# Insig-mediated degradation of HMG CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol

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## Summary

**Feedback control of cholesterol synthesis is mediated in part by sterol-induced binding of HMG CoA reductase to Insig proteins in the endoplasmic reticulum (ER). Binding leads to ubiquitination and proteasomal degradation of reductase, a rate-controlling enzyme in cholesterol synthesis. Using in vitro and in vivo assays, we show that lanosterol, the first sterol intermediate in cholesterol synthesis, potently stimulates ubiquitination of reductase, whereas cholesterol has no effect at 10-fold higher concentrations. Lanosterol is not effective in mediating the other action of Insigs, namely to promote ER retention of SCAP-SREBP complexes, a reaction that is mediated directly by cholesterol. A pair of methyl groups located in the C4 position of lanosterol confers this differential response. These data indicate that buildup of cholesterol synthesis intermediates represses the pathway selectively at reductase and reveal a previously unappreciated link between feedback inhibition of reductase and carbon flow through the cholesterol synthetic pathway.**

## Introduction

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG CoA reductase) catalyzes the reduction of HMG CoA to mevalonate (Figure 1), a rate-limiting step in the synthesis of cholesterol and nonsterol isoprenoids (Goldstein and Brown, 1990). HMG CoA reductase is anchored to membranes of the endoplasmic reticulum (ER) through its hydrophobic $NH_2$-terminal domain, which consists of eight membrane-spanning regions separated by short loops (Roitelman et al., 1992). The membrane domain of reductase precedes a large, cytosolic COOH-terminal domain that exerts all of the catalytic activity of the enzyme (Gil et al., 1985; Liscum et al., 1985). The levels and activity of reductase are governed by a complex, multivalent regulatory system mediated by products of mevalonate metabolism (Brown and Goldstein, 1980). Part of this regulatory system involves sterol-induced ubiquitination of reductase (Ravid et al., 2000), a reaction that is mediated by the reductase membrane domain and leads to its ER-associated degradation (Sever et al., 2003a, 2003b).

As demonstrated by three observations, accelerated degradation of reductase requires at least one of a pair of ER membrane proteins called Insig-1 and Insig-2. First, when reductase is overexpressed in Chinese hamster ovary (CHO) cells by transfection, the enzyme is no longer subjected to sterol-accelerated degradation (Sever et al., 2003b). Overexpression of Insig-1 or Insig-2 restores regulated degradation of overexpressed reductase. Second, through the use of RNA interference, the combined knockdown of Insig-1 and Insig-2 abolishes sterol-induced ubiquitination of endogenous reductase, an obligatory reaction in accelerated degradation of the enzyme (Sever et al., 2003a). Third, in mutant cells lacking Insig-1, sterols fail to stimulate sterol-dependent ubiquitination and degradation of reductase (Sever et al., 2004).

Insigs also mediate sterol regulation of another ER membrane protein called SREBP cleavage-activating protein (SCAP) (Yang et al., 2002). SCAP controls the activity of SREBPs, transcription factors that enhance transcription of all of the genes required for cholesterol synthesis, as well as the LDL receptor (Brown and Goldstein, 1999). Excess sterols promote the ER retention of complexes between SCAP and SREBPs, thus preventing their translocation to the Golgi where the SREBPs are activated by sequential proteolytic cleavages (DeBose-Boyd et al., 1999; Nohturfft et al., 2000). As a result of this ER retention, mRNAs encoding SREBP target genes decline and cholesterol synthesis is suppressed. Insigs bind to SCAP through a region of the protein that comprises five of its eight membrane-spanning helices (Nohturfft et al., 1998; Yang et al., 2002), and the amino acid sequence of this region bears significant identities with the corresponding region of reductase. This region has become known as the sterol-sensing domain (Brown and Goldstein, 1999). Point mutations within their sterol-sensing domains prevent SCAP and reductase from binding to Insigs, thereby abolishing sterol-mediated ER retention of mutant SCAP-SREBP complexes and sterol-dependent ubiquitination and degradation of reductase (Sever et al., 2003a; Yabe et al., 2002; Yang et al., 2002).

The oxygenated cholesterol derivative, 25-hydroxycholesterol (25-HC), has been utilized as a general tool in dissecting the mechanism for sterol regulation, owing to its ability to potently stimulate binding of both SCAP and reductase to Insigs. The potency of 25-HC and other oxysterols raises the intriguing question as to whether an oxygenated metabolite of cholesterol or cholesterol itself is the physiologic mediator of sterol regulation.

In the case of SCAP, support for a direct role of cholesterol in its regulation has been provided by several observations. The in vitro addition of cholesterol to isolated membranes induced conformational changes in SCAP that exposed a cryptic trypsin cleavage site (Brown et al., 2002), and coexpression

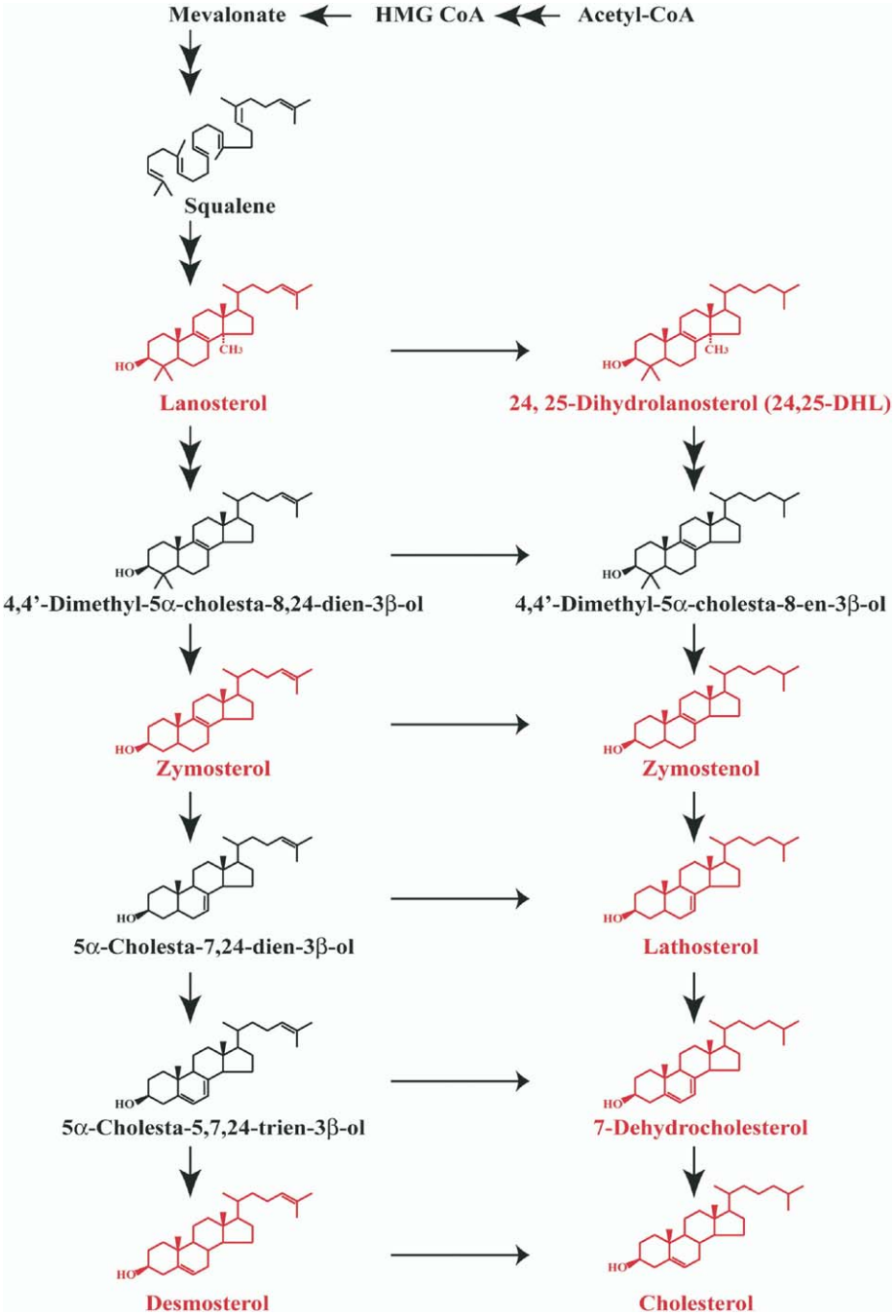



**Figure 1**. Schematic representation of the cholesterol biosynthetic pathway in animal cells

Sterols evaluated for HMG CoA reductase-ubiquitinating activity are highlighted in red.

