Identification of Farnesol as the Non-sterol Derivative of Mevalonic Acid Required for the Accelerated Degradation of 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase*

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The degradation of the microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is highly regulated and is dependent on both a sterol and non-sterol derivative of mevalonic acid (MVA). We recently proposed that the non-sterol component is derived from farnesyl diphosphate (FPP), presqualene pyrophosphate, or squalene (Correlli, C. C. and Edwards, P. A. (1994) J. Biol. Chem. 269, 633–638). In the current study, we have used digitonin-permeabilized cells to further define this MVA-derived non-sterol component required for the regulated degradation of HMG-CoA reductase. The addition of either FPP or farnesol to digitonin-permeabilized cells resulted in a rapid and dose-dependent degradation of HMG-CoA reductase. The effect of FPP, but not farnesol, was blocked by the phosphatase inhibitor sodium fluoride. The enhanced degradation of HMG-CoA reductase in permeabilized cells specifically required farnesol, since the addition of any of the structurally related isoprenoids geraniol, geranyl diphosphate, gerrnylgeranyl diphosphate, and all-cis-farnesol, or of the non-sterol squalene to the permeabilized cells did not stimulate enzyme degradation. The present studies demonstrate for the first time that the accelerated degradation of HMG-CoA reductase can be initiated in vitro. Further, since farnesol is shown to be specifically required for the enhanced degradation of the enzyme in vitro, we propose that this isoprenoid alcohol is important in this process in intact cells.

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase catalyzes the reduction of HMG-CoA to mevalonic acid (MVA) (1,2). The expression and cellular activity of HMG-CoA reductase are regulated at many levels including transcription, translation, thiol status of the cell, enzyme phosphorylation, and enzyme stability (1,2). The active enzyme is a 77-kDa glycoprotein that is localized to the endoplasmic reticulum (3). A short non-conserved sequence links the multiple transmembrane domains to the highly conserved catalytic domain, which extends out into the cytosol (3,4). The transmembrane domains of HMG-CoA reductase are necessary for the regulated degradation of the enzyme (5,6) that is known to occur in the endoplasmic reticulum (7–9). This latter conclusion is based on studies using brefeldin A (7), permeabilized cells (8,9), and isolated microsomes (9). However, the specific role that these domains play in this degradative process is not understood.

HMG-CoA reductase has a relatively long half-life (9–11 h) in cells that have been deprived of MVA and sterols (9–13). In contrast, cells incubated in the presence of high levels of MVA and sterols exhibit a 3–5-fold increase in the rate of degradation of HMG-CoA reductase (9,11,12). This rapid and specific degradation has been demonstrated to be dependent on both a sterol and a non-sterol derivative of MVA (9,13). However, it has also been proposed that the non-sterol can activate enzyme degradation in the absence of excess sterols (14). Neither of these derivatives of MVA have been identified. Recent studies have shown that the addition of tocotrienols (15) or either farnesyl acetate or ethyl farnesyl ether (16) to intact cells results in stimulated degradation of HMG-CoA reductase. It was proposed that these compounds may act as mimetics of the physiological regulatory isoprenoid (15,16). Based on studies utilizing inhibitors of squalene synthase and squalene epoxidase, we recently proposed that the non-sterol derivative of MVA required for the degradation of HMG-CoA reductase is derived from either farnesyl diphosphate (FPP), presqualene pyrophosphate, or squalene (9).

In the current studies, we have utilized a digitonin-permeabilized cell system and have been able to identify farnesol as the non-sterol derivative that can specifically initiate and promote the degradation of HMG-CoA reductase in permeabilized cells.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine lipoprotein-deficient serum (LPDS) was purchased from Organon Teknika Biotechnology Research Institute. Trypsin was purchased from Life Technologies, Inc. Tran32P-label was purchased from ICN Flow. Digitonin and Pansorbin were purchased from Calbiochem. Digitonin was dissolved in dimethyl sulfoxide (Me2SO) and stored at −20°C. Farnesol, geraniol, nerolidol, and squalene were purchased from Sigma. All-trans-geranylgeraniol, geranyl diphosphate, and all-cis-farnesol were generous gifts from Kuraaya Co., Japan, Dr. H. C. Rilling (University of Utah), and Dr. Johan Ericsson, respectively. Farnesyl diphosphate and geranylgeranyl diphosphate were synthesized as described (17). All other materials are from commercially available sources or have been given previously (9).

Tissue Culture—Met-18b-2 cells were stably transfected with a β-galactosidase expression plasmid (CMV-β-gal) (18) and grown as described (9).

Cell Permeabilization by Digitonin—Met-18b-2 cells were permeabilized with 60 μg/ml digitonin as described by Plutner et al. (19) with minor modifications. The permeabilized cells obtained from the 100-mm dish were resuspended in 175 μl of KH buffer (90 mM potassium acetate and 50 mM Hepes, pH 7.2). Cell permeabilization was confirmed by release of soluble enzymes from the cell and by loss of the ability to exclude trypan blue. Preliminary experiments utilized digitonin at concentrations ranging from 10 to 80 μg/ml. The value of 60 μg/ml digitonin used in all subsequent experiments was chosen based on the release of cytosolic enzymes, trypan blue staining of the cells, and the

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This paper is dedicated to Dr. George Poppik on his 80th birthday.

