The Ubiquitin-Proteasome Pathway Mediates the Regulated Degradation of Mammalian 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase*

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3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), the key regulatory enzyme in the mevalonate (MVA) pathway, is rapidly degraded in mammalian cells supplemented with sterols or MVA. This accelerated turnover was blocked by N-acetyl-leucyl-leucyl-norleucinal (ALLN), MG-132, and lactacystin, and to a lesser extent by N-acetyl-leucyl-leucyl-methioninal (ALLM), indicating the involvement of the 26 S proteasome. Proteasome inhibition led to enhanced accumulation of high molecular weight polyubiquitin conjugates of HMGR and of HMGal, a chimera between the membrane domain of HMGR and β-galactosidase. Importantly, increased amounts of polyubiquitinated HMGR and HMGal were observed upon treating cells with sterols or MVA. Cycloheximide inhibited the sterol-stimulated degradation of HMGR concomitantly with a marked reduction in polyubiquitination of the enzyme. Inhibition of squalene synthase with zaragozic acid blocked the MVA- but not sterol-stimulated ubiquitination and degradation of HMGR. Thus, similar to yeast, the ubiquitin-proteasome pathway is involved in the metabolically regulated turnover of mammalian HMGR. Yet, the data indicate divergence between yeast and mammals and suggest distinct roles for sterol and nonsterol metabolic signals in the regulated ubiquitination and degradation of mammalian HMGR.

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), catalyzes the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A to MVA, the major regulatory step in the MVA pathway that leads to the synthesis of cholesterol and a variety of essential nonsterol isoprenoids. The content of HMGR is controlled according to the cellular demands for sterols and nonsterol products derived from MVA (see Ref. 1 for review). This metabolic control is achieved through changes in the rate of HMGR gene transcription (2–5), altered stability and translational efficiency of its mRNA (6–10), and by accelerated degradation of the enzyme when these requirements have been satisfied (7, 11, 12).

HMGR is a 97-kDa integral membrane glycoprotein of the ER, which consists of a C-terminal catalytic domain that faces the cytoplasm and a non-catalytic N-terminal membrane domain that anchors the enzyme in the ER (13, 14). This hydrophobic domain, with its eight membrane spans (15), constitutes the cis-acting element that is necessary and sufficient to regulate the enzyme’s stability (16–19).

The signaling pathway(s) and proteolytic machinery that is responsible for the degradation of mammalian HMGR in response to metabolic cues remain largely unknown. Studies by Simoni and coworkers (20, 21) have revealed that degradation of HMGR takes place in a pre-Golgi compartment in a process that requires ongoing protein synthesis. Moreover, HMGR degradation is blocked by ALLN and ALLM, known inhibitors of calpains, which also inhibit proteasome activity, as well as by the more specific proteasome inhibitor lactacystin (22–24). These latter observations suggested that the proteasome, or an as yet unidentified lactacystin-sensitive protease(s), is involved either directly or indirectly in the degradation of HMGR.

Genetic studies in Saccharomyces cerevisiae have identified several factors that are involved in the degradation of HMGR. Similar to the mammalian enzyme, the stability of the yeast HMGR isozyme Hmg2p is controlled by the intracellular levels of MVA-derived metabolite(s) (25, 26). At least three genes, HRD1, HRD2, and HRD3 were implicated in Hmg2p degradation. Hrd1p/Der3p and Hrd3p are generally involved in the reverse translocation of ER membrane and luminal proteins for their disposal in the cytoplasmic ubiquitin-proteasome pathway (27–30), and Hrd2p was identified as a subunit of the 26 S proteasome (26). Hmg2p degradation is also strongly dependent on the activity of the ubiquitin-conjugating enzyme Ubc7p (26, 31). Together, these results demonstrated that the ubiquitin-proteasome pathway mediates the degradation of Hmg2p in yeast. Indeed, regulated attachment of polyubiquitin chains to Hmg2p was directly demonstrated, with farnesol being implicated as the MVA-derived metabolic product that signals for this process (32, 33). Both ubiquitination and regulated turnover of Hmg2p required specific structural determinants within the membrane domain of the enzyme (34–36).

