoefer TE Series blot transfer unit for 4 h at 300 mA. The membrane was stained with the appropriate primary antibody (anti-reductase or anti-calnexin), washed, and then stained with alkaline phosphatase-conjugated secondary antibody. After additional washes, chemiluminescent substrate (Bio-Rad) was added, and membranes were exposed to x-ray film for 5–30 min. Bands were quantified by densitometry.

Lipid Saponification and Farnesol Extraction—For each experimental condition we used ten 150-mm plates of cells grown to near confluency. Cells were washed with PBS, harvested with trypsin, and resuspended in fresh MEM/1% lipid-poor serum. Cell suspensions were placed in 40 ml of preheated glass centrifuge tubes and centrifuged at 800 × g for 5 min at 4 °C, and then pellets were washed with PBS and resuspended in 3 ml of PBS. A small aliquot was removed for cell number estimation using a Coulter counter and a hemacytometer and also for protein determination by the method of Lowry et al. (22). 0.2 μCi of [3H]farnesol was added as an internal standard to determine recovery, and 1/100 of each sample was reserved for scintillation counting. Next 300 μl of 10 N sodium hydroxide was added, samples were frozen overnight and then thawed, and 15 ml of a 4:1 mixture of ethan:hexanes was added and mixed well. Samples were then heated to 80 °C for 1 h to saponify lipids. Samples were evaporated to 3–4 ml under N2, and then 10 ml water and 10 ml diethyl ether were added and mixed vigorously. After phases separated the ether was removed to another tube. This extraction was repeated twice more, and then 1 g of anhydrous sodium sulfate was added to the pooled ether to remove any traces of aqueous solution. Ether was filtered through cotton and then evaporated under N2 to roughly 0.5 ml. Methanol was added to 10 ml and then evaporated to less than 400 μl under N2. Methanol was added to bring volume to 400 μl, and 1/100 of volume was removed for scintillation counting (Beckman LS-750) to determine recovery. Samples were stored at −20 °C under N2.

Thin Layer Chromatography—[1-3H]Farnesyl pyrophosphate, either subjected to the above saponification procedure or not, as well as [3H]farnesol were spotted onto a 250 μm Silica Gel G plate, and chromatography was performed using a 4:1 mixture of hexanes:ethyl acetate. The plate was dried, and 1-cm increments were scraped into scintillation vials and counted for radioactivity.

High Performance Liquid Chromatography—Extractions from the above saponification procedure and farnesol standards were analyzed by reverse phase HPLC using a Hewlett Packard Amincoquant 1090 Series II, complete with a diode array detector for UV light absorption analysis and outfitted with an Altex UltraSphere C18 column (particle size, 5 μm; internal diameter, 5 μm; mobile phase: 50 μM methanol-acetonitrile gradient, mobile phase with a flow rate of 2 ml/min. For each run, 25 μl of sample was injected, and column output was detected by UV absorbance at 194 nm. Peaks of absorbance were integrated and quantified using the Amincoquant data analysis software and compared with standard curves.

RESULTS

Effect of Farnesol on Degradation of HMG-CoA Reductase in Vivo—Previous work from our laboratory revealed that added farnesyl acetate and farnesyl ethyl ether caused accelerated degradation of reductase in intact CHO cells (17), but no similar effect was found at the concentrations of farnesol used in that study (approximately 5, 50, and 100 μM). In the current study we tested a wider range of farnesol concentrations and found that as the concentration is raised above 20 μM but kept well below the toxic level of 50 μM, added farnesol causes an acceleration of reductase turnover. As shown in Fig. 1, pulse-chase studies revealed that addition of 30 μM farnesol accelerated reductase degradation, with a reduction in half-life from about 9.8 to 5.2 h in six experiments. This effect is comparable with the acceleration induced by added mevalonate under the same experimental conditions (12, 23) and suggests that farnesol, as a natural isoprenoid pathway product, could be a mevalonate-derived, nonsterol regulatory molecule in-
Closedsymbols or the absence (open symbols) of 30 μM farnesol for 0, 4, 6, 8, or 10 h, and then cells were solubilized. Reductase was immuno-precipitated and subjected to SDS-PAGE, and then bands were quantified by Bio-Rad Molecular Imager. The image presented is from one representative experiment of six, and the graph is a compilation of these experiments.

In vivo reductase inhibits the endoplasmic reticulum Ca\(^{2+}\) ATPase transmembrane pump. Both of these agents have been shown to inhibit the effect of mevalonate on reductase degradation (24, 25). As can be seen in Fig. 2, the regulatory effect of farnesol is inhibited by ALLN and by thapsigargin, both used at concentrations known to inhibit mevalonate-induced degradation.

