Ubiquitination of 3-Hydroxy-3-methylglutaryl-CoA Reductase in Permeabilized Cells Mediated by Cytosolic E1 and a Putative Membrane-bound Ubiquitin Ligase*

Bao-Liang Song and Russell A. DeBose-Boyd‡

From the Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9046

The endoplasmic reticulum (ER) enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase, catalyzes the production of mevalonate, a rate-controlling step in cholesterol biosynthesis. Excess sterols promote ubiquitination and subsequent degradation of reductase as part of a negative feedback regulatory mechanism. To characterize the process in more detail, we here report the development of a permeabilized cell system that supports reductase ubiquitination stimulated by the addition of sterols in vitro. Sterol-dependent ubiquitination of reductase in permeabilized cells is dependent upon exogenous cytosol, ATP, and either Insig-1 or Insig-2, two membrane-bound ER proteins shown previously to mediate sterol regulation of reductase degradation in intact cells. Oxytetracyclines, but not cholesterol, promote reductase ubiquitination under our conditions. Finally, we show that ubiquitin-activating enzyme (E1) can efficiently replace cytosol to ubiquitinate reductase in response to sterol treatment, suggesting that other molecules required for ubiquitination of reductase, such as the ubiquitin-conjugating and -ligating enzymes (E2 and E3), are localized to ER membranes.

The enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase produces mevalonate, an important intermediate in the synthesis of cholesterol and nonsterol isoprenoids (1). Sterol and nonsterol end-products of mevalonate metabolism accelerate degradation of reductase as part of a complex, multivalent feedback mechanism that ensures cellular sterol homeostasis (2). HMG-CoA reductase is an endoplasmic reticulum (ER)-localized glycoprotein that consists of two distinct domains (3). The NH2-terminal domain of 339 amino acids, which contains eight membrane-spanning regions separated by short loops, anchors the protein to ER membranes (4, 5). The COOH-terminal domain of 548 amino acids projects into the cytosol and contains all of the catalytic activity of the enzyme. Two independent observations demonstrate the crucial role of the NH2-terminal domain in sterol-accelerated degradation of reductase. First, the expression of the truncated cytosolic COOH-terminal domain of reductase resulted in the production of a stable, catalytically active protein whose degradation was not accelerated by sterols (6). Second, a chimeric protein that consisted of a fusion protein between the transmembrane domain of reductase and soluble β-galactosidase exhibited sterol-regulated degradation in a manner similar to wild-type reductase (7). Sterol-accelerated degradation of reductase is believed to occur in ER membranes and can be blocked by inhibitors of the 26 S proteasome, which leads to the accumulation of ubiquitinated forms of the enzyme (8–10).

Recently, we found that sterol-accelerated degradation of HMG-CoA reductase is dependent upon the presence of at least one member of a pair of ER-resident membrane proteins called Insig-1 and Insig-2 (10, 11). When reductase was overexpressed in Chinese hamster ovary cells by transfection, the enzyme was no longer degraded rapidly in response to sterol treatment. Overexpression of Insig-1 restored regulated degradation of overexpressed reductase, and this degradation coincided with sterol-dependent binding of Insig to the membrane attachment region of reductase. Similar results were obtained with Insig-2.2 Expanding on these initial observations, we recently used RNA interference to demonstrate that sterol-dependent ubiquitination and subsequent degradation of endogenous reductase in human fibroblasts requires Insig-1 or Insig-2 (10). Consistent with this finding, sterol-regulated ubiquitination of reductase displayed an absolute Insig-dependence in transient transfection assays. Insig binding appears to be a prerequisite for reductase ubiquitination and degradation as indicated by alanine substitution experiments that revealed that formation of Insig-reductase complexes required the tetrapeptide sequence YIYF located in the second transmembrane segment of reductase (10). When the YIYF sequence was mutated, reductase no longer bound to Insigs, and the enzyme was no longer subject to sterol-regulated ubiquitination or degradation. The substitution of arginine for lysine 248 in reductase did not affect Insig binding but abolished detectable reductase ubiquitination and slowed its degradation, strongly implicating lysine 248 as the major site for Insig-dependent, sterol-regulated ubiquitination.

The data summarized above support a model in which sterols promote binding of HMG-CoA reductase to Insigs in a reaction mediated by the reductase transmembrane domain. The reductase-Insig complex then recruits other proteins, presumably components of the ubiquitin-proteasome pathway, culminating in ubiquitination of reductase, which marks the protein for proteasomal degradation. The apparent site of reductase ubiq-

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‡ Recipient of National Institutes of Health Mentored Minority Faculty Development Award HL70441. To whom correspondence should be addressed. Tel.: 214-648-8894; Fax: 214-648-8894; E-mail: Russell.DeBose-Boyd@utsouthwestern.edu.