of Insigs significantly lowered the concentration of cholesterol required to induce the response (Adams et al., 2003). Recently, it has been demonstrated that [$^3$H]cholesterol binds to the membrane domain of SCAP in detergent solution. The binding of cholesterol was stereospecific and saturable, indicating that it modulates SCAP activity through direct interaction (Radhakrishnan et al., 2004). Oxysterols neither altered SCAP conformation in vitro nor bound the purified membrane domain of SCAP, indicating that oxysterols act through an indirect mechanism to block SCAP activity. This conclusion is supported by recent experiments demonstrating that a photoactivated derivative of cholesterol, but not that of 25-HC, crosslinked to the membrane domain of SCAP (Adams et al., 2004).

In striking contrast to the results with SCAP, analysis of reductase ubiquitination in vitro revealed that the reaction was potently stimulated by oxysterols but not by cholesterol (Song and DeBose-Boyd, 2004). These results suggest that oxysterols derived from cholesterol, but not cholesterol itself, are physiologic regulators of reductase degradation. Furthermore, these findings indicate that selective recognition of sterol ligands by SCAP and reductase may contribute to the ability of Insigs to mediate regulation of both proteins through distinct

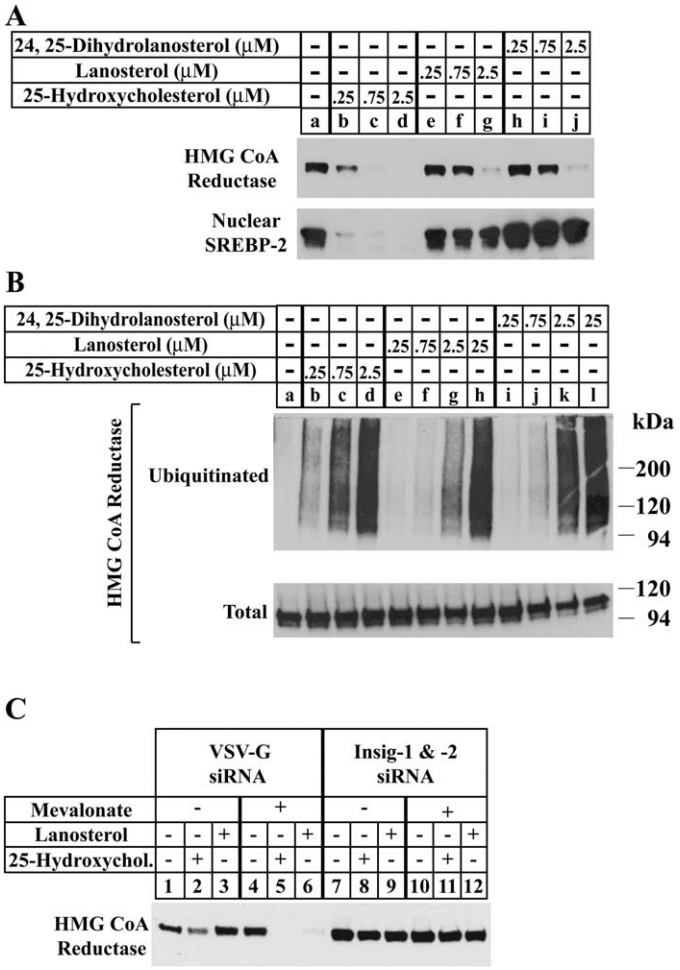



**Figure 2**. Lanosterol and 24,25-dihydrolanosterol stimulate degradation and ubiquitination of HMG CoA reductase, but do not inhibit processing of SREBPs, through an Insig-dependent mechanism

**A** and **B)** SV-589 cells were set up for experiments on day 0 at 2 × $10^5$ cells per 100-mm dish in medium A supplemented with 10% FCS. On day 2, the cells were washed with PBS and refed medium A containing 10% lipoprotein-deficient serum, 50 μM sodium compactin, and 50 μM sodium mevalonate. In (**B**), the medium also contained 10 μM MG-132.

**A)** After 16 hr at 37°C, the cells were switched to medium A containing 10% lipoprotein-deficient serum, 50 μM compactin, 10 mM mevalonate, and the indicated concentration of 25-hydroxycholesterol, lanosterol, or 24,25-dihydrolanosterol. After incubation for 5 hr at 37°C, the cells were harvested and subjected to cell fractionation. Aliquots of the membrane and nuclear extract fractions (10–45 μg protein/lane) were subjected to SDS-PAGE, transferred to nylon membranes, and immunoblot analysis was carried out with 5 μg/ml monoclonal IgG-A9 (against reductase) or 5 μg/ml monoclonal IgG-1D2 (against SREBP-2). The filters were exposed to film at room temperature for 2–30 s.

**B)** After incubation for 1 hr at 37°C, the cells were harvested, lysed, and subjected to immunoprecipitation with polyclonal anti-HMG CoA reductase as described in Experimental Procedures. Aliquots of the immunoprecipitates were subjected to SDS-PAGE, transferred to nylon membranes, and immunoblot analysis was carried out with 5 μg/ml IgG-A9 (against reductase) or 0.2 μg/ml monoclonal IgG-P4D1 (against ubiquitin). Filters were exposed to film for 2 s–3 min at room temperature.

**C)** SV-589 cells were set up for experiment at 5 × $10^4$ cells per 60-mm dish in medium B with 10% FCS. On days 1 and 3, cells were transfected with 400 pmol/dish of VSV-G siRNA (lanes 1–6) or Insig-1 and Insig-2 siRNA (lanes 7–12) as described in Experimental Procedures. After the second transfection on day 3, cells were incubated for 16 hr at 37°C in medium A containing 10% lipoprotein-deficient serum, 50 μM compactin, and 50 μM mevalonate. On day 4, the cells were switched to medium A containing 10% lipoprotein-deficient serum, 50 μM compactin in the absence (–) or presence (+) of various combinations of 2.5 μM 25-hydroxycholesterol, 2.5 μM lanosterol, and 10 mM mevalonate as indicated. After 5 hr, cells were harvested for preparation of whole-cell extracts. Aliquots of the extracts (63 μg) were subjected to SDS-PAGE and immunoblot analysis was carried out with 5 μg/ml IgG-A9 (against reductase). The filter was exposed to film at room temperature for 15 s.

mechanisms. It is noteworthy that the 1,1-bisphosphonate ester SR-12813 differentially replaces sterols to promote reductase ubiquitination and degradation, but not ER retention of SCAP (Sever et al., 2004), lending credence to the notion of selective recognition of sterols by SCAP and reductase.