Sensitivity of Farnesol-induced Degradation to Inhibitors of Mevalonate-induced Degradation—To begin to test the hypothesis of farnesol as the nonsterol mevalonate-derived regulator involved in reductase degradation, we utilized inhibitors that are known to blunt the accelerated degradation caused by added mevalonate. If farnesol is a mevalonate-derived regulatory molecule, one would expect that the effect of farnesol would exhibit sensitivity to these agents as well. For these experiments we tested ALLN, an inhibitor of cysteine proteases and the proteasome, and thapsigargin, a Ca\(^{2+}\) perturbant that inhibits the endoplasmic reticulum Ca\(^{2+}\) ATPase transmembrane pump. Both of these agents have been shown to inhibit the effect of mevalonate on reductase degradation (24, 25). As can be seen in Fig. 2, the regulatory effect of farnesol is inhibited by ALLN and by thapsigargin, both used at concentrations known to inhibit mevalonate-induced degradation.

Sensitivity of Farnesol-induced Degradation to a Mutation in Squalene Synthase—Our laboratory previously characterized a mutant CHO cell line lacking the enzyme squalene synthase (26), which catalyzes the condensation of two farnesyl pyrophosphate molecules to form squalene, which is the first step of the sterogenic branch of the mevalonate pathway. Consistent with this previous study, we found that these SSD cells failed to exhibit accelerated degradation of reductase after mevalonate addition (Fig. 3). This cell line thus provided another useful criterion for testing the physiological relevance of the effect of farnesol. As also shown in Fig. 3, the accelerated degradation of farnesol was likewise inhibited in SSD cells as compared with wild type cells. The sensitivity of both mevalonate and farnesol regulation to this mutation lends further credence to the hypothesis that farnesol is a mevalonate-derived nonsterol regulator.

Requirement of Farnesol and Sterols in Causing Regulated Degradation—The primary reason for using SSD cells in this study was as a criterion to determine whether farnesol shows the characteristics expected of a putative mevalonate-derived regulator (see Fig. 3). However, some of our findings in SSD cells had interesting and important implications regarding the issue of a synergistic requirement between the nonsterol and sterol regulators involved in accelerated reductase degradation. Although it is generally well accepted that the sterol component cannot cause accelerated degradation without the nonsterol component (14, 15), previous reports have disagreed whether the nonsterol component reciprocally requires the sterol component. Our group (15) and others (8) have reported that the sterol product is not required, citing that inhibitors within the sterol branch of the isoprenoid pathway do not prevent a mevalonate-induced response. On the contrary, Correll and Edwards (16) have found that inhibitors in the sterol branch do block the effect of mevalonate, suggesting that the sterol component is required. Since the characterization of the SSD mutation (26), it has been clear that added mevalonate has a stunted regulatory effect in these cells. In agreement with Bradfute et al. (26), we found that exogenous 25-hydroxycholesterol triggers accelerated reductase degradation in SSD cells to the same degree as in wild type cells (data not shown). This is not surprising, given that all isoprenoid metabolites prior to squalene synthase, including the putative nonsterol regulator, would be expected to accumulate in SSD cells and...
provide the added sterol component with its necessary non-sterol complement. However, we wished to determine whether exogenous farnesol could amplify the accelerated degradation caused by exogenous 25-hydroxycholesterol in SSD cells. We therefore tested a range of concentrations of this regulatory sterol and found that its effect begins to lose potency below 0.1 μM. But interestingly, as shown in Fig. 4, farnesol acts synergistically with 25-hydroxycholesterol to cause a greater acceleration of reductase degradation, which became clear at sterol concentrations of 0.1 μM or less. Based on these results, we conclude that the nonsterol regulatory product does require sterols in order to cause regulated degradation of reductase.

Furthermore, this cooperativity of added farnesol and added sterols in inducing accelerated reductase degradation further supports the hypothesis of farnesol as a nonsterol regulatory molecule.

Change in Intracelllar Farnesol Levels Following Mevalonate Addition—Although our findings indicate that farnesol satisfies a variety of criteria one would expect of a putative mevalonate-derived nonsterol regulator, we felt it important to determine whether intracellular levels of farnesol increased after mevalonate addition as would be required. For this purpose we utilized HPLC to analyze the farnesol levels in cells treated with 20 mM mevalonate. As shown in Fig. 5, farnesol levels in both CHO and SSD cells increased by approximately 4.5-fold after 3 h of mevalonate treatment. In a separate experiment, a 3-h treatment with 50 μM compactin caused farnesol levels in CHO cells to drop 43% (data not shown). To ensure that the detected levels of farnesol were not partially due to hydrolysis of endogenous farnesyl pyrophosphate during the saponification procedure, we repeated the experiments with radiolabeled farnesyl pyrophosphate added to the cell suspension before saponification of lipids. Thin layer chromatography revealed that just after saponification, all radioactivity comigrated with farnesyl pyrophosphate and not farnesol, and after subsequent ether extraction essentially no radioactivity was present in the extract (data not shown). Although these findings demonstrate that intracellular levels of farnesol increase after the addition of exogenous mevalonate and decrease after blockage of mevalonate production, they admittedly reveal only a correlation between farnesol levels and reductase degradation rate not a cause and effect relationship. However, we feel they are an important contribution to the accumulating body of evidence suggesting that farnesol is a regulator of reductase degradation.