1 The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ER, endoplasmic reticulum; MjCJD, methyl-b-cyclodextrin; siRNA, small interfering RNA; VSV-G, vesicular stomatitis virus glycoprotein; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.

2 B.-L. Song and R. A. DeBose-Boyd, unpublished observations.
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EXPERIMENTAL PROCEDURES

Materials—We obtained MG-132 and digitonin from Calbiochem; hors eradish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgG (affinity-purified) from Jackson ImmunoResearch Laboratories; ubiquitin, ubiquitin-aldehyde, Fraction I, Fraction II, and S100 isolated from HeLa cells, and ubiquitin activating-enzyme (E1) from Boston Biochem (Cambridge, MA); sterols from Steraloids, Inc. (Newport, RI); and NHH-terminal FLAG-tagged ubiquitin from Sigma. Stock solutions of digitonin were prepared by dissolving 1 g of solid in 10 ml of boiling H2O. After cooling to room temperature, the solutions were filtered, Aliquots were prepared, and stored at −20 °C until use. Other reagents were obtained from previously described sources (14). Lipoprotein-deficient serum (d > 1.215g/ml) was prepared from newborn calf serum by ultracentrifugation (15).

Cell Culture—Monolayers of SV-589 cells, an immortalized line of human fibroblasts expressing the SV40 large T antigen (16), were grown at 37 °C in 5% CO2. Stock cultures of SV-589 were maintained 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).

Preparation of Rat Liver Cytosol—Male Sprague-Dawley rats (350–400 g) were housed in colony cages, maintained on a 12-h light/12-h dark cycle, and fed a Harlan Teklad 4% Mouse/Rat Diet 7001. At 3 h into the dark cycle, the rats were anesthetized by halothane inhalation following by intraperitoneal injection of Nembutal, after which their livers were perfused with 0.9% (w/v) NaCl through the portal vein at room temperature. The livers were excised, cut into small pieces, and adjusted to 2 ml/g with ice-cold buffer containing 50 mm Hepes-KOH at pH 7.2, 250 mm sorbitol, 70 mm potassium acetate, 5 mm potassium EGTA, 2.5 mm magnesium acetate, plus protease inhibitors (20 μM N-acetyl-leucinal-leucinal-norleucinal, 1 mm dithiothreitol). The resuspended livers were then disrupted in a Polytron homogenizer followed by 10 strokes in a 50-ml Dounce homogenizer fitted with a Teflon pestle. All subsequent steps were carried out at 4 °C. Homogenates were centrifuged at 1,000 × g for 10 min. The supernatants from this spin were subjected sequentially to centrifugation at 20,000 × g for 20 min, 186,000 × g for 1 h, and 186,000 × g for 45 min. The final supernatant, designated as cytosol (30–40 mg of protein/ml), was divided into multiple portions and stored at −80 °C. For experiments, tubes were thawed in a 37 °C water bath and placed on ice until use.

Ubiquitination of HMG-CoA Reductase in Permeabilized SV-589 Cells—The protocol used to analyze HMG-CoA reductase ubiquitination in permeabilized cells was adapted from procedures described by the laboratories of Rapoport and Ploegh (17, 18). 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 ice-cold phosphate-buffered saline containing 0.9 mm NaCl. The washed cells were resuspended in 0.5 ml of permeabilization buffer (25 mm Hepes-KOH at pH 7.3, 115 mm potassium acetate, 5 mm sodium acetate, 2.5 mm MgCl2, 0.5 mm sodium EGTA) containing 0.025% (w/v) digitonin, an ATP-regenerating system (2 mm Hepes-KOH at pH 7.3, 1 mm magnesium acetate, 1 mm ATP, 30 mm creatine phosphate, and 0.05 mg/ml creatine kinase) (19), and protease inhibitors (20 μM leupeptin, 10 μM MG-132, 5 μg/ml pepstatin A, and 2 μg/ml aprotinin). After rotation for 10 min at 4 °C, the cells were collected by centrifugation for 10 min at 4000 rpm at 4 °C, resuspended in 0.5 ml of permeabilization buffer containing protease inhibitors, the ATP-regenerating system, but no digitonin, and subjected to a second centrifugation for 10 min at 4000 rpm at 4 °C. The resulting pellets of permeabilized cells were then subjected to ubiquitination assays in a final volume of 1 ml of permeabilization buffer containing the ATP-regenerating system, 0.1 mg/ml ubiquitin, 0.01 mg/ml ubiquitin-aldehyde, and 30–3000 μg of protein of rat liver cytosol. Sterols were added to reactions in a final concentration of 1% (v/v) ethanol. Typical reactions were carried out at 37 °C for 20–30 min, unless otherwise stated in the figure legends. Reactions were terminated by centrifuging samples at 4,000 rpm at 4 °C, and the resulting cell pellets were lysed in detergent-containing buffer, clarified, and subjected to immunoprecipitation as described below.

