Degradation of HMG-CoA Reductase in Vitro
CLEAVAGE IN THE MEMBRANE DOMAIN BY A MEMBRANE-BOUND CYSTEINE PROTEASE*

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We have recently shown that the endoplasmic reticulum (ER) membrane protein, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, is cleaved in isolated membrane fractions enriched for endoplasmic reticulum. Importantly, the cleavage rate is accelerated when the membranes are prepared from cells that have been pretreated with mevalonate or sterols, physiologically regulators of the degradation process in vivo (McGee, T. P., Cheng, H. H., Kumagai, H., Omura, S., and Simoni, R. D. (1996) J. Biol. Chem. 271, 25630–25638). In the current study, we further characterize this in vitro cleavage of HMG-CoA reductase. E64, a specific inhibitor of cysteine-proteases, inhibits HMG-CoA reductase cleavage in vitro. In contrast, lactacystin, an inhibitor of the proteasome, inhibits HMG-CoA reductase degradation in vivo but does not inhibit the in vitro cleavage. Purified ER fractions contain lactacystin-sensitive and E64-insensitive proteasome activity as measured by succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin hydrolysis. We removed the proteasome from purified ER fractions by solubilization with heptylthioglucoside and observed that the detergent extracted, proteasome-depleted membrane fractions retain regulated cleavage of HMG-CoA reductase. This indicates that ER-associated proteasome is not involved in degradation of HMG-CoA reductase in vitro.

In order to determine the site(s) of proteolysis of HMG-CoA reductase in vitro, four antisera were prepared against peptide sequences representing various domains of HMG-CoA reductase and used for detection of proteolytic intermediates. The sizes and antibody reactivity of the intermediates suggest that HMG-CoA reductase is cleaved in the in vitro degradation system near the span 8 membrane region, which links the N-terminal membrane domain to the C-terminal catalytic domain of the protein.

We conclude that HMG-CoA reductase can be cleaved in the membrane-span 8 region by a cysteine protease(s) tightly associated with ER membranes.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is a key enzyme in the sterol and nonsterol isoprenoid biosynthesis pathway (1, 2). This enzyme is highly regulated at the level of synthesis as well as at the level of degradation of the protein (1–3).

HMG-CoA reductase is a 97-kDa integral membrane protein of the endoplasmic reticulum (ER) with eight transmembrane spans (4). Previous studies demonstrated that the membrane-spanning regions of this enzyme are necessary for the regulated degradation of the enzyme (5–8).

Recent studies show that some proteins in the endoplasmic reticulum are degraded by the ubiquitin-proteasome pathway (9–13, 32–36). These studies suggest that both the ubiquitin-conjugating enzyme(s) and the proteasome are localized to the cytosolic surface of the endoplasmic reticulum, where both ER membrane and luminal proteins can be degraded. In the case of HMG-CoA reductase, however, there is evidence that the protein is not ubiquitinated (14, 15), so that it remains an open question whether the proteasome is responsible for the degradation of HMG-CoA reductase in mammalian cells.

HMG-CoA reductase degradation has been extensively studied in yeast, where genetic and biochemical evidence indicates that degradation occurs by the ubiquitin-proteasome pathway (35, 36).

Early attempts to dissect the process of regulated ER proteolysis of HMG-CoA reductase succeeded in demonstrating degradation in digitonin-permeabilized cells (16, 17). This degradation is accelerated by prior treatment of cells with regulatory molecules and is inhibited by the cysteine protease/proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN); an inhibitor of the in vivo proteolysis of HMG-CoA reductase (16). These results demonstrate that cytosolic proteins are not required for proteolysis of HMG-CoA reductase once the process has been initiated by mevalonate or sterols in vivo. To extend this work, we recently developed an in vitro degradation assay of reductase using purified ER fractions and showed this process too is physiologically relevant (15). In the present study, we further characterize the degradation of HMG-CoA reductase in vitro and present evidence that an ER membrane-bound cysteine protease(s), not the proteasome, is responsible for the cleavage of HMG-CoA reductase. A cleavage region is determined by epitope mapping of cleavage fragments to be in the span 8 region of the membrane domain.