Previous indirect studies implicated that lanosterol, the first sterol produced in the synthesis of cholesterol (Figure 1), or one of its metabolites play a major role in feedback inhibition of reductase (Chen et al., 1988; Frye et al., 1994; Leonard et al., 1994; Trzaskos, 1995). These observations prompted us, in the current paper, to directly evaluate lanosterol as a regulator of reductase ubiquitination and degradation. When added to intact cells, lanosterol stimulated ubiquitination and degradation of reductase in a reaction that required Insigs. The activity of lanosterol in accelerating reductase ubiquitination and degradation appeared specific inasmuch as it did not suppress SREBP processing. This is consistent with the inability of lanosterol to directly bind to the membrane domain of SCAP (Radhakrishnan et al., 2004). In vitro, lanosterol promoted reductase ubiquitination as efficiently as oxysterols and its activity was traced to a pair of C4 methyl groups. Taken together, the current results demonstrate a direct role for lanosterol as a selective, physiologic regulator of reductase ubiquitination and degradation.

## Results

The experiment shown in Figure 2A was designed to determine the effects of lanosterol, and its 24,25-reduced metabolite 24,25-dihydrolanosterol (24,25-DHL) (Figure 1), on the steady-state levels of reductase and nuclear SREBP-2. SV-589 cells, a line of transformed human fibroblasts (Yamamoto et al., 1984), were depleted of sterols by incubation for 16 hr in lipoprotein-deficient serum containing the reductase inhibitor, compactin (Brown et al., 1978), and the lowest level of mevalonate (50 μM) that assures viability. Subsequent to sterol depletion, the cells were treated for an additional 5 hr with various concentrations of 25-HC, lanosterol, and 24,25-DHL. In addition, all of the cells received a high level of mevalonate (10 mM) to provide nonsterol mevalonate metabolites that augment sterol-accelerated degradation of reductase (Sever et al., 2003a). Following treatments, the cells were harvested and separated into membrane and nuclear extract fractions. Aliquots of the fractions were then subjected to SDS-PAGE and immunoblotted with anti-reductase (Figure 2A, top panel) and anti-SREBP-2 (Figure 2A, bottom panel) monoclonal antibodies. In untreated cells, we observed bands of immunoreactive protein for reductase (top panel, lane a) and nuclear SREBP-2 (bottom panel, lane a). In a dose-dependent fashion, 25-HC accelerated both the degradation of reductase, as indicated by the disappearance of the reductase band in membranes isolated from treated cells (top panel, lanes b–d), and the disappearance of nuclear SREBP-2 (bottom panel, lanes b–d). Similarly, lanosterol and 24,25-DHL treatment led to a decrease of anti-reductase reac-

tivity (top panel, lanes e–j); however, neither sterol blocked the processing of SREBP-2 (bottom panel, lanes e–j). In addition, lanosterol and 24,25-DHL also failed to inhibit the processing of SREBP-1 (unpublished data).

We next evaluated lanosterol and 24,25-DHL for the ability to promote ubiquitination of reductase, a reaction that is essential for its accelerated degradation (Sever et al., 2003a) (Figure 2B). Sterol-depleted SV-589 cells were incubated with various concentrations of 25-HC, lanosterol, and 24,25-DHL in the presence of the proteasome inhibitor, MG-132 (to prevent degradation of ubiquitinated reductase). Following treatments, the cells were harvested, lysed in detergent-containing buffer, and the lysates were subjected to immunoprecipitation with polyclonal antibodies against reductase. The resulting immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti-ubiquitin (top panel) or anti-reductase (bottom panel) monoclonal antibodies. When the cells were treated with 25-HC, reductase became ubiquitinated in a dose-dependent fashion. This was indicated by the appearance of high molecular weight smears in anti-ubiquitin immunoblots of the reductase immunoprecipitates (top panel, lanes b–d). Lanosterol and 24,25-DHL stimulated ubiquitination of reductase, although with reduced efficiency (approximately 3- and 10-fold for 24,25-DHL and lanosterol, respectively) (compare lanes b–d with e–l). Thus, the reductase-ubiquitinating activity of the methylated sterols coincides with their ability to diminish steady-state levels of the enzyme.

We next addressed the requirement of Insig-1 and Insig-2 for lanosterol-dependent downregulation of endogenous reductase by reducing their expression through RNA interference. In the experiment of Figure 2C, SV-589 cells were transfected with duplexes of small-interfering RNA (siRNA) targeting the control gene, vesicular stomatitis virus glycoprotein (VSV-G), which is not expressed in the cells, or the combination of Insig-1 and Insig-2. Subsequent to sterol depletion, the transfected cells were treated for 5 hr with 25-HC or lanosterol in the absence or presence of 10 mM mevalonate. In control transfected cells, the combination of 25-HC and 10 mM mevalonate led to complete disappearance of reductase (lane 5), and similar results were obtained with lanosterol plus 10 mM mevalonate (Figure 2C, lane 6). However, when siRNAs targeting Insig-1 and Insig-2 were introduced into the cells, the reductase band persisted regardless of treatment with either sterol (lanes 7–12). Under these conditions, we observed a 90% and 60% decrease in mRNA for Insig-1 and Insig-2, respectively (unpublished data). The requirement for nonsterol mevalonate-derived products in downregulating reductase indicates that 25-HC and lanosterol act to stimulate degradation of the enzyme through a common, Insig-dependent mechanism (lanes 1–6). Moreover, unpublished transfection studies revealed that lanosterol-stimulated degradation of reductase required Insigs and amino acid residues in reductase necessary for the covalent attachment of ubiquitin and Insig binding (Sever et al., 2003a).

We recently developed a permeabilized cell system that supports ubiquitination of reductase stimulated by the in vitro addition of sterols (Song and DeBose-Boyd, 2004). Thus, we next sought to determine whether the permeabilized cell system could also support lanosterol-dependent ubiquitination of reductase (Figure 3A). SV-589 cells were depleted of sterols so as to provide an abundant supply of nonubiquitinated reductase. Following sterol depletion, the cells were harvested, washed, and permeabilized with a low concentration of the mild detergent digitonin. We previously found that permeabilizing cells in this manner resulted in the release of a substantial portion of cytosolic proteins into the supernatant after centrifugation, while membrane proteins such as reductase remained associated with the pellet fraction (Song and DeBose-Boyd, 2004). Pellets of permeabilized cells were subsequently subjected to in vitro treatments with a mixture of FLAG-tagged ubiquitin, ubiquitin-aldehyde (to inhibit de-ubiquitinating enzymes) (Hershko and Rose, 1987), an ATP-regenerating system, and various concentrations of 25-HC, lanosterol, 24,25-DHL, or cholesterol. The reactions were also supplemented with rat liver cytosol to facilitate the activation of FLAG-ubiquitin and provide factors necessary for possible modifications required for the reductase-ubiquitinating activity of lanosterol and 24,25-DHL. Following incubation at 37°C, pellets of permeabilized cells were collected by centrifugation, solubilized in detergent-containing buffer, and subjected to immunoprecipitation with polyclonal antibodies against reductase. The resulting immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti-FLAG (Figure 3A, top panel) or anti-reductase (Figure 3A, bottom panel). In reactions containing 25-HC, reductase became ubiquitinated in a dose-dependent fashion (top panel, lanes b–d), and similar results were obtained with lanosterol and 24,25-DHL (top panel, lanes e–g and h–j, respectively). Cholesterol only modestly stimulated the reaction and was at least 10-fold less potent than lanosterol and 24,25-DHL (top panel, lanes k and l). Importantly, it should be noted that while lanosterol and 24,25-DHL were found to be 3- to 10-fold less potent than 25-HC in stimulating reductase ubiquitination in intact cells (see Figure 2), in permeabilized cells, the activities of both methylated sterols were equivalent to or greater than that of 25-HC.