RNA Interference—Duplexes of small interfering RNA targeting human Insig-1, human Insig-2, and an irrelevant control gene, vesicular stomatitis virus glycoprotein (VSV-G), were synthesized by Dharmacon Research (Lafayette, CO), and RNA interference experiments were carried out as previously described (10).

HMG-CoA Reductase Immunoprecipitation and Immunoblot Analysis—Immunoprecipitation of HMG-CoA reductase from detergent lysates was carried out with polyclonal antibodies directed against the 60-kDa COOH-terminal domain of human reductase as previously described (10, 20). Aliquots of the immunoprecipitates were subjected to SDS-PAGE on 6% gels, transferred to nylon membranes, and subjected to immunoblot analysis.

RESULTS

To dissect the mechanism for sterol-dependent ubiquitination of HMG-CoA reductase, we began by analyzing the process in a permeabilized cell system. Recent experiments from the laboratories of Ploegh and Rapoport described a permeabilized cell system that recapitulates the ubiquitination and proteasome-mediated destruction of major histocompatibility complex Class I heavy chain molecules induced by the human cytomegalovirus protein US11 (17, 18). In the Ploegh/Rapoport assays, permeabilization was carried out with a low concentration of the mild detergent digitonin, such that the cells retained their subcellular organelle integrity, yet became permeable to molecules such as Trypan blue, ATP, and proteins. In our experiments it was first necessary to deplete cells of sterols so as to assure an abundant supply of nonubiquitinated reductase. For this purpose SV589 cells, a line of SV-40-transformed human fibroblasts, were incubated for 16 h in lipoprotein-deficient serum containing the reductase inhibitor, compactin, and the lowest level of mevalonate (50 μM) that assures viability. Cells were then harvested, washed, and permeabilized with buffer containing a low concentration of digitonin, an ATP-regenerating system, and protease inhibitors to prevent degradation of any ubiquitinated reductase. The samples were subsequently subjected to centrifugation to separate them into supernatant and pellet fractions. The pellet fraction was resuspended in buffer containing the ATP-regenerating system and protease inhibitors, but no digitonin, and re-isolated by centrif-
ubiquitination. As shown in Fig. 1A, the pellet fraction of permeabilized cells contained numerous proteins (lane 4), including reductase (Fig. 1B, top panel, lane 4). Similarly, the supernatant fraction also contained a large number of proteins with a range of molecular masses, as would be expected of cytosolic proteins (Fig. 1A, lane 3). Several proteins that were associated with the pellet fraction of nonpermeabilized cells were absent from that of permeabilized cells, but appeared in the supernatant upon permeabilization (Fig. 1A, compare lanes 2–4). This suggests that the majority of cytosolic proteins are released into the supernatant upon permeabilization, and we confirmed this by immunoblotting the fractions with antibodies against two cytosolic proteins: the chaperone Hsp90 and ubiquitin-activating enzyme (E1). Permeabilization of the cells with digitonin resulted in the complete release of Hsp90 and E1 immunoreactivity into the supernatant fraction after centrifugation (Fig. 1A, bottom two panels). Thus, permeabilization of sterol-depleted SV-589 cells results in the release of a substantial amount of cytosolic proteins into the supernatant fraction after centrifugation while membrane-associated proteins, such as reductase, remain associated with the pellet fraction.