In the current study, we demonstrate that, similar to Hmg2p, the metabolically regulated degradation of mammalian HMGR involves attachment of polyubiquitin chains to the protein as well as proteasomal activity. However, unlike yeast, both polyubiquitination and degradation of the mammalian reductase are largely driven by the sterol products of MVA, in a process that requires ongoing protein synthesis.
EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. Genetin was from Life Technologies. Lactacystin and MG-132 were purchased from Calbiochem Corp. ALLN and ALLM were obtained from Roche Molecular Biochemicals. Pro-mix 35S cell labeling mix was from Amersham Pharmacia Biotech. Mevalonolactone was from Fluka, and 25-hydroxycholesterol was from Steraloids. Immunobilized recombinant Protein A was obtained from RepliGen. MicroBCA protein reagent and SuperSignal chemiluminescent substrate were from Pierce. Lovastatin and ZA were kindly provided by Merck Laboratories. Lipoprotein-deficient fetal calf serum (LPDS; d = 1.25) was prepared by ultracentrifugation (37).

Antibodies—HMGal was immunoprecipitated with rabbit polyclonal anti-HMGal (Cytosolic antibodies (Cortex Biochemicals Inc.). Ubiquitin was detected with a monoclonal antibody from BabCo (clone P4D1). Jerry Faust (Tufts University) kindly provided the A9 anti-HMG monoclonal antibody, and specific antisera against the membrane domain of HMGal was prepared in rabbits, as described previously (15). HRP-conjugated goat anti-mouse or anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories.

Cells and Media—CHO cells were grown in MEM containing 5% (v/v) FCS, and HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS. All media also included 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. The media of cells stably transfected with pHMIGal (Neo-38) also contained Genetin at 250 µg/ml. To induce high levels of HMGal, 24 h before the experiments, cells were switched to media in which LPDS substituted for FCS and was also supplemented with 2 µM compactin and 100 µM MVA (20, 21, 38). HMGal-overexpressing LP-90 cells (38) were continuously maintained and assayed in MEM containing 5% (v/v) LPDS and 90 µM lovastatin. Where indicated, steroids were added to final concentrations of 2 µM 25-hydroxycholesterol plus 20 µg/ml MVA, and 20 mM mevalonolactone, to a final concentration of 20 mM (38).

Metabolic Labeling, Immunoprecipitation, and Immunoblotting—These procedures were performed as described previously (38), with the exception that lysis buffer (21) also contained 5 mM N-ethylmaleimide. Blots were stripped by a brief immersion in 0.1 M glycine-HCl, pH 2, before reprobing with different primary antibodies.

RESULTS AND DISCUSSION

The possible involvement of ubiquitin-proteasome pathway in HMGal-regulated turnover in mammalian cells was initially evaluated by examining the effect on this process of compounds known to inhibit proteasome activities. As shown in Fig. 1, HMGal-overexpressing LP-90 cells (38) were pulse-labeled with 35S-labeled methionine/cysteine and chased either in the absence (Fig. 1, compare lanes 1, 17, and 18) or presence (Fig. 1, lanes 2–8, 19–21, and 22–28) of sterols and the indicated concentrations of the inhibitors. Cell lysates were prepared in lysis buffer containing 1% Nonidet P-40 and 1% sodium deoxycholate (21, 38), and centrifuged at 16,000 × g for 30 min. HMGal was immunoprecipitated from the supernatant fraction with anti-membrane domain antisera and analyzed by 5–15% SDS-PAGE and fluorography. Quantitative results, obtained by densitometric scanning of the gels, are described in the text.