EXPERIMENTAL PROCEDURES

Materials—Minimal essential medium (MEM) without methionine and cysteine was obtained from ICN Biomedicals, Inc. 25-Hydroxycholesterol and phenylmethylsulfonyl fluoride (PMSF) were purchased carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI, carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI, carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI, carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI, carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI, carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI, carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI, carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI, carboxenzyo-i-isoleucyl-yl-butyl-t-glutamyl-t-alanylt-t-leucinal; MG115, carboxenzyo-i-isoleucyl-t-leucyl-t-norvalinal; E64, N-(L-3-trans-carboxy-2-carbonyl-1-leucyl-7-amino-4-methylcoumarin; NEM, N-ethylmaleimide; AMC, 7-amino-4-methylcoumarin; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSF, phenylmethylsulfonyl fluoride; PSI,
from Sigma. ALLN was purchased from Calbiochem, and E64 was obtained from Boehringer Mannheim. Carbobenzoxzy-1-isoleucyl-γ-buty1-L-glutamyl-L-alanyl-L-leucinal (PSI) and carbobenzoxzy-1-isoleucyl-γ-leucyl-L-leucyl-L-norvalinal (MG115) were purchased from Peninsula Laboratory. Proteasome substrate peptide, succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-7-amino-4-methylcoumarin (sLLVY-AMC), was kindly provided by Dr. Martin Rechsteiner and Dr. Katherine Ferrell (University of Utah). Polyvinylidene difluoride blotting membrane was kindly provided by Dr. Martin Rechsteiner and Dr. Katherine Ferrell (University of Utah). Polyvinylidene difluoride blotting membrane was purchased from Millipore Corp. Donkey anti-rabbit IgG conjugated to horseradish peroxidase, ECL Western blotting detection reagents, and Hyperfilm-MP were purchased from Amersham Pharma Biotech.

**Cell Culture—**C100 cells, a compactin-resistant SV40-transformed baby hamster kidney cell line that overexpresses HMG-CoA reductase (18), were maintained in minimal essential medium supplemented with nonessential amino acids and 5% fetal calf serum (MEM, 5% FCS) or 5% lipid-poor serum (MEM, 5% LPS). LPS was prepared as described previously (19). In order to increase expression of HMG-CoA reductase, compactin (25 μg/ml) was added to cells grown in MEM, 5% LPS. Endoplasmic Reticulum Membrane Fractionation—ER membrane was isolated as described previously (20). Briefly, C100 baby hamster kidney cells grown in MEM, 5% FCS to 80% confluency in 850-cm² roller bottles. The medium was then changed, and the cells were incubated overnight in MEM, 5% LPS supplemented with compactin (10 μM) and sodium mevalonate (100 μM). Cells were then starved in methionine-, cysteine-, and glutamine-free media for 1 h and pulse-radio-labeled with Trans-35S-label (ICN). Labeling media were removed, and the cells were chased in MEM, 5% LPS supplemented with 10 μM compactin, 2 mM methionine, and 2 mM cysteine. Accelerated degradation was observed by supplementing the chase medium with 25-hydroxycholesterol (2.5 μM). At the indicated time points, samples were washed with ice-cold phosphate-buffered saline, and the labeled cells were lysed and collected in solubilization buffer (21). Lysates were clarified by centrifugation at 16,000 × g, and immunoreactive proteins were precipitated with specific antisera and protein A-Sepharose. Immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography.

**Detection of Proteolytic Intermediates—**To estimate the cleavage site(s) of HMG-CoA reductase in vitro, the degradation assay was carried out as described above, and proteolytic intermediates were detected by blotting using anti-224, anti-284, anti-717, and anti-825 antibodies. These antibodies were prepared in rabbits against synthetic peptides corresponding to residues Arg224 through Leu242 (anti-224), Arg284 through Leu302 (anti-284), Arg717 through Leu736 (anti-717), and Ala825 through Cys832 (anti-825) of HMG-CoA reductase, respectively. The anti-224 and anti-284 antibodies have been reported previously (22). The new antibodies, anti-717 and anti-825, were prepared and carefully characterized as described previously (22).