We next sought to determine whether the pellet fraction of permeabilized cells could support sterol-regulated ubiquitination of HMG-CoA reductase. The experiment in Fig. 2 shows the basic features of reductase ubiquitination in the permeabilized cell system. Permeabilized SV-589 cells were subjected to in vitro treatments with a mixture of ubiquitin, ubiquitin-aldehyde (to inhibit de-ubiquitinating enzymes) (22), an ATP-regenerating system, and rat liver cytosol in the absence or presence of sterols (10 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol) (Fig. 2A). Following incubation at 37 °C for various periods of time, membrane pellets were collected by centrifugation, solubilized in detergent-containing buffer, and subjected to immunoprecipitation with polyclonal antibodies against reductase. The resulting immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-ubiquitin (Fig. 2A, top panel) or anti-reductase (Fig. 2A, lower panel) monoclonal antibodies. Incubation of permeabilized cells with

![Image](image-url)
Sterols led to the appearance of ubiquitinated reductase in a time-dependent fashion, as indicated by high molecular weight smear in the anti-ubiquitin immunoblots of the reductase immunoprecipitates (Fig. 2A, top panel, lanes 3–10). The amount of ubiquitinated reductase rose with time and reached a plateau by 30 min. When cytosol (Fig. 2B, top panel, lanes 1 and 2; Fig. 2C, top panel, lanes 3 and 4) or the ATP-regenerating system (Fig. 2C, top panel, lanes 5 and 6) were omitted from reaction mixtures, no ubiquitinated reductase was observed upon the addition of sterols. Importantly, the amount of immunoprecipitated reductase remained constant throughout the assay, regardless of treatment or time of incubation (lower panels, Fig. 2, A–C). As previously noted in studies of reductase ubiquitination in living cells (10), the anti-reductase immunoblots did not show a smear of ubiquitinated reductase. This suggests that only a small proportion of reductase was ubiquitinated in the in vitro permeabilized cell system as well as the living cell system. Taken together, the results of Fig. 2 demonstrate that reductase ubiquitination in permeabilized cells is stimulated upon the in vitro addition of sterols and the ubiquitination reaction is time- and cytosol-dependent and requires an exogenous source of energy.

Fig. 3 shows a competition experiment between ubiquitin containing an NH2-terminal FLAG epitope tag (FLAG-ubiquitin) and untagged ubiquitin for sterol-induced ubiquitination of HMG-CoA reductase in permeabilized SV-589 cells. Permeabilized cell pellets were incubated with increasing amounts of FLAG-ubiquitin in the absence (lanes 1–6) or presence (lanes 7–12) of 20 μM untagged ubiquitin. After incubation for 20 min at 37 °C, the reactions were terminated, and detergent extracts of the permeabilized cells were prepared and immunoprecipitated with anti-reductase polyclonal antibodies. Anti-FLAG immunoblots revealed sterol-regulated ubiquitination of reductase that increased in parallel with the amount of FLAG-ubiquitin in reaction mixtures (Fig. 3, top panel, lanes 2, 4, and 6). Virtually identical results were observed when the immunoprecipitates were probed with anti-ubiquitin, which recognizes both FLAG-tagged and untagged versions of ubiquitin (middle panel, lanes 2, 4, and 6). Untagged ubiquitin effectively competed with FLAG-ubiquitin for reductase ubiquitination, as indicated by the ablation of immunoreactivity in anti-FLAG immunoblots when reactions contained untagged ubiquitin in addition to FLAG-ubiquitin (top panel, compare lanes 2, 4, and 6 with lanes 8, 10, and 12). These results demonstrate that sterol-dependent ubiquitination of reductase in the permeabilized cell system can be influenced by at least two factors added in vitro: ubiquitin and sterols.