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The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; FPP, farnesyl diphosphate; LPDS, lipoprotein-deficient serum.
were solubilized in radioimmune precipitation buffer and used for the outlined under "Experimental Procedures" in the absence or presence of Ham's F-12 medium containing 10% LPDS and 5 µM mevinolin. Where appropriate, the overnight incubation was followed by metabolic labeling of the cells for 3 h in the presence of 10% LPDS, 50 µg/ml mevinolin, and 100 µCi/ml Tran35S-label. Equal aliquots of cells were treated as outlined under "Experimental Procedures" in the absence or presence of 60 µg/ml digitonin. Permeabilization was verified by the inability of greater than 98% of the cells to exclude trypan blue. Phosphoglucone isomerase and β-galactosidase activities were assayed as described under "Experimental Procedures." Radiolabeled and permeabilized cells were solubilized in radioimmune precipitation buffer and used for the immunoprecipitation of HMG-CoA reductase or the determination of trichloroacetic acid-precipitable proteins as described under "Experimental Procedures." Control values of 100% were determined from intact cells, which had not been treated with digitonin. The data presented are from three identical experiments (average ± standard deviation). TCA, trichloroacetic acid.

general morphology of the permeabilized cells. Enzyme Assays—β-Galactosidase (20) and phosphoglucone isomerase (21) activities were assayed as described. Measurement of Protein Degradation in Permeabilized Cells—Met-18b-2 cells were grown to confluence in 100-mm dishes and radiolabeled as described (9). After 3 h, the cells were washed twice with phosphate-buffered saline and permeabilized as described above. The radiolabeled cell suspension (170 µl) was diluted with 425 µl of Buffer A (50 mM HEPES, pH 7.2, 178 mM potassium acetate, 5.9 mM magnesium acetate, 4.27 mM calcium chloride, 0.6 mg/ml soybean trypsin inhibitor, 5.9 mM methionine/cysteine, 60 µg/ml leupeptin, 9.4 µM reduced glutathione). Aliquots of cells (24 µl) were transferred to microcentrifuge tubes and supplemented with an NADPH-generating system (5.1 mM glucose 6-phosphate, 2.0 mM NADP, 2.0 mM NAD, and 0.2 units of glucose-6-phosphate dehydrogenase) up to a final volume of 39 µl. The in vitro degradation assay was initiated by the addition of 1 µl of carrier or of various isoprenoids in Me2SO or isoprenoid diphosphates in 2 mM ammonium hydroxide, followed by incubation at 37 °C.

At the end of each incubation period, the permeabilized cells were solubilized in 1 ml of ice-cold radiimmune precipitation buffer and HMG-CoA reductase was immunoprecipitated and analyzed as described (9).

The incorporation of Tran35S-label into total cellular proteins was quantified by precipitation of proteins with trichloroacetic acid followed by scintillation counting.

RESULTS AND DISCUSSION

Met-18b-2 Cells Permeabilized by Digitonin Release Cytosolic Enzymes but Retain Membrane-bound HMG-CoA Reductase—Digitonin permeabilization of Met-18b-2 cells that stably express β-galactosidase resulted in a greater than 90% decrease in the cell-associated activity of the cytosolic enzymes phosphoglucone isomerase and β-galactosidase as compared to cells that had not been treated with digitonin (Fig. 1). Further, digitonin permeabilization of cells radiolabeled with Tran35S-label resulted in more than 50% loss of trichloroacetic acid-precipitable protein (Fig. 1). In contrast, there was no significant loss in the level of radiolabeled HMG-CoA reductase immunoprecipitated from digitonin-treated cells as compared to untreated cells (Fig. 1). These results indicate that permeabilization of Met-18b-2 cells with digitonin at 60 µg/ml results in almost complete loss of cytosolic macromolecules without a significant decrease in the cell-associated level of membrane-bound HMG-CoA reductase.

FPP or Farnesol Stimulates the Degradation of HMG-CoA Reductase in Permeabilized Cells—Cells were radiolabeled, permeabilized with digitonin, and incubated at 37 °C as described under "Experimental Procedures." The amount of radiolabeled HMG-CoA reductase declined less than 25% when the permeabilized cells were incubated for 4 h under standard conditions that included either an NADPH-generating system (Figs. 2-4), HMG-CoA (0.5 mM), or NADPH and HMG-CoA (data not shown). However, omission of both enzyme substrates (NADPH and HMG-CoA) resulted in a variable loss of immunoprecipitable HMG-CoA reductase, over the 4 h, that ranged from 20 to 50% (data not shown). The finding that either of the substrates of HMG-CoA reductase increases the stability of the protein in permeabilized cells may indicate that protein conformation plays a role in the susceptibility of the enzyme to degradation. All subsequent degradation assays contained the NADPH-generating system.

In agreement with earlier studies (8, 9), incubation of permeabilized cells for 4 h in the presence of rat liver cytosol (0.5 mg/ml) or an ATP-generating system did not significantly affect the amount of radiolabeled HMG-CoA reductase protein (data not shown).