**RESULTS**

**Degradation of HMG-CoA Reductase in Vitro—**We prepared ER membranes from homogenates of C100 cells by sucrose gradient fractionation for the analysis of HMG-CoA reductase degradation according to the procedure of Urbani and Simoni (20) and as described previously (15). Two hours prior to harvesting the cells for homogenization, 25-hydroxycholesterol was added to cultures for accelerated degradation studies. Incorporation of pure ER membranes at 37 °C results in the in vitro degradation of the intact 97-kDa HMG-CoA reductase protein. As shown in Fig. 1, ER fractions prepared from C100 cells degrade HMG-CoA reductase relatively slowly. Supplementation of the cells with 25-hydroxycholesterol prior to cell lysis, however, results in a significant acceleration in the rate of HMG-CoA reductase degradation. The in vitro degradation of reductase under both conditions was inhibited by ALLN (Fig. 1).

**In Vitro Degradation of HMG-CoA Reductase Is Sensitive to Inhibitors of Cysteine Proteases—**We performed inhibitor studies to characterize the sterol-accelerated proteolytic degradation of HMG-CoA reductase in vitro. As shown in Fig. 2, the extracted pellet was resuspended in 10 mM Tris-HCl (pH 7.5), 150 mM sucrose. The concentrations of HTG in the reaction mixtures of each fraction for the in vitro degradation of the reductase were adjusted to be the same.

**Pulse-Chase Analysis of Protein Degradation—**C100 cells were analyzed in a pulse-chase regimen as described previously (21). Briefly, cells were grown to near confluency in MEM, 5% FCS medium. The medium was then changed, and the cells were incubated overnight in MEM, 5% LPS supplemented with compactin (10 μM) and sodium mevalonate (100 μM). Cells were then starved in methionine-, cysteine-, and glutamine-free media for 1 h and pulse-radio-labeled with Trans-35S-label (ICN). Labeling media were removed, and the cells were chased in MEM, 5% LPS supplemented with 10 μM compactin, 2 mM methionine, and 2 mM cysteine. Accelerated degradation was observed by supplementing the chase medium with 25-hydroxycholesterol (2.5 μM). At the indicated time points, samples were washed with ice-cold phosphate-buffered saline, and the labeled cells were lysed and collected in solubilization buffer (21). Lysates were clarified by centrifugation at 16,000 × g, and immunoreactive proteins were precipitated with specific antisera and protein A-Sepharose. Immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography.

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sterol-accelerated reductase degradation was blocked by ALLN (50 μM), PSI (50 μM), MG115 (50 μM), and E64 (50 μg/ml), but not by lactacystin (50 μM) or APMSF (100 μM). We had previously reported that lactacystin inhibits the in vitro degradation of reductase, but that result has subsequently not been reproducible. The lack of inhibition of reductase degradation in vitro reported here is highly reproducible with many different preparations of ER membranes. ALLN, PSI, and MG115 are known to be relatively nonspecific proteasome inhibitors, which also inhibit cysteine proteases including calpain and cathepsins (24, 25). Lactacystin, on the other hand, is reported to be a specific protease inhibitor (27). These results suggest that a cysteine protease(s) and not the proteasome is involved in the proteolytic degradation of HMG-CoA reductase in vitro.

Effect of SH Reagents on Degradation of HMG-CoA Reductase in Vitro—Previous work has indicated that ER degradation of HMG-CoA reductase is inhibited by the cysteine protease inhibitors as well as by general thiol reagents in vivo (28). In this work, therefore, we tried to confirm that thiol reagents inhibit in vitro degradation of reductase. As shown in Fig. 3, NEM (100 μM) inhibits reductase degradation in vitro. Incubation with 1 mM dithiothreitol prevents this inhibition, suggesting that a free thiol group is necessary for reductase degradation and supporting the notion that a cysteine protease is involved in the intravesosomal degradation of HMG-CoA reductase in vivo.

Lactacystin Inhibits the Degradation of HMG-CoA Reductase in Vivo—Lactacystin is thought to be a specific inhibitor of the proteasome (26). The degradation of HMG-CoA reductase in vivo was examined by pulse-chase analysis, and the effect of lactacystin was tested in this system. As shown in Fig. 4, basal and sterol-accelerated degradation of HMG-CoA reductase of C100 cells are inhibited by lactacystin in vivo, confirming our earlier report (15). This suggests that the proteasome or some other lactacystin-sensitive protease(s) is involved in the degradation of HMG-CoA reductase in vivo.