We previously demonstrated that a pair of ER membrane proteins, Insig-1 and Insig-2, plays critical roles in sterol-dependent ubiquitination and degradation of HMG-CoA reductase in intact cells (10, 11). To demonstrate their requirement in permeabilized cells, we decreased the amount of Insigs through the use of RNA interference (Fig. 4). SV-589 cells were transfected with duplexes of small interfering RNAs (siRNAs) targeting a control mRNA encoding vesicular stomatitis virus glycoprotein (VSV-G, lanes 1 and 2), which is not present in the cells, or Insig-1 and Insig-2 mRNAs (lanes 3 and 4). Following transfections the cells were permeabilized and subjected to the ubiquitination assay, and detergent extracts were immunoprecipitated with polyclonal anti-reductase antibodies and immunoblotted for FLAG-ubiquitin (upper panel) or reductase (lower panel). In the control cells receiving VSV-G siRNA, sterol treatment caused the appearance of ubiquitinated reductase (upper panel, lane 2). Ubiquitination was greatly diminished in cells transfected with the combination of siRNAs targeting Insig-1 and Insig-2 (upper panel, lane 4). These results demonstrate that Insigs are required for reductase ubiquitination mediated by the in vitro addition of sterols to permeabilized cells.
FIG. 5. Oxysterol specificity for HMG-CoA reductase ubiquitination in permeabilized SV-589 cells. On day 0, SV-589 cells were set up and refed as described in Fig. 1. On day 3, cells were harvested, washed, and permeabilized with digitonin as described under “Experimental Procedures.” The permeabilized cells were resuspended in permeabilization buffer containing protease inhibitors, an ATP-regenerating system, 0.1 mg/ml ubiquitin, 0.01 mg/ml ubiquitin aldehyde, and 3 mg/ml rat liver cytosol. Incubations were carried out with 0.3, 1, 3, or 10 µg/ml of 25-hydroxycholesterol (lanes 2–5 and 7–10, respectively) in the absence (−) or presence (+) of 10 µg/ml cholesterol. The reactions were terminated by centrifugation, and the resulting cell pellets were lysed and subjected to immunoprecipitation and immunoblot analysis as in Fig. 2.
In experiments presented thus far, we used mixtures of 25-
hydroxycholesterol and cholesterol to induce HMG-CoA reductase ubiquitination in permeabilized SV-589 cells. The experi-
ment of Fig. 5A shows ubiquitination assays in which 25-
hydroxycholesterol and cholesterol were individually added to the reactions. In the absence of cholesterol, 25-hydroxychole-
sterol stimulated ubiquitination of reductase in a dose-dependent fashion (top panel, lanes 2–5). The addition of cholesterol alone had no significant effect (top panel, lane 6) and did not potentiate the dose-dependent increase in reductase ubiquitination stimu-
lated by 25-hydroxycholesterol (top panel, lanes 7–10). To determine whether other modified sterols could enhance reductase ubiquitina-
tion in permeabilized cells, a panel of sterols were added to reactions at concentrations of 10 μg/ml in ethanol (Fig. 5, B and C). Of the oxyysterols tested, only 25-hydroxycholesterol, 5-cho-
lesterol-3β,16β,27-triol, 24(S)-27-hydroxysterol, and 27-hy-
droxycholesterol stimulated reductase ubiquitination to appreci-
able levels when added to reactions in ethanol (Fig. 5C; top gel lanes a, c, d, and i, respectively).

The possibility exists that the sterol specificity of reductase ubiquitina-
tion observed in Fig. 5C reflects differences in the solubilities and/or membrane partition rates among the vari-
ous sterols tested. To circumvent this problem, we assessed the ability of those ineffective sterols in Fig. 5C to stimulate reductase ubiquitination by adding them in complex with methyl-β-
cyclodextrin (MβCD) rather than from ethanolic solutions (Fig.
5, D–F). A similar procedure was recently used to demonstrate that cholesterol and other structurally related sterols induce a conformational change in SREBP cleavage-activating protein, a sterol-responsive escort protein, when added to isolated ER membranes in vitro (23). When added as MβCD complexes, 25-hydroxycholesterol, 24(S)-25-epoxycholesterol, and 19-
hydroxycholesterol stimulated reductase ubiquitination in the permeabilized cells (Fig. 5, D and F, respectively). The activi-
ties of 24(S),25-epoxycholesterol and 19-hydroxycholesterol in MβCD complexes contrasts with their activities in ethanol, and
this is likely due to increased membrane partitioning of the sterols as a result of their enhanced delivery from MβCD. It
should be noted that cholesterol (Fig. 5, A, C, and F) and certain oxyysterols, such as 17β-hydroxycholesterol (Fig. 5, C and D), 25-oxo-hydroxycholesterol, and 22(E)-hydroxycholesterol (Fig. 5, C and E), failed to stimulate ubiquitination of reductase
in permeabilized cells regardless of method for sterol delivery. These results are representative of at least two other experi-
ments performed at sterol concentrations of 10 μg/ml. To-
gether, these results demonstrate that reductase ubiquitina-
tion in the permeabilized cell system is stimulated by a specific set of oxyysterols.

Ubiquitination of proteins is carried out through the sequen-
tial action of three distinct enzymes: the ubiquitin-activating
enzyme (E1), ubiquitin conjugating enzymes (E2s), and ubiqui-
tin ligases (E3s) (24, 25). Ubiquitin is first activated by E1, which forms a thiol ester between a reactive cysteine residue in E1 and the COOH terminus of ubiquitin. Next, ubiquitin is transferred from E1 to a catalytic cysteine residue of E2 as a thiol ester. In the final step, E3 facilitates the transfer of activated ubiquitin from E2 to a lysine residue in the substrate (or ubiquitin). Only a single E1 is believed to exist, and it is found in the cytosol (Fig. 1) (24, 25). In contrast, a variety of E2s and E3s exist, both soluble and membrane-bound, have
been described (24, 26). The specificity of substrate ubiquitina-
tion is usually determined by the E3, either alone or in combi-
nation with its cognate E2. Considering our current data, which indicates that the site of Insig-dependent reductase ubiquitination is predicted to lie immediately adjacent to a transmembrane region (10), the possibility exists that the re-
ductase E3 is a membrane-bound enzyme.