We next determined whether other intermediates of cholesterol synthesis could promote ubiquitination of reductase in permeabilized cells. Of particular interest were zymosterol and zymostenol, which are demethylation products of lanosterol and 24,25-DHL, respectively (Figure 1). As shown in Figure 3B, lanosterol and 24,25-DHL were the only cholesterol synthesis intermediates that stimulated reductase ubiquitination to appreciable levels (top panel, lanes 5 and 6). In addition, zymosterol, zymostenol, desmosterol, and 7-dehydrocholesterol neither promoted reductase ubiquitination and degradation nor inhibited SREBP processing in intact cells (unpublished data). Thus, the failure of zymosterol and zymostenol to influence reductase ubiquitination in permeabilized cells establishes the importance of C4 and/or C14 methylation in the recognition of lanosterol and 24,25-DHL by the regulatory system that mediates ubiquitination and degradation of reductase.

Previously, we found that a specific set of oxysterols stimulated reductase ubiquitination when added in vitro at concentrations less than 25 μM (Song and DeBose-Boyd, 2004). The experiment of Figure 4B shows an extended analysis of the sterol specificity for reductase ubiquitination in permeabilized cells (see Figure 4A). Of the sterols tested, only 25-HC, lanosterol, 27-hydroxylanosterol, 7-keto-25-hydroxycholesterol, and 27-hydroxycholesterol stimulated reductase ubiquitination to appreciable levels (Figure 4B, top panels, lanes A, B, C, E, and H). In particular, 27-hydroxylanosterol strongly stimulated reductase ubiquitination, prompting us to compare its ubiquiti-

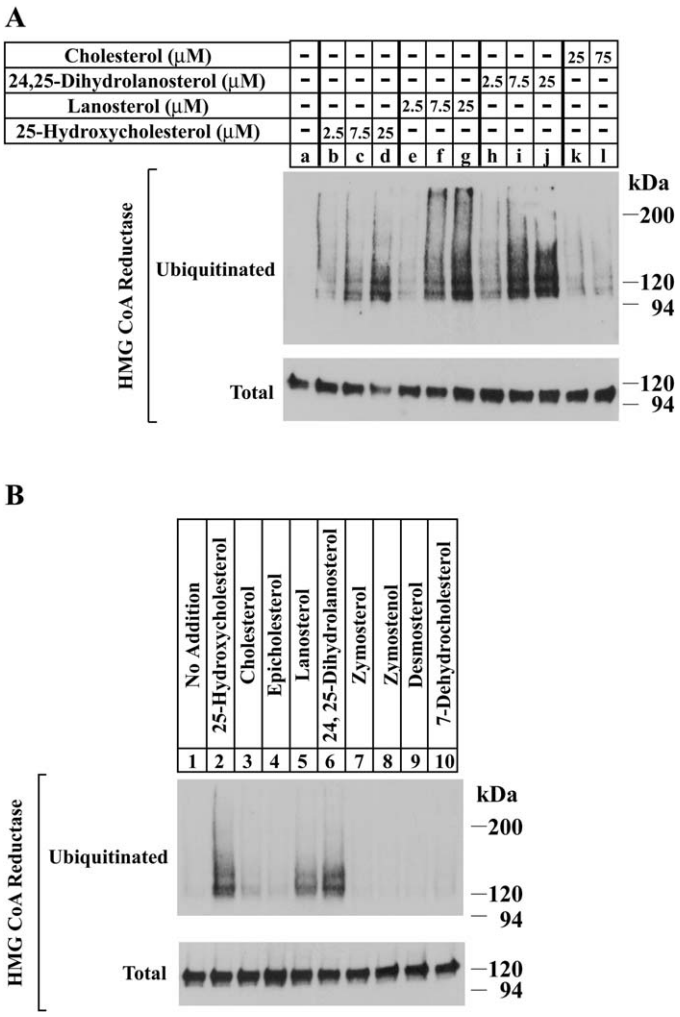



**Figure 3**. Lanosterol and 24,25-dihydrolanosterol promote ubiquitination of HMG CoA reductase in permeabilized cells

SV-589 cells were set up and refed as described in the legend to Figure 2. On day 3, the cells were harvested and permeabilized with digitonin as described in Experimental Procedures. The permeabilized cells were resuspended in permeabilization buffer containing protease inhibitors, an ATP-regenerating system, 0.1 mg/ml FLAG-ubiquitin, 0.01 mg/ml ubiquitin-aldehyde, and 3 mg/ml rat liver cytosol.

**A)** Reactions containing the indicated concentration of 25-hydroxycholesterol (lanes b–d), lanosterol (lanes e–g), 24,25-dihydrolanosterol (lanes h–j), or cholesterol (lanes k–l) were incubated for 30 min at 37°C, after which they were terminated by centrifugation for 10 min at 4°C. The resulting cell pellets were lysed and subjected to immunoprecipitation with polyclonal anti-HMG CoA reductase as described in Experimental Procedures. Aliquots of the immunoprecipitates were subjected to SDS-PAGE, transferred to nylon membranes, and immunoblotted with 5 μg/ml IgG-A9 (against reductase) or 1 μg/ml monoclonal IgG-M2 (against FLAG-ubiquitin). Filters were exposed to film at room temperature for 30 s.

**B)** Reactions containing 25 μM of the indicated sterol were carried out for 30 min at 37°C, after which they were terminated by centrifugation. The resulting cell pellets were lysed and subjected to immunoprecipitation and immunoblot analysis as in (**A**). Filters were exposed to film at room temperature for 1–30 s.

nating activity at lower concentrations to that of other positive sterols at 25 μM. As shown in Figure 4C, 2.5 μM 27-hydroxylanosterol stimulated approximately equivalent levels of reductase ubiquitination as 25 μM lanosterol (top panel, compare lanes 3 and 7). Together, these results indicate that hydroxyl groups in the sterol side chain and methyl groups at the C4 and/or C14 position are key determinants for recognition in the sterol-sensing phase of reductase degradation.