Addition of either FPP or farnesol, the dephosphorylated form of FPP, to digitonin-permeabilized cells resulted in rapid degradation of HMG-CoA reductase protein in a dose-dependent manner (Fig. 2). The addition of FPP or farnesol, at a
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Previously, permeabilized cells have been used to demonstrate that, following the initiation of HMG-CoA reductase degradation in intact cells, the accelerated degradation of the enzyme persists in permeabilized cells in the absence of ATP or cytosol (8, 9). However, it is unclear why it was not possible to initiate the degradation of HMG-CoA reductase in the permeabilized cell systems used in these prior studies (8, 9). One earlier report demonstrated that the addition of ATP to permeabilized cells resulted in the accumulation of a 68-kDa fragment of HMG-CoA reductase (23). However, since normal degradation of this enzyme in vivo does not lead to the accumulation of proteolytic fragments (9, 11–13, 22) and since ATP is not necessary for either the initiation or the continued degradation of HMG-CoA reductase (8, 9) (Figs. 2–4), the relevance of this finding to normal enzyme degradation is not clear.

The Degradation of HMG-CoA Reductase in Permeabilized Cells Is Specific for Farnesol—The FPP-induced, but not the farnesol-induced, degradation of HMG-CoA reductase was blocked by the addition of sodium fluoride, a general phosphatase inhibitor (Fig. 4). These data suggest that FPP is converted to farnesol in permeabilized cells, that this conversion is inhibited by sodium fluoride, and that farnesol is the active component necessary for the degradation of HMG-CoA reductase. The finding that the farnesol-induced degradation occurred with no lag period (Fig. 3), but that the addition of either FPP to permeabilized cells (Fig. 3) or MVA to intact cells (9, 11, 13) was followed by a 1–2-h lag period, is also consistent with farnesol having a more direct role in enzyme degradation.

In order to further define the specificity of MVA-derived components necessary for the degradation of HMG-CoA reductase, permeabilized cells were incubated in the presence of other isoprenoid derivatives having structural similarities to the 15-carbon isoprenol farnesol. The degradation of HMG-CoA reductase was not stimulated by the addition of 50 μM geraniol, squalene, the farnesol isomers all-cis-farnesol or norerolidol (Fig. 4), geranyl diphosphate, or geranylgeranyl diphosphate (data not shown). Geranylgeraniol at a concentration of 50 μM resulted in variable increases in the degradation of HMG-CoA reductase; as compared to controls, the loss of immunoprecipitable protein varied from 0 to 40% (Fig. 4 and data not shown). However, in all studies the loss of HMG-CoA reductase protein was 2–3-fold greater in permeabilized cells incubated with farnesol as compared to cells incubated with geranylgeraniol (Fig. 4 and data not shown).

Thus, the accelerated degradation of HMG-CoA reductase is specifically dependent on the correct isomer form of the fifteen carbon isoprenol, farnesol. Furthermore, since the addition of isoprenols having structural similarities to farnesol did not induce the degradation of HMG-CoA reductase, it is unlikely that farnesol was acting as a nonspecific detergent to promote the proteolysis of the protein (Fig. 4). The finding that geranylgeraniol could partially mimic the effect of farnesol (Fig. 4) would also be consistent with a specific protein-isoprenol interaction. This lack of complete specificity may be analogous to the situation known to exist with protein prenyltransferases; the enzymes are specific for either FPP or geranylgeranyl diphosphate but can, under non-physiological in vitro conditions, utilize either substrate (24). Taken together, all the present data indicate that farnesol is specifically required for the accelerated degradation of HMG-CoA reductase.

Other indirect support for the role of farnesol comes from
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REFERENCES


studies in which the activity of cholesterogenic enzymes distal to farnesyl diphosphate synthase were reduced as a result of a mutation (25) or the addition of nonspecific inhibitors of the cholesterol biosynthetic pathway (16). Under these conditions farnesol levels increased in the media, presumably as a result of dephosphorylation of FPP and secretion of the resulting alcohol (16, 25). This increase in farnesol and other sterol intermediates was accompanied by an increase in the degradation of HMG-CoA reductase (16). The current studies do not address the role of the sterol component in the enzyme degradation. Since both a sterol and non-sterol derivative of sterols may be involved in the regulated degradation of sterols may indicate that intracellular pools of sterols change following permeabilization of the cells.

A number of proteins, including the α chain of the T cell receptor (26), the asialoglycoprotein receptor (27), apoprotein B (28), and HMG-CoA reductase (7-9), are known to be degraded in the endoplasmic reticulum. Of these, only HMG-CoA reductase is a normal resident protein of the endoplasmic reticulum whose rate of degradation is physiologically regulated. The current studies indicate that farnesol plays a role in this regulated degradation; farnesol may intercalate into endoplasmic reticulum membrane and interact with HMG-CoA reductase, thereby altering the enzyme conformation and result in degradation of the protein. Further studies utilizing the current permeabilized cell system should allow us to define the mechanism involved in the regulated degradation of HMG-CoA reductase.