We next sought to determine which components of the HMG-
CoA reductase ubiquitinating machinery (E1, E2, and possibly the E3) are provided by rat liver cytosol in our permeabilized cell system. To achieve this, we evaluated the ability of frac-
tionated HeLa cell S100 to replace rat liver cytosol in the ubiquitination reaction (Fig. 6A). The fractions tested were first described by Herskho et al. (27, 28) and were generated by separating S100 derived from HeLa cells into fractions that bind (Fraction II) or do not bind (Fraction I) an anion exchange resin. It was subsequently found that Fraction I contains ubiqui-
tin and Fraction II contains E1 (25, 27, 28). As demonstrated in Fig. 6A, when permeabilized cells were incubated in the absence of cytosol, sterols did not cause the appearance of ubiquitinated reductase (top panel, lanes 1 and 2), whereas the addition of cytosol at 1 or 3 mg/ml resulted in the ubiquitina-
tion of reductase in the presence of sterols (top panel, lanes 4 and 6). In contrast, when permeabilized cells were incubated with Fraction I, sterols failed to stimulate reductase ubiquiti-
nation (top panel, lane 8), yet those reactions containing Frac-
tion II or S100 supported sterol-dependent ubiquitination of the enzyme (top panel, lanes 10 and 12). Immunoblotting the reaction supernatants with anti-E1 polyclonal antibody reve-
aled that effective amounts of cytosol, Fraction II, and S100 contained approximately equivalent levels of E1 (lower panel, lanes 3–6, 9–10, and 11–12, respectively); E1 was absent from Fraction I (lower panel, lanes 7 and 8).

Fig. 6B shows an experiment designed to evaluate whether purified E1 could replace rat liver cytosol for HMG-CoA reduc-
tase ubiquitination. Low concentrations of E1 (less than 16 μg/ml) supported sterol-stimulated ubiquitination of reductase (top panel, lanes 4, 6, and 8) as well as 1000-fold higher con-
centrations of cytosol (lanes 10 and 12). To directly compare the activity of purified E1 to that of rat liver cytosol, an aliquot of the supernatant of the reaction mixture was analyzed (bottom panel). Immunoblotting these samples with anti-E1 revealed that reactions containing 1.0 mg/ml rat liver provided about 1.6 μg/ml E1 (compare lanes 3 and 4 with 9 and 10). These levels of E1 and cytosol produced approximately equivalent levels of reductase ubiquitination in the presence of sterols (top panel, compare lanes 3 and 4 with 9 and 10). Thus, it appears that the role of rat liver cytosol for reductase ubiquitination in the permeabilized cell system is to replace E1 activity released into the supernatant fraction after centrifugation as a result of permeabilization (see Fig. 1).

We next performed a depletion experiment to confirm the necessity for E1 in the permeabilized cell system (Fig. 7). Al-
quois of rat liver cytosol were immunoprecipitated with anti-E1 polyclonal antibodies or an irrelevant antiserum. The resulting supernatants were then assayed for their ability to support sterol-
dependent ubiquitination of reductase. In the absence of exoge-
nous cytosol, reductase failed to become ubiquitinated in perme-
abilized cells regardless of treatment (top gel, lanes 1 and 2). Cytosol that was immunodepleted with the control antiserum