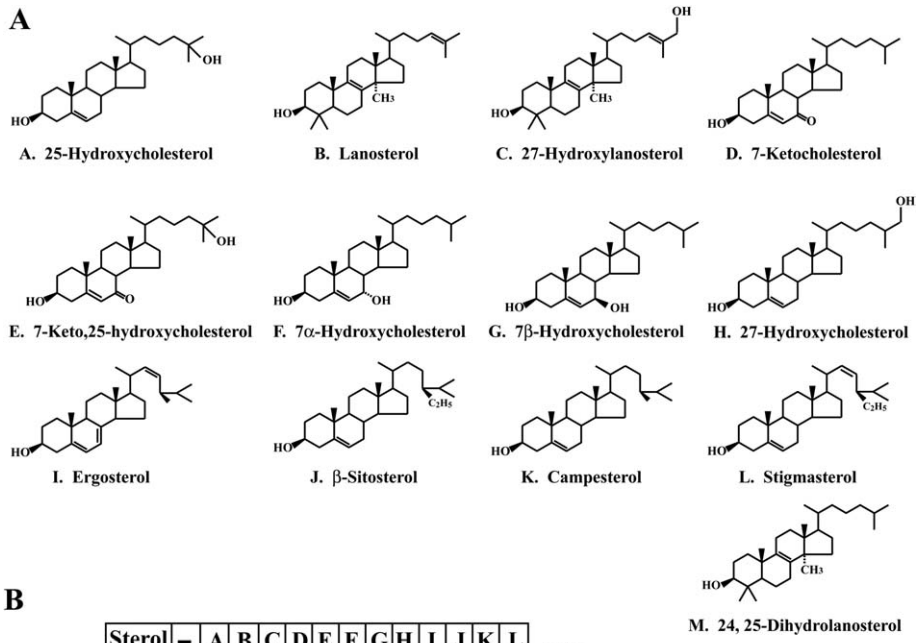
We next designed experiments to determine the relative contribution of the methyl groups in lanosterol for stimulating reductase ubiquitination and degradation. For this purpose, we compared the activities of several sterols with or without C4 methyl substitutions to those of lanosterol and 24,25-DHL (Figure 5A). In intact cells, the 4,4-dimethylated analog of cholesterol (4,4-dimethylcholesterol) stimulated reductase ubiquitination (Figure 5B, top panel, lane E) and degradation (Figure 5C, lane E) with an activity approximately equivalent to that of lanosterol and 24,25-DHL (Figures 5B and 5C, lanes B and C). Reduced levels of reductase ubiquitination and degradation were observed with C4 dimethylated cholestenone (Figures 5B and 5C, lane I), suggesting that optimal activity requires a hydroxyl group at the 3β position in addition to the 4,4-dimethyl moiety. Lophenol, which contains a single methyl group in the C4 position, modestly promoted the ubiquitination and degradation of reductase (Figures 5B and 5C, lane G), highlighting the importance of the two C4 methyl groups. Importantly, 4,4-dimethylcholesterol, 4,4-dimethylcholestenone, and lophenol did not inhibit processing of SREBP-2 (Figure 5D, lanes E, G, and I).

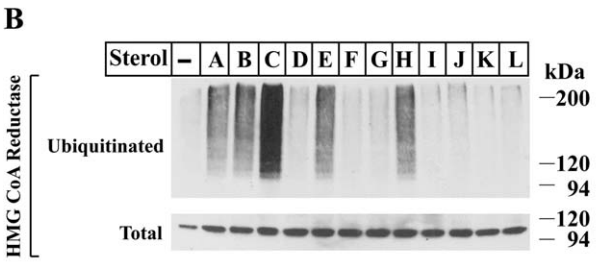
We next evaluated the methylated sterols for their ability to stimulate reductase ubiquitination in vitro. Ubiquitination reactions of isolated membrane fractions were supplemented with purified ubiquitin-activating enzyme (E1), rather than rat liver cytosol, as a source of ubiquitin-activating activity (Song and DeBose-Boyd, 2004). As depicted in Figure 5E, 4,4-dimethylcholesterol was a potent agonist of E1-mediated reductase ubiquitination with an efficiency similar to 25-HC, lanosterol, and 24,25-DHL (top panel, compare lane E with lanes A–C). As we observed in intact cells, the activities of lophenol and 4,4-dimethylcholestenone in promoting the ubiquitination of reductase were reduced in comparison to 4,4-dimethylcholesterol (top panel, lanes G and I, respectively).

Finally, we sought to determine whether protein phosphorylation plays a role in regulated ubiquitination of reductase. In the experiment of Figure 6, ATP and its nonhydrolyzable analog, adenylyl-imidodiphosphate (AMP-PNP), were utilized as energy sources in ubiquitination reactions conducted with isolated membranes. In the absence of an energy source, no ubiquitinated reductase was observed upon the addition of 25-HC, lanosterol, or 24,25-DHL to reaction mixtures (top panel, lanes 1–4). Regulated ubiquitination of reductase was restored when the reactions were supplemented with ATP (top panel, lanes 5–8) or AMP-PNP (top panel, lanes 9–12). Given that AMP-PNP allows ubiquitin activation by E1 (Johnston and Cohen, 1991; Pickart et al., 1994) but cannot function as a protein kinase substrate, these results indicate that neither 25-HC nor methylated sterols require protein phosphorylation in order to stimulate ubiquitination of reductase in vitro.

## Discussion

The current results provide evidence for a major role of the cholesterol synthesis intermediate lanosterol in posttranscriptional regulation of HMG CoA reductase. When delivered to intact SV-589 cells, lanosterol triggered rapid degradation and



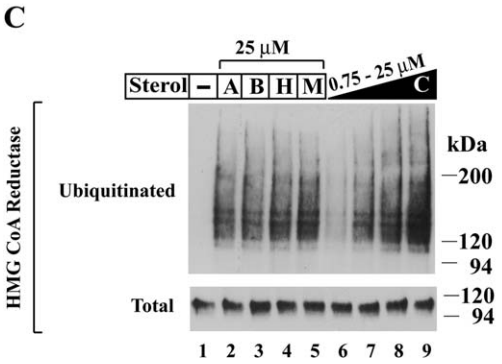




**Figure 4**. Sterol specificity for HMG CoA reductase ubiquitination in permeabilized cells

**A)** Structures of the sterols evaluated for stimulating ubiquitination of HMG CoA reductase in (**B**) and (**C**). **B** and **C)** SV-589 cells were set up and refed as described in the legend to Figure 2. On day 3, the cells were harvested and permeabilized with digitonin as described in Experimental Procedures. The permeabilized cells were resuspended in permeabilization buffer containing protease inhibitors, an ATP-regenerating system, 0.1 mg/ml FLAG-ubiquitin, 0.01 mg/ml ubiquitin-aldehyde, and 3 mg/ml rat liver cytosol. Incubations were carried out for 30 min in the presence of 25 μM of the indicated sterol in (**B**). In (**C**), reactions contained 25 μM 25-hydroxycholesterol, lanosterol, 27-hydroxycholesterol, and 24,25-dihydrolanosterol or 0.75, 2.5, 7.5, and 25 μM 27-hydroxylanosterol as indicated. After 30 min at 37°C, the reactions were terminated by centrifugation. The resulting cell pellets were lysed and subjected to immunoprecipitation and immunoblot analysis as described in the legend to Figure 3. Filters were exposed to film at room temperature for 2–20 s.

ubiquitination of reductase (Figures 2A and 2B). The characteristics of lanosterol-mediated regulation of reductase mimicked those of 25-HC with the following respects: (1) nonsterol end products of mevalonate metabolism augmented both lanosterol- and 25-HC-dependent degradation of reductase (Figure 2C, lanes 1–6) and (2) the combined knockdown of Insig-1 and Insig-2 abolished 25-HC and lanosterol-mediated degradation of reductase (Figure 2C, lanes 7–12). Despite its potent activity in promoting reductase degradation, lanosterol was not effective in suppressing SREBP processing in intact cells as did 25-HC (Figure 2). The possibility remains that in permeabilized cells, lanosterol could influence the activity of SCAP with much lower potency than that for reductase. Unfortunately, the permeabilized cell system is not amenable to the study of SREBP processing or sterol-mediated retention of SCAP-SREBP. However, the failure of lanosterol to modulate SREBP processing in intact cells is consistent with its inability to directly bind the SCAP membrane domain (Radhakrishnan et al., 2004) and modulate SCAP conformation in vitro (Brown et al., 2002).

The potency of lanosterol-induced ubiquitination of reductase was 3- to 10-fold less than that of 25-HC in intact cells (Figure 2B). We reasoned that in aqueous culture medium, the reduced solubility of the methylated sterols in comparison to 25-HC limits their access to membranes of intracellular organelles. In light of this, lanosterol and 24,25-DHL should achieve a higher effective concentration when delivered directly to ER membranes in vitro and thus exhibit an enhanced potency. We found this to be the case as the methylated sterols stimulated reductase ubiquitination as potently as 25-HC when added to permeabilized cells and isolated membranes (Figures 3 and 5).