Characterization of ER-associated Proteasome Activity—Given that lactacystin inhibits degradation of reductase in vivo, implicating the proteasome, we examined the ER fractions for proteasome activity. We previously reported that purified ER fractions contain proteasome subunits by Western blotting using antibodies against 20 and 26 S proteasome subunits (15). However, these experiments did not test whether our ER fractions contained enzymatically active proteasome, although this has been reported by others (30). In this study, we measured proteasome activity in the purified ER membranes using sLLVY-AMC peptide as a substrate. As shown in Fig. 5, purified ER membranes contain lactacystin-sensitive sLLVY-AMC hydrolysis activity. Other proteasome inhibitors, ALLN, PSI, and MG115, also inhibit this activity. A cysteine protease inhibitor, E64, or a serine-protease inhibitor, APMSF, however, has little inhibitory effect. The dose dependence of these inhibitors for the purified preparations of 20 and 26 S proteasome and purified ER membrane fraction were compared. As shown in Fig. 6, lactacystin inhibits the activity of purified 20 and 26 S proteasome in the same dose-dependent manner as it inhibits the proteasome activity of the purified membranes, but E64 (up to 100 μg/ml) does not inhibit either activity. The dose-dependent curves of these two protease inhibitors on the purified proteasomes and purified ER fraction are quite similar (Fig. 6, A versus B). These results demonstrate that purified ER contains lactacystin-sensitive, E64-insensitive proteasome activity.

HTG Extraction Removes Proteasome and Luminal Proteins—The inhibitor studies presented above suggest that the proteasome is not responsible for the degradation of HMG-CoA reductase in vitro. To obtain further evidence that this is true in the isolated ER system, we tried to remove the proteasome from ER membranes and then test the in vitro degradation of reductase. As a first attempt to remove the proteasome from the ER membranes, we washed the membranes three times with 10 mM Tris (pH 7.5), 150 mM sucrose as had been suggested earlier (30). The proteasome subunits, however, were stably associated with the ER membranes, i.e. remained sedimentable, under these washing conditions (data not shown). High concentrations of salt (2 M KCl) also failed to remove proteasome subunits completely (data not shown). Next, we tried to remove the proteasome from the ER membranes with
lyzing activity of purified proteasomes (centrations of lactacystin or E64. The reaction mixture (200 proteasome was measured in the presence or absence of various protease inhibitors. The reaction was carried out as described under “Experimental Procedures.” Data were represented as percentage of activity. Error bars indicate the range of deviation in three independent experiments using three independent preparations of ER.

HTG, a mild nonionic detergent. ER membranes were solubilized with HTG and separated into supernatant and pellet subfractions as described under “Experimental Procedures.” Equal volumes of total ER (Total ER), supernatant (HTG sup), and pellet (HTG pellet) were mixed with sample buffer, and the solubilized proteins were resolved by SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride membrane and probed with anti-S4 (upper column), anti-20 S (middle column), or anti-ER60 (lower column) antibodies. The same polyvinylidene difluoride membrane blot was used for each detection by stripping and repробing cycles.

Degradation of HMG-CoA Reductase in the HTG-extracted Pellet—The in vitro degradation of HMG-CoA reductase was measured in the HTG-extracted pellet material to further investigate the possible role of the proteasome in reductase degradation. The rate of degradation of reductase in the HTG-extracted pellet was compared with that observed with intact ER. As shown in Fig. 7A, the rate of loss of reductase in the HTG-extracted pellet is almost the same as the loss in intact ER. Degradation of HMG-CoA reductase in HTG-extracted pellet from cells grown in the absence of sterols is slower than in that from cells supplemented with sterol, verifying that the degradation of reductase in HTG-extracted pellet continues to reflect the action of physiological regulatory molecules. (Fig. 8B). The sensitivities of degradation to various protease inhibitors in HTG-extracted pellet are the same as observed with intact ER (Fig. 8C). These results indicate that the degradation of reductase in HTG-extracted ER membranes is the same proteolytic process as that observed in intact ER and that the proteasome and luminal proteins are not required for the degradation of the reductase in vitro.