exposed to film for 2 min. B, schematic representation of oxyysterols evaluated for stimulation of reductase ubiquitination in C. C, incubations containing the indicated sterol were carried out for 20 min at 37 °C. The reactions were terminated by centrifugation and the resulting cell pellets were lysed and subjected to immunoprecipitation and immunoblot analysis as in Fig. 2. Filters were exposed to film for 2 min.
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**Fig. 6.** Ubiquitin-activating enzyme (E1) can replace rat liver cytosol to promote sterol-dependent ubiquitination of HMG-CoA reductase in permeabilized SV-589 cells. A and B, on day 0, SV-589 cells were set up and refed as described in Fig. 1. On day 3, cells were harvested, washed, and permeabilized with digitonin as described under "Experimental Procedures." A, permeabilized cells were incubated with 1 or 3 mg/ml rat liver cytosol (lanes 3–4 and 5–6, respectively), 1 mg/ml Fraction I (lanes 7–8), Fraction II (lanes 9–10), or S-100 fraction (lanes 11–12) in addition to the ATP-regenerating system, 0.1 mg/ml ubiquitin, and 0.01 mg/ml ubiquitin aldehyde. Reactions were carried out for 20 min at 37 °C in the absence or presence of sterols, after which they were terminated by centrifugation. An aliquot of the supernatants was saved for further analysis, and the resulting cell pellets were lysed and subjected to immunoprecipitation with polyclonal anti-reductase. Aliquots of the immunoprecipitates, along with the supernatants obtained from the terminating centrifugation step, were subjected to SDS-PAGE, transferred to nylon membranes, and immunoblot analysis was carried with 5 µg/ml monoclonal IgG-A9 (against HMG-CoA reductase), 1:1000 dilution of polyclonal IgG-P4D1 (against ubiquitin). Filters were exposed to film for 30 s (top gel) and 10 s (bottom gel).

**Fig. 7.** Immunodepletion of ubiquitin-activating enzyme (E1) from rat liver cytosol abolishes sterol-stimulated ubiquitination of HMG-CoA reductase in permeabilized cells. Rat liver cytosol was subjected to immunoprecipitation with anti-E1 polyclonal antibodies or control antiserum. Following incubation for 16 h at 4 °C, immunoprecipitated proteins were removed by centrifugation, and the resulting supernatants were assayed for their ability to support reductase ubiquitination in permeabilized cells as described below. On day 0, SV-589 cells were set up and refed as described in Fig. 1. On day 3, cells were harvested, washed, and permeabilized with digitonin as described under "Experimental Procedures." The permeabilized cells were resuspended in permeabilization buffer containing protease inhibitors, the ATP-regenerating system, 0.1 mg/ml ubiquitin, 0.01 mg/ml ubiquitin aldehyde, and 3 mg/ml of rat liver cytosol immunodepleted with the control antiserum, E1-depleted cytosol, or 1.6 µg/ml purified E1, as indicated. After 20 min at 37 °C in the absence (−) or presence (+) of sterols, the reactions were terminated by centrifugation. An aliquot of the supernatant from this spin was saved for further analysis, and the resulting cell pellets were lysed and subjected to immunoprecipitation and immunoblot analysis as in Fig. 6. Filters were exposed to gels for 1.5 min (top gel), 15 s (middle gel), and 5 s (bottom gel).

**DISCUSSION**

The results presented in this report, describing the analysis of sterol-regulated ubiquitination of HMG-CoA reductase in a permeabilized cell system, provide new insights into the mechanism underlying this aspect of sterol regulation. Our initial characterization of reductase ubiquitination in permeabilized cells reveals: 1) Reductase ubiquitination is stimulated by the *in vitro* addition of sterols in a reaction that requires rat liver cytosol and an energy source and occurs in a time-dependent fashion (Fig. 2). 2) Reductase ubiquitination in permeabilized cells is promoted by oxysterols, including 25-hydroxycholesterol, 5-cholesten-3β,16α,27-triol, 24(S)-hydroxycholesterol, 27-hydroxycholesterol, 24(S),25-epoxycholesterol, and 19-hydroxycholesterol at concentrations less than 10 µg/ml (see Fig. 5), whereas cholesterol appears negative at similar concentrations. 3) The ubiquitin-activating enzyme (E1) is the only cytosolic protein required for sterol-dependent ubiquitination of reductase, indicating that supported sterol-dependent ubiquitination of reductase (top gel, lane 4), but reductase ubiquitination was completely ablated when the reactions were supplemented with cytosol immunodepleted with anti-E1 (top gel, lanes 5 and 6). E1 depletion was essentially complete, as revealed by immunoblots of the reaction supernatants probed with anti-E1 antibody (bottom panel, compare lanes 3–4 with 5–6). Purified E1 restored sterol-regulated ubiquitination of reductase when incubated with permeabilized cells alone (top gel, lane 7 and 8) or in combination with cytosol from which E1 was immunodepleted (top gel, lanes 11 and 12). Taken together, the results of Figs. 6 and 7 demonstrate that the role of rat liver cytosol in our assay is to provide E1 for the activation of ubiquitin, suggesting that E2 and E3, which mediate sterol-dependent ubiquitination of reductase in permeabilized cells are membrane-associated proteins.
other components of the ubiquitin-proteasome pathway that contribute to reductase ubiquitination are membrane-associated proteins (Figs. 6 and 7). 4) The ability of E1 to replace cytosol indicate that in these permeabilized cells, a cytosolic transport molecule is not necessary to deliver oxysterols to the ER, where they promote Insig binding to reductase, a prerequisite for recognition of reductase by the ubiquitinating enzymes.