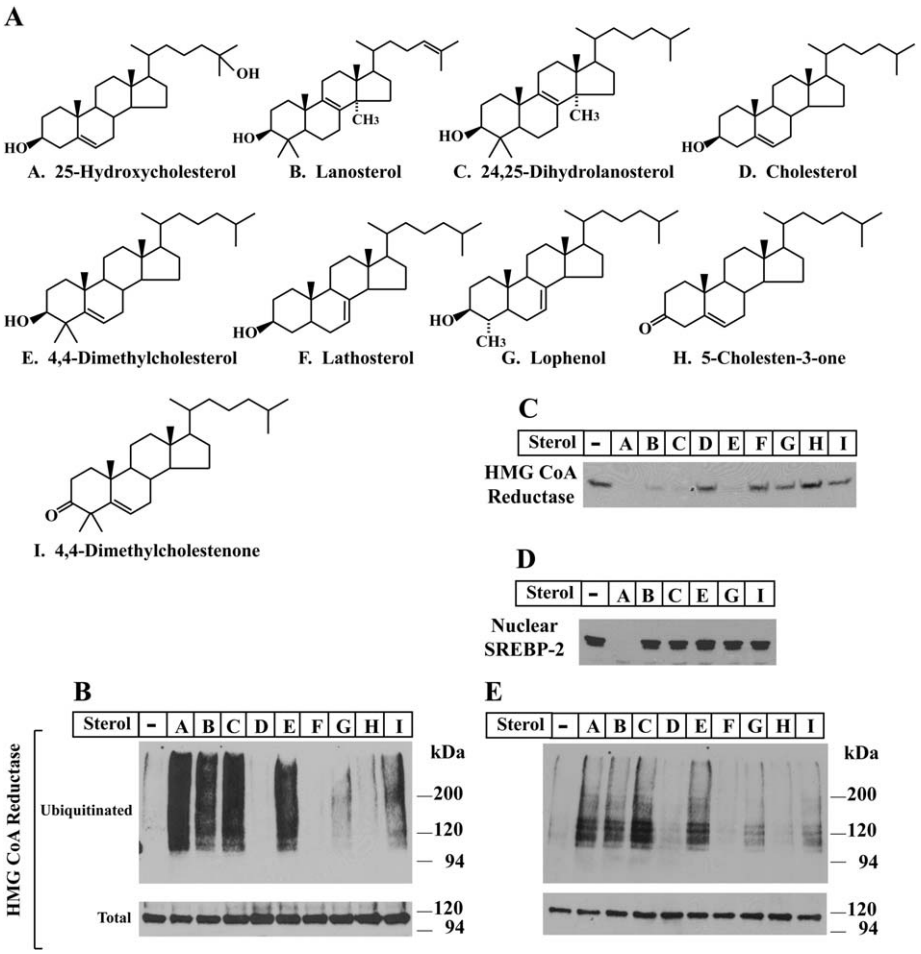



**Figure 5**. 4,4-dimethyl moiety of lanosterol is sufficient to promote ubiquitination and degradation of HMG CoA reductase

**A)** Structures of the sterols evaluated for stimulating ubiquitination of HMG CoA reductase in (**B**)–(**E**). SV-589 cells were set up and refed as described in the legend to Figure 2.

**B–D)** On day 3, the cells were switched to medium A containing 10% lipoprotein-deficient serum, 50 μM compactin, either 10 μM MG-132 (**B**) or 10 mM mevalonate (**C** and **D**), and 2.5 μM of the indicated sterol. (**B**) Following incubation at 37°C for 1 hr, the cells were harvested, lysed, and subjected sequentially to immunoprecipitation, SDS-PAGE, and immunoblot analysis as described in the legend to Figure 2. Filters were exposed to film for 1 s. (**C** and **D**) Following incubation at 37°C for 5 hr, the cells were harvested and sequentially subjected to cell fractionation. Aliquots of the membrane fractions (**C**, 10 μg protein/lane) and nuclear extracts (**D**, 35 μg protein/lane) were subjected to SDS-PAGE and immunoblot analysis as described in the legend to Figure 2. Filters were exposed to film at room temperature for 1 s–2 min.

**E)** On day 3, the cells were harvested, washed, and subjected to cell fractionation as described in Experimental Procedures. Aliquots of isolated membrane fractions were resuspended in permeabilization buffer containing protease inhibitors, the ATP-regenerating system, 0.1 mg/ml FLAG-ubiquitin, 0.01 mg/ml ubiquitin aldehyde, and 5 μg/ml purified ubiquitin-activating enzyme. Incubations were carried out at 37°C in the absence (–) or presence (+) of 25 μM of the indicated sterol. After 30 min, the reactions were terminated by centrifugation, and the resulting cell pellets were subjected to immunoprecipitation and immunoblot analysis as described in the legend to Figure 4. Filters were exposed to film at room temperature for 15 s–5 min.

The 4,4-dimethyl moiety of lanosterol appears to be the major determinant for reductase regulation as indicated by the ability of 4,4-dimethylcholesterol, but not cholesterol, to promote reductase ubiquitination in intact cells and in vitro (Figure 5). On the other hand, the 3β-hydroxyl group seems to play a secondary role in recognition of lanosterol since 4,4-dimethylcholestenone, which contains a 3-keto group in place of the 3β-hydroxyl group, exhibited reduced reductase-ubiquitinating activity in comparison to 4,4-dimethylcholesterol. The importance of 4,4-dimethylation is somewhat surprising considering that a comparative analysis of the molecular structures of cholesterol and lanosterol reveals that lanosterol is structurally flatter than cholesterol in its hydrophobic region (Miao et al., 2002). In both sterols, this region consists of a planar steroid ring and a short hydrocarbon tail, and in particular, the C14 methyl group of lanosterol protrudes from the steroid ring. While evidence for the participation of C14 methylation is lacking, the ability of 4,4-dimethylcholesterol to stimulate reductase ubiquitination indicates that the C14 methyl group plays a minor role in the process.

Stimulation of reductase ubiquitination by lanosterol could be mediated solely by ubiquitin-activating enzyme (E1) (Figure 5D). Thus, lanosterol does not require modifications by cytosolic enzymes in order to stimulate in vitro ubiquitination of reductase, indicating that lanosterol is directly recognized in the sterol-sensing reaction. This is an important conclusion in view of previous studies demonstrating that oxylanosterols, endogenous oxysterols generated during the demethylation of lanosterol, can also accelerate reductase degradation (Frye et al., 1994; Trzaskos, 1995). Inasmuch as oxylanosterols are transient intermediates in lanosterol demethylation, they would not be expected to accumulate within cells, suggesting that their role in the feedback control of reductase would be minor at best. Moreover, in vitro, E1-mediated ubiquitination of reductase was stimulated by lanosterol in reactions that were not supplemented with NADPH, an essential cofactor required for the generation of oxylanosterols (Gaylor, 2002).