Proteolytic Intermediates of HMG-CoA Reductase Degradation—The topological aspects of reductase degradation are complex given that the protein exists in three compartments, the ER lumen, the membrane, and the cytosol. Thus, it would be informative to determine the cleavage site(s) for reductase degradation in vitro. It has not been possible to detect proteolytic intermediates of reductase degradation in vitro. To analyze the cleavage site of HMG-CoA reductase in vitro, we carried out in vitro incubation and checked for immunoreactive intermediate bands using several antibodies raised to various domains of the reductase. The antibodies were prepared for synthetic peptides and are named for the number of the N-terminal residue of the 18–20 amino acids in the peptide (see “Experimental Procedures”). The antibodies used were anti-224, anti-284, anti-717, and anti-825. Anti-224 and anti-284 were pre-

![FIG. 5. Inhibitor study of sLLVY-AMC hydrolyzing activity of isolated ER fraction. The proteasome activity of isolated ER was measured using sLLVY-AMC as a substrate in the presence or absence of various protease inhibitors. The reaction was carried out as described under “Experimental Procedures.” Data were represented as percentage of activity. Error bars indicate the range of deviation in three independent experiments using three independent preparations of ER.](image)

![FIG. 6. Effect of lactacystin or E64 on the sLLVY-AMC hydrolyzing activity of purified proteasomes (A) and purified ER fraction (B). A, the sLLVY-AMC hydrolyzing activity of purified 20 or 26 S proteasome was measured in the presence or absence of various concentrations of lactacystin or E64. The reaction mixture (200 μl) contained 0.11 μg of purified 26 S proteasome protein or 0.28 μg of purified 20 S proteasome protein. The reaction was carried out as described under “Experimental Procedures.” Data were represented as percentage of activity. B, the sLLVY-AMC hydrolyzing activity of purified ER fraction was measured in the presence or absence of various concentrations of lactacystin or and E64. The reaction mixture (200 μl) contained about 70 μg of ER proteins. The reaction was carried out as described under “Experimental Procedures.” Data were represented as percentage of activity.](image)

![FIG. 7. Solubilization of ER membranes with HTG. ER membranes prepared from cells grown in MEM, 5% LPS plus compactin (20 μg/ml) supplemented with 2.5 μg 25-hydroxycholesterol were solubilized with HTG and separated into supernatant and pellet subfractions as described under “Experimental Procedures.” Equal volumes of total ER (Total ER), supernatant (HTG sup), and pellet (HTG pellet) were mixed with sample buffer, and the solubilized proteins were resolved by SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride membrane and probed with anti-S4 (upper column), anti-20 S (middle column), or anti-ER60 (lower column) antibodies. The same polyvinylidene difluoride membrane blot was used for each detection by stripping and repробing cycles.](image)
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In the current studies, we characterize the in vitro cleavage of HMG-CoA reductase and identify the kind of protease(s) involved in this process. Since the 26 S proteasome and the ubiquitin system have recently been implicated in the degradation of many cellular proteins, including the degradation of HMG-CoA reductase, we initiated the experiments reported here with a focus on the probable involvement of the proteasome in reductase degradation.

Nevertheless, our experiments indicate that a cysteine protease is involved in reductase degradation both in vivo and in vitro. Several observations support this view: 1) the inhibition of reductase degradation in vivo by E64-d, a cysteine protease inhibitor that does not inhibit the proteasome (31); 2) the in vitro protease inhibitor sensitivity, particularly inhibition of cleavage by E64 but not lactacystin (Fig. 2); 3) inhibition of reductase cleavage by NEM (Fig. 3); and 4) persistent cleavage of reductase in vitro after removal of detectable proteasome from the preparation by detergent extraction (Fig. 8). In addition, we have previously reported that ubiquitination is not required for reductase degradation in vitro (15). These data suggest a membrane-bound cysteine protease and argue against the involvement of the proteasome.