Of further interest is a comparison between intracellular farnesol levels in SSD cells and wild type cells. The initial studies of SSD cells (26) revealed that these cells secrete a considerable amount of farnesol into the culture medium. Consistent with this observation, we found intracellular farnesol levels to be approximately twice as high in SSD cells compared with wild type cells, with or without mevalonate addition (Fig. 5).

Degradation of Reductase In Vitro—We found farnesol to cause an extremely rapid loss of reductase in vitro (data not shown), as was reported by Correll et al. (19). However, this abrupt farnesol-induced loss of reductase protein (t1/2, approximately 1.5 h) was anomalously rapid when compared with farnesol-induced degradation in vivo (t1/2, approximately 5 h, see Fig. 1), and therefore led us to question the physiological significance of this result. The finding that Triton X-100, a
nonionic detergent, also caused very rapid disappearance of reductase in vitro (data not shown) further suggested that the effect caused by farnesol in this permeabilized cell system might not be physiological.

To further examine this matter, we tested another protein as an internal standard to determine whether the effect caused by farnesol in vitro is specific for the loss of reductase. Calnexin, a chaperone protein implicated in protein folding and retention in the endoplasmic reticulum, is a good control based on the fact that, like reductase, it is an integral membrane-spanning resident protein of the endoplasmic reticulum (31). The effect of farnesol on the loss of calnexin was measured both in permeabilized cells and in intact cells. As shown in Fig. 6A, the addition of farnesol to permeabilized cells results in very rapid loss of calnexin protein in a dose-dependent manner. However, following farnesol addition to intact cells, calnexin remains stable, whereas the degradation of reductase is accelerated (Fig. 6B). These results suggest that in vitro, the loss of reductase caused by farnesol is nonspecific and nonphysiological, whereas in vivo the effect is specific for reductase and physiologically meaningful.

Farnesol-induced Detergent Insolubility of HMG-CoA Reductase and Calnexin in Permeabilized Cells—We also utilized a CHO cell line stably transfected with a plasmid encoding the protein HMGal. HMGal is a chimera comprised of the transmembrane regulatory domain of reductase fused to Escherichia coli β-galactosidase, and it has been shown to exhibit regulated turnover in the same manner as endogenous reductase (12, 27).

As can be seen in Fig. 7A, farnesol caused a rapid loss of HMGal protein in vitro. Surprisingly, however, farnesol did not cause a corresponding loss of β-galactosidase activity in the permeabilized cells, as shown in Fig. 7B. In an attempt to resolve these paradoxical findings, we noted that in the β-galactosidase activity determinations the total cell lysate was assayed, whereas in the pulse-chase procedure the lysates were centrifuged at a low speed to remove unlysed cells, nuclei, and detergent-insoluble material prior to immunoprecipitation of reductase. Therefore, following this centrifugation step we assayed the resulting pellet and the supernatant for HMGal activity and found that a significant amount of HMGal activity was detectable in the pellet (Fig. 7C). The amount of activity in the pellet was found to increase with time and with increasing concentrations of farnesol (data not shown). An immunoblot analysis of the two fractions verified that farnesol caused a significant fraction of reductase protein to appear in the pellet and further revealed that a significant fraction of calnexin protein was rendered insoluble by farnesol (Fig. 8). By comparison, we tested lysates from cells treated with farnesol in vivo, and in these virtually all of the HMGal activity was detected in the supernatant and none was detected in the pellet (data not shown), demonstrating that the in vivo effect of farnesol is distinct from the apparently nonphysiological effect responsible for the in vitro results.

**Fig. 5.** Intracellular farnesol levels rise following mevalonate addition. CHO and SSD cells were grown in ten 150-mm plates per sample and then were left untreated (open columns) or treated with 20 mM mevalonate (shaded columns) for 3 h. Cells were then subjected to lipid saponification and extraction as described under “Experimental Procedures.” Farnesol levels were determined by HPLC as described under “Experimental Procedures” using a set of known quantities of farnesol as standards.