The lack of cytosol requirement also indicates that lanosterol does not require presentation to membranes by cytosolic proteins in order to promote reductase ubiquitination, supporting our previous notion that the sterol-sensing mechanism occurs at the membrane (Song and DeBose-Boyd, 2004). Interestingly, the sterol 27-hydroxylanosterol was found to be the most potent of all sterols tested for reductase ubiquitination. The methylation and side chain hydroxylation of 27-hydroxylanosterol appear to act synergistically in promoting reductase ubiquitination, indicating that side chain hydroxylation is a third structural feature recognized by the reductase sterol-sensing machinery. It should be noted that while C4-dimethylated sterols are specific for reductase, side chain hydroxylated sterols regulate

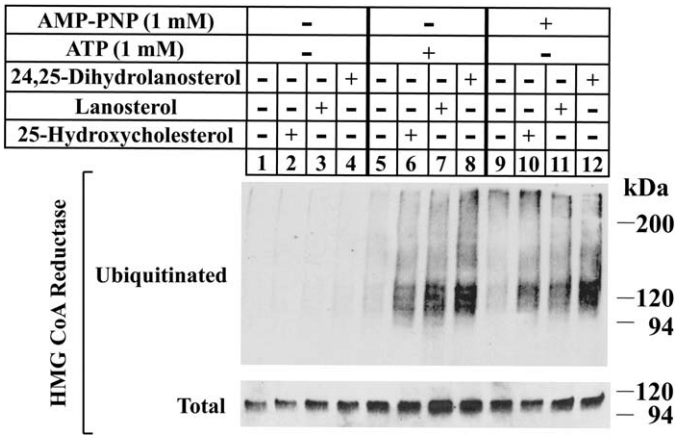



**Figure 6**. Protein phosphorylation is not a requirement for sterol-dependent ubiquitination of HMG CoA reductase in vitro

On day 0, SV-589 cells were set up and refed as described in the legend to Figure 2. On day 3, the cells were harvested, washed, and subjected to cell fractionation as described in Experimental Procedures. Aliquots of isolated membrane fractions were resuspended in permeabilization buffer containing protease inhibitors, 0.1 mg/ml FLAG-ubiquitin, 0.01 mg/ml ubiquitin aldehyde, 5 μg/ml purified ubiquitin-activating enzyme, and 1 mM ATP or AMP-PNP substituted for the ATP-regenerating system. Incubations were carried out at 37°C in the absence (–) or presence (+) of 25 μM of the indicated sterol. After 30 min, the reactions were terminated by centrifugation, and the resulting cell pellets were subjected to immunoprecipitation and immunoblot analysis as described in the legend to Figure 3. Filters were exposed to film at room temperature for 3–15 s.

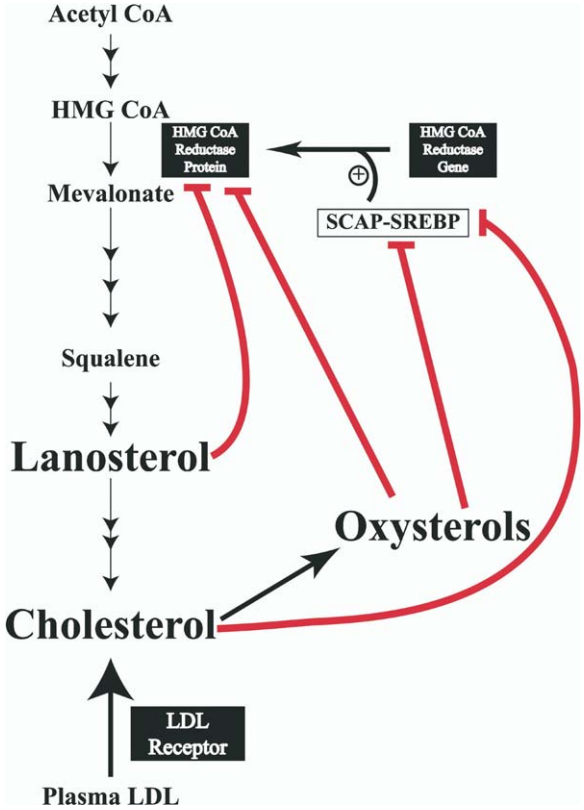



**Figure 7**. Model for sterol-mediated regulation of HMG CoA reductase

As discussed in the text, HMG CoA reductase is subject to feedback inhibition by cholesterol, oxysterols, and methylated sterols such as lanosterol. Cholesterol derived either from endogenous synthesis or via LDL receptors inhibits reductase activity by suppressing the activation of SREBPs through SCAP. Oxysterols derived from the conversion of endogenous and/or exogenous cholesterol inhibit reductase by accelerating its degradation and by suppressing the activation of SREBPs. Lanosterol, through its C4-dimethylation, downregulates reductase solely by accelerating degradation of the enzyme. Through these reactions, cholesterol synthesis is maintained such that important by-products of the synthetic pathway are continually provided, while at the same time avoiding the toxic overaccumulation of cholesterol.

both reductase and SCAP. It is presently unclear whether sterol-sensing involves direct binding of methylsterols and oxysterols to reductase itself or to another membrane bound protein that in turn acts on the reductase. Furthermore, it is unclear as to whether oxysterols and methylated sterols are recognized through the same mechanism in the sensing reaction. Interestingly, 25-HC and cholesterol appear to modulate SCAP through distinct mechanisms: oxysterols act indirectly through another protein, whereas cholesterol acts by directly binding to SCAP (Adams et al., 2004). Perhaps 25-HC and other oxysterols act on reductase and SCAP through a shared mechanism while cholesterol and lanosterol act by direct binding to the membrane domains of SCAP and reductase, respectively. Efforts are currently being undertaken to resolve these issues and characterize the reductase sterol-sensing reaction in more detail.

The importance of reductase to the maintenance of cholesterol homeostasis is highlighted by its tight control through a complex, multivalent regulatory system (Brown and Goldstein, 1980; Goldstein and Brown, 1990). Sterol and nonsterol byproducts of the cholesterol biosynthetic pathway reduce activity of reductase by inhibiting transcription of the reductase gene, blocking translation of reductase mRNA, and accelerating degradation of the reductase protein. While the mechanism for the translational regulation of reductase is unknown, the current results provide a clearer understanding of how cells coordinate transcriptional control of reductase, which is mediated by SCAP-SREBP, with the regulation of reductase stability.

Figure 7 shows a model for Insig-dependent regulation of reductase mediated by three types of sterols: oxysterols, cholesterol, and methylated sterols such as lanosterol. Oxysterols derived either by the conversion of endogenous or LDL-derived cholesterol downregulate reductase through two mechanisms: accelerated degradation of the reductase protein and inhibition of reductase gene transcription by blocking the ER to Golgi transport of SCAP-SREBP. Cholesterol does not influence reductase stability directly but acts indirectly to downregulate reductase by blocking the activity of SCAP-SREBP. On the other hand, lanosterol selectively accelerates the degradation of reductase without effect on ER to Golgi transport of SCAP-SREBP.

The aforementioned findings are significant in the following respects. First, the selective recognition of cholesterol and lanosterol by SCAP and reductase, respectively, helps to explain how Insigs mediate regulation of both proteins through distinct mechanisms. Second, the current results point to the synthesis of lanosterol, the first sterol produced in the cholesterol synthetic pathway, as a key focal point in sterol regulation. Lanosterol demethylation has been implicated as a rate-limiting step

in cholesterol synthesis (Spence and Gaylor, 1977; Williams et al., 1977), and it seems reasonable for lanosterol to inhibit its own synthesis through the downregulation of reductase, thus reducing flux through the sterol pathway. It should be noted that a nonsterol mevalonate metabolite, most likely derived from geranylgeranyl pyrophosphate (Sever et al., 2003a), enhances sterol-accelerated degradation of reductase. Thus, an intricate link between reductase stability and carbon flux through the cholesterol biosynthetic pathway is attainted, and we envision this process to be the first in a series of reactions that guard against sterol overaccumulation. The accumulation of lanosterol is prevented, owing to its inability to mediate suppression of SREBP processing through SCAP. Under such conditions, mRNAs encoding enzymes that catalyze reactions subsequent to lanosterol synthesis remain elevated, allowing metabolism of lanosterol to cholesterol. The necessity of this conversion is highlighted by the ability of cholesterol, rather than lanosterol, to optimize certain physical properties of cell membranes with regard to biological functions (Miao et al., 2002; Smondyrev and Berkowitz, 2001). Finally, the specificity of lanosterol for reductase may permit the identification of proteins selectively recruited to the reductase-Insig complex.