The role for the ubiquitin-proteasome pathway in the control of HMG-CoA reductase degradation was first established in the yeast Saccharomyces cerevisiae (30). HMG2p, one of two reductase isoforms expressed in yeast, undergoes accelerated degradation when flux through the mevalonate pathway is high (30, 31). The genetic analysis of mutant yeast strains incapable of accelerating HMG2p degradation revealed that the process is mediated by HRD1p, an integral ER membrane protein that displays E3 activity in vitro (32), and a membrane-associated E2, Ubc7p (31). The membrane localization of HRD1p and Ubc7p is consistent with our conclusion that E2 and E3, mediating mammalian reductase ubiquitination, are membrane-associated enzymes. Mammalian versions of HRD1p and Ubc7p are known to exist (33, 34), but whether they participate in the sterol-dependent regulation of mammalian reductase remains to be determined. In this regard, it should be noted that, although it contains multiple membrane-spanning segments, the membrane domain of yeast HMG2p shows no resemblance to that of mammalian reductase (3, 35). Moreover, in yeast degradation of reductase is not sterol-mediated, as in mammalian cells, and no orthologues of Insigs have been identified in the yeast genome.

We envision that the pathway for reductase degradation in mammalian cells is divided into three phases. In the sterolsensing phase, designated Phase I, intracellular levels of sterols are monitored by either direct binding to reductase, or a membrane-bound sterol receptor that subsequently transmits signals to the nucleus. Sterol signaling may induce conformational changes in the reductase membrane domain that promotes Insig binding, resulting in the diversion of reductase into the ubiquitin-proteasome pathway. The second phase of the reductase degradative pathway, Phase II, encompasses the recruitment and action of enzymes that polyubiquitinate reductase. Such enzymes include ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s), which act in concert to ubiquitinate reductase, thus targeting it for proteasomal recognition and degradation (26). Phase III, the final phase of the reductase degradative pathway, constitutes post-ubiquitination steps that include the recognition and delivery of ubiquitinated reductase to the proteasome for destruction. The demonstration that E1 can replace cytosol for reductase ubiquitination in permeabilized cells indicates that the pathway for reductase degradation can be interrupted in Phase II by removing the source of activated ubiquitin. This observation forms a possible basis for the biochemical isolation of the reductase E3, and its cognate E2, by virtue of their recruitment to reductase upon complex formation with Insig in response to sterol sensing.

Another potential avenue for investigation in the permeabilized cell system centers on the elucidation of molecular events underlying the third phase of the reductase degradative pathway. Although the beginning of the ubiquitin-proteasome pathway (ubiquitin activation by the E1, substrate recognition, and ubiquitination by E2s and E3s) and the end result, proteasomal degradation, are well defined, how proteins such as reductase are recognized by and presented to proteasomes is poorly understood. Considering the cytosolic localization of proteasomes, it seems likely that an intermediary exit that recognizes and shuttles proteins from the membrane of the ER to the cytosol for proteasomal degradation. Thus, the permeabilized cell system developed in the current report presents an opportunity to dissect post-ubiquitination steps of the reductase degradative pathway and may lead to the identification and isolation of factors that mediate recognition of ubiquitinated reductase and present it to proteasomes for degradation. For example, it is well demonstrated that nonsterol, mevalonate-derived products further accelerate degradation of reductase when sterols are abundant (12, 29, 36). Our previous data indicated that geranylgeranyl pyrophosphate is the major nonsterol product that mediates this effect (10). These observations suggested that geranylgeranyl pyrophosphate is incorporated into a protein that is required for the maximal rate of reductase degradation. We hypothesized geranylgeranylated Rab proteins as possible candidates, and, considering the cytosolic localization of these proteins, their participation in reductase degradation could be readily determined in the context of the permeabilized cell system. In conclusion, the experiments presented in this report mark the beginning of the biochemical characterization of the pathway through which Insigs mediate sterol-stimulated ubiquitination and degradation of reductase and may shed light on the poorly understood process by which polytopic proteins are extruded from membranes and directed to proteasomes for destruction.

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