**Fig. 6.** Farnesol causes calnexin loss in permeabilized cells but not in intact cells. A, protein loss in vitro is shown. Pulse-labeled CHO cells were permeabilized as described under “Experimental Procedures” and then incubated 4.5 h with 0, 25, 50, or 100 μM farnesol. Calnexin was immunoprecipitated, and samples were subjected to SDS-PAGE. Bands were quantified by Molecular Imager, and values are shown relative to a 100% value at time zero of the chase period. B, protein loss in vivo is shown. Pulse-labeled CHO cells were chased in the absence (open symbols) or the presence (closed symbols) of 30 μM farnesol and then solubilized. Samples were immunoprecipitated with antibodies either to reductase (HMGR, circles) or calnexin (CNX, triangles) and then subjected to SDS-PAGE, and the bands were quantified by Molecular Imager. Reductase and calnexin bands shown are from two different exposure times of the same gel. The antibodies used to immunoprecipitate calnexin were successfully characterized in an immuno inhibition experiment using a peptide corresponding to the calnexin epitope (data not shown).
DISCUSSION

Studies aimed toward determining the identity of the regulatory nonsterol, mevalonate-derived metabolite involved in HMG-CoA reductase degradation have been subject to certain limitations when performed in living cells. This is because many of these candidate pathway metabolites, such as isopentenyl pyrophosphate, geranyl pyrophosphate, and farnesyl pyrophosphate, are polar molecules and are thus not permeant to cells. This has led to the employment of other strategies, one of which is the use of nonpolar artificial isoprenoid analogues, which were added to intact cells to determine whether reductase degradation could be accelerated, presumably by these artificial products mimicking some natural products.

This approach showed some success, as farnesylated tocopherol analogs (18) and farnesyl acetate and farnesyl ethyl ether (17) were shown to accelerate reductase degradation in vivo. These results suggested that some farnesyl metabolite(s) in the isoprenoid pathway is the regulatory molecule.

Another strategy has involved the use of permeabilized cells, which do not present a permeability barrier. We showed previously (13) that mevalonate-induced accelerated reductase degradation persisted in cells after permeabilization with digitonin, but only if cells were pretreated with mevalonate before permeabilization. Presumably this period was necessary for production/accumulation of adequate levels of the mevalonate-derived regulator(s) so that regulated degradation could be underway at the time of permeabilization. Using a modification of our permeabilized cell system, Correll et al. (19) were able to demonstrate apparent accelerated degradation of reductase by addition of farnesol, without the pretreatment period required for mevalonate.

Farnesol is produced from farnesyl pyrophosphate in cells, in a reaction catalyzed by an allyl pyrophosphatase (28–30), which diverts some farnesyl pyrophosphate from its primary metabolic route toward cholesterol. We had not tested farnesol in our permeabilized cell system (13), and in the studies of farnesyl acetate in vivo (17), farnesol had been tested but at a very limited set of concentrations, which in hindsight were either too low to elicit a regulatory response or so high that toxicity resulted. In the current study we have more rigorously tested farnesol in vivo, using concentrations low enough to be nontoxic to cells but higher than those previously found to be ineffectual and have found that farnesol indeed causes accelerated degradation of reductase. This is an important finding, because it is the first evidence of a natural nonsterol mevalonate-derived metabolite causing this effect when added to cells in vivo. Also of importance are our findings that this effect of exogenous farnesol in accelerating reductase degradation can be blocked by ALLN, by thapsigargin, and in a CHO cell line missing the enzyme squalene synthase (see Figs. 2 and 3).

Accelerated degradation by exogenous mevalonate has been shown to be sensitive to all three of these conditions, so our hypothesis regarding the role of farnesol is supported by these findings.

Although the in vitro results of Correll et al. (19) were controlled for degradation of total cell protein in their study, for the following reasons we feel that their reported loss of reductase in vitro is not due to physiologically meaningful causes. One, the farnesol-induced degradation of reductase in permeabilized cells is extremely rapid, considerably more so than observed in mevalonate-pretreated permeabilized cells (13) or even intact cells treated with mevalonate or farnesol. Two, in order to achieve significant acceleration of reductase loss in
vitro, farnesol must be used at concentrations of 50 μM and higher, which are levels we have found to be highly toxic when added to intact cells. Three, our studies show that farnesol causes a second resident endoplasmic reticulum membrane protein, calnexin, to be rapidly lost in permeabilized cells. This loss of calnexin is not observed in farnesol-treated intact cells. Four, our studies show that farnesol, when added to permeabilized cells, causes a significant fraction of reductase (and calnexin) to become detergent insoluble, and therefore not recoverable in the usual immunoprecipitation from cell lysates. Although the amount of reductase and calnexin protein that is rendered detergent insoluble by farnesol is significant, it does not account for the total amount of each protein that is lost (compare fig. 8 with Figs. 7A and 6A). We suggest that a certain degree of nonspecific proteolysis occurs in vitro, perhaps brought about by farnesol disrupting the endoplasmic reticulum.

We believe that our findings, which demonstrate physiologically meaningful reductase degradation in vivo by a natural mevalonate product, serve as an important extension of previous in vivo studies utilizing farnesyl analogs and show that farnesol is a nonsterol mevalonate product with a key role in the regulated degradation of reductase.

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