The novel behavior of lanosterol and 24,25-DHL in uncoupling regulation of reductase from the regulation of SCAP-SREBP is important from clinical as well as the aforementioned biological perspectives. Statins, a group of competitive inhibitors of reductase, reduce the incidence of heart attacks and prolong the lives of subjects with pre-existing coronary artery disease (Heart Protection Study Collaborative Group, 2002; Scandinavian Simvastatin Study Group, 1994). However, this inhibition reduces mevalonate-derived products that govern reductase activity through the multivalent feedback mechanism. The absence of such regulatory molecules contributes to a major increase in the amount of active reductase in statin-treated animals (and presumably humans) that becomes progressively harder to inhibit (Kita et al., 1980; Singer et al., 1984). In theory, this compensatory increase of reductase should be reduced by an agent that accelerates the degradation of the accumulated enzyme. Thus, understanding how C4-dimethylation of lanosterol specifically triggers reductase degradation, but not ER retention of SCAP-SREBP (which ultimately governs LDL receptor expression), may lead to the development of novel hypocholesterolemic agents. Considering the recent recommendation for more aggressive cholesterol-lowering strategies (Grundy et al., 2004), these new agents may be utilized to potentiate the therapeutic effectiveness of statins.

## Experimental procedures

### Materials

We obtained MG-132 and digitonin from Calbiochem; horseradish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgG (affinity purified) from Jackson ImmunoResearch Laboratories; ubiquitin, ubiquitin-aldehyde, and ubiquitin-activating enzyme from Boston Biochem (Cambridge, Massachusetts); sterols (see Figures 1, 4, and 5) from Steraloids, Inc. (Newport, Rhode Island), Research Plus, Inc. (South Plainfield, New Jersey), and Sigma; and $NH_2$-terminal FLAG-tagged ubiquitin from Sigma. Other reagents were obtained from described sources (DeBose-Boyd et al., 1999). Lipoprotein-deficient serum (d > 1.215 g/ml) was prepared from newborn calf serum by ultracentrifugation (Goldstein et al., 1983).

27-hydroxylanosterol (25R,26-hydroxylanosterol) was synthesized from lanosterol (Sigma-Aldrich, St. Louis, Missouri) by selective selenium dioxide oxidation using minor modifications of previous procedures (Camps et al., 1978). The final product has a mass spectrum identical to that of the naturally occurring diol (Pikuleva and Javitt, 2003), and NMR analysis (Byron Arison) confirmed the stereospecificity of the 25R,26-hydroxylation.

### Cell culture

Monolayers of SV-589 cells, an immortalized line of human fibroblasts expressing the SV40 large T antigen (Yamamoto et al., 1984), were maintained in tissue culture at 37°C in 5% $CO_2$. Stock cultures of SV-589 cells were grown in medium A (Dulbecco's modified Eagle's medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) fetal calf serum (FCS).

### Cell fractionation and immunoblot analysis

Pooled cell pellets were used to isolate nuclear extracts, $2 \times 10^5$ g membrane fractions, or whole-cell lysate fractions, and subjected to 8% SDS-PAGE and immunoblot analysis as previously described (Sever et al., 2003b). Primary antibodies used for immunoblotting were as follows: mouse monoclonal anti-T7-Tag ($IgG_{2b}$) (Novagen); mouse monoclonal anti-Myc (IgG fraction) from the culture medium of hybridoma clone 9E10 (American Type Culture Collection); IgG-A9, a mouse monoclonal antibody against the catalytic domain of hamster HMG CoA reductase (amino acids 450–887) (Liscum et al., 1983); IgG-1D2, a mouse monoclonal antibody against the $NH_2$ terminus of human SREBP-2 (amino acids 48–403) (Yabe et al., 2002); IgG-P4D1, a mouse monoclonal antibody against bovine ubiquitin (Santa Cruz Biotechnology); and IgG-M2, a mouse monoclonal antibody against the FLAG epitope (Sigma).

### Ubiquitination of HMG CoA reductase in intact cells

The conditions of incubations prior to harvesting of cells are described in the figure legends. At the end of the incubations, the cells were harvested, lysed in detergent-containing buffer, and immunoprecipitations were carried out with polyclonal antibodies directed against the 60 kDa COOH-terminal domain of human HMG CoA reductase as previously described (Sato et al., 1993; Sever et al., 2003a). Aliquots of the immunoprecipitates were subjected to 6% SDS-PAGE, transferred to nylon membranes, and subjected to immunoblot analysis.

### Ubiquitination of HMG CoA reductase in permeabilized cells and isolated membranes

The conditions of incubations prior to harvesting of cells are described in the figure legends. SV-589 cells were harvested into the medium by scraping and collected by centrifugation, after which pooled cell pellets from triplicate dishes were washed with phosphate-buffered saline and either permeabilized with 0.025% (w/v) digitonin or subjected to cell fractionation for the isolation of membrane fractions as described above. Permeabilized cells and membranes were resuspended in 300 μl and 150 μl, respectively, of permeabilization buffer (25 mM Hepes-KOH at pH 7.3, 115 mM potassium acetate, 5 mM sodium acetate, 2.5 mM magnesium chloride, 0.5 mM sodium EGTA) containing protease inhibitors (20 μM leupeptin, 10 μM MG-132, 5 μg/ml pepstatin A, and 2 μg/ml aprotinin), an ATP-regenerating system (2 mM Hepes-KOH at pH 7.3, 1 mM magnesium acetate, 1 mM sodium ATP, 30 mM creatine phosphate, and 0.05 mg/ml creatine kinase), 0.1 mg/ml FLAG-ubiquitin, and 0.01 mg/ml ubiquitin-aldehyde. The source of ubiquitin-activating activity was provided by the addition of either 3 mg/ml rat liver cytosol (Song and DeBose-Boyd, 2004) or 5 μg/ml purified ubiquitin-activating enzyme (E1). Sterols were added to reactions in a final concentration of 1% (v/v) ethanol. Typical reactions were carried out at 37°C for 30 min, unless otherwise stated in the figure legends. Reactions were terminated by centrifugation at 4,000 rpm (permeabilized cells) or $2 \times 10^5$ g (isolated membranes) at 4°C, and the resulting pellets were lysed in detergent-containing buffer, clarified, and subjected to immunoprecipitation with polyclonal anti-HMG CoA reductase as described above.

### RNA interference

Duplexes of small-interfering RNA targeting Insig-1, Insig-2, and the irrelevant control gene VSV-G were synthesized by Dharmacon Research (Lafayette,

Colorado), and RNA interference experiments were carried out as previously described (Sever et al., 2003a).

**Acknowledgments**

We thank Drs. Michael S. Brown and Joseph L. Goldstein for their continued encouragement and critical reading of the manuscript. We also thank Tammy Dinh and Kristi Garland for excellent technical assistance and Marissa Hodgin for help with tissue culture. The work was supported by research grants from the National Institutes of Health (HL20948), Perot Family Foundation, and W.M. Keck Foundation. R.A.D.-B. is the recipient of a National Institutes of Health Mentored Minority Faculty Development Award (HL70441) and an Established Investigator Award from the American Heart Association.

Received: November 3, 2004
Revised: December 15, 2004
Accepted: January 5, 2005
Published: March 15, 2005

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