In addition, HgCl₂ and diamide also block degradation in vitro (data not shown). These results demonstrate that the presence of a free SH group(s) is crucial to the cleavage of HMG-CoA reductase in vitro. It is notable that the ER degradation of HMG-CoA reductase in vitro is also inhibited by general thiol reagents (28). While it is possible that thiol reagents and dithiothreitol are exerting an effect on reductase itself, it seems more likely that they are affecting the putative cysteine protease(s). In addition, EGTA (5 mM) had no effect on the degradation of HMG-CoA reductase (data not shown), suggesting that calpain is not involved in this process, since calpain requires calcium for activity. From these results, it seems more likely that they are affecting the putative cysteine protease(s).
The results reported suggest that the proteasome might be involved either indirectly or in events specific to reductase degradation but not demonstrable in vitro. Many possibilities exist for an indirect role including a pathway where the proteasome is necessary to activate a step directly involved in reductase degradation. This kind of sequential pathway would explain why in vivo reductase degradation is inhibited by both E64-d, a cysteine protease inhibitor, and lactacystin, a proteasome inhibitor. It is also possible that the in vitro system reflects only a portion of the degradation process. In this regard, it is important to keep in mind that the cells must be preprogrammed with sterols before the purified ER fractions reflect physiologically accelerated rates of degradation. It is also noteworthy that we are able to detect proteolytic intermediates in the span 8 region between residues 313–339 with the membrane domain of bacteriorhodopsin dramatically extends both the basal and sterol-accelerated half-life of reductase in vivo, suggesting that this mutant protein is resistant to proteolysis. More recently, we have been able to show that replacement of 5 amino acids in this region results in a protein with an extended half-life (data not shown). This information, including the results obtained in the current study, suggests that the span 8 region might be the target of an initial cleavage. Previous studies (39) have shown that hypotonic lysis of CHO cells results in a degraded reductase and the size of the fragments detected in these studies roughly agrees with the fragment size reported here. We doubt these are the same fragments, because we can also see the “hypotonically induced” fragments if we are not careful in preparation of our extracts for membrane isolation. Further analysis will be required to clearly determine if the fragments are different.

The lack of inhibition of reductase degradation by lactacystin in vitro argues that the proteasome is not involved in the in vitro cleavage process. Yet lactacystin effectively blocks degradation in vivo. Yet again, E64-d blocks reductase degradation in vivo, and it is not a proteasome inhibitor. These paradoxical results suggest the following possibilities: 1) the in vitro, lactacystin-insensitive cleavage is a nonphysiological process; 2) the lactacystin-sensitive step in the in vivo degradation is only indirectly related to reductase degradation; and/or 3) the in vitro cleavage is detecting one step, maybe the first, in a series of reactions that lead to reductase degradation in vivo.

We feel that the in vitro cleavage we describe indicates a physiological process, because it is accelerated by pretreatment of cells with a physiological regulatory molecule, 25-hydroxycholesterol. In addition, we detect intermediate cleavage products that indicate that one step in the in vitro process is cleavage in the span 8 region of the membrane domain, a region that previous mutagenesis experiments have indicated is necessary for rapid proteolysis in vivo (7). It is also possible, however, that the treatment of cells with 25-hydroxycholesterol alters the structure of reductase and results in increased sensitivity to a nonphysiological, endogenous protease, and the increased rate of in vitro cleavage reflects this altered structure/increased protease sensitivity.

Importantly, data have been presented recently demonstrating a lack of degradation of HMG-CoA reductase in yeast cells defective in the proteasome (35). In addition, both genetic and biochemical data have been reported showing that yeasts defective in a ubiquitin-conjugating enzyme, UBC7, do not degrade reductase, and moreover the ubiquitination of reductase was directly demonstrated and shown to be regulated by the mevalonate pathway (36). The reasons for the apparent differences in the proteolysis systems for reductase degradation between yeast and mammalian cells will no doubt be clarified with further work.

The results reported suggest that the proteasome might be involved either indirectly or in events specific to reductase degradation but not demonstrable in vitro. Many possibilities exist for an indirect role including a pathway where the proteasome is necessary to activate a step directly involved in reductase degradation. This kind of sequential pathway would explain why in vivo reductase degradation is inhibited by both E64-d, a cysteine protease inhibitor, and lactacystin, a proteasome inhibitor. It is also possible that the in vitro system reflects only a portion of the degradation process. In this regard, it is important to keep in mind that the cells must be preprogrammed with sterols before the purified ER fractions reflect physiologically accelerated rates of degradation. It is also noteworthy that we are able to detect proteolytic intermediates in vitro but not in vivo. It seems possible that the in vitro system reflects the final step in a regulatory sequence and the initial step in a multistep degradation pathway. It is our hope that this simpler biochemically tractable in vitro system will allow us to characterize the ER-bound cysteine protease and eventually resolve the currently paradoxical situation of possible proteasome involvement in vivo but not in vitro.

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