![Fig. 1. Inhibition of HMGal degradation by peptide aldehydes.](http://www.jbc.org/)

**Fig. 1. Inhibition of HMGal degradation by peptide aldehydes.** LP-90 cells were pulse-labeled for 30 min with 150 µCi of Pro-mix 35S cell labeling mix and either lysed immediately (lanes 1, 17, and 18) or chased for 5 h in the absence (lanes 19 and 19) or presence (lanes 2, 10–16, and 20–28) of sterols and the indicated concentrations of the inhibitors. Cell lysates were prepared in lysis buffer containing 1% Nonidet P-40 and 1% sodium deoxycholate (21, 38), and centrifuged at 16,000 × g for 30 min. HMGal was immunoprecipitated from the supernatant fraction with anti-membrane domain antisera and analyzed by 5–15% SDS-PAGE and fluorography. Quantitative results, obtained by densitometric scanning of the gels, are described in the text.
(denoted by the bracket) that, in some experiments, appeared to be banded at intervals of ~10 kDa (for example, see Fig. 4). These results suggested that ubiquitinated species of HMGR are accumulating in MG-132-treated cells. To directly examine whether HMGR was modified by polyubiquitin chains and to study the relationship between enzyme ubiquitination and regulated degradation, anti-membrane domain antibodies were used to immunoprecipitate HMGR from CHO cells that were treated with sterols and/or MG-132. The immune complexes were resolved by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with anti-ubiquitin monoclonal antibody (Fig. 3). In untreated cells (Fig. 3A, lane 1) there was very little high molecular weight material that reacted with the ubiquitin antibody. The intensity of this material increased upon addition of sterols; its levels peaked within 30 min of sterol treatment and gradually declined in the following 4.5 h (Fig. 3A; lanes 1–4). During this time course, HMGR immunoreactivity rapidly diminished and by 5 h its total levels decreased 3-fold (Fig. 3B, lanes 1–4). When MG-132 was added (lanes 5–7), the intensity of the ubiquitin-containing material was markedly enhanced (Fig. 3A, lanes 5–7), and there was also a noticeable increase in the amounts of HMGR (Fig. 3B, lane 5–7). Treatment with sterols together with MG-132 caused massive accumulation of ubiquitinated material that was precipitated with anti-HMGR antibodies, even more pronounced than with MG-132 alone (Fig. 3A, lanes 8–10). As expected, MG-132 blocked the sterol-dependent decrease in HMGR levels (Fig. 3B, lanes 8–10). Significantly, sterols neither affected the extent nor the general pattern of total protein ubiquitination in the cells (Fig. 3C, compare lane 1 to 2). MG-132, on the other hand, augmented the intensity of ubiquitinated cellular proteins (Fig. 3C, lane 3). It should be noted (Fig. 3A) that the electrophoretic mobility of the immunoprecipitated ubiquitin-decorated material corresponded to proteins larger than the 97-kDa size marker, indicating that it originated from the 97-kDa HMGR. Indeed, reprobing the blot with anti-reductase antibodies demonstrated the presence of HMGR at the leading edge of this ubiquitinated material (Fig. 3A, lane 11). No ubiquitin-containing material could be detected when we used preimmune serum as the precipitating antibody, or when anti-reductase antiserum was omitted altogether (data not shown). These results demonstrated that HMGR is specifically subjected to polyubiquitination in a sterol-regulated manner. Moreover, HMGR appears to be ubiquitinated in a transient manner unless proteasomal activity is blocked. Under the latter conditions, the amounts of polyubiquitin chains that are attached to HMGR greatly increase and persist. Similar results were obtained when we used 20 mM MVA, instead of sterols, to accelerate the degradation of HMGR (data not shown; see also Fig. 9).

Two lines of experimental evidence corroborated these conclusions. First, we examined whether an acute increase in intracellular flux of MVA affected ubiquitination of HMGR. For this, we utilized LP-90 cells that were thoroughly washed to remove lovastatin. At various time points HMGR was precipitated with anti-HMGR antibodies, and the immune complexes were blotted and probed with anti-ubiquitin antibodies (Fig. 4). As can be seen, HMGR was modified with ubiquitin to some extent upon treatment with high concentrations of sterols. Cell lysates were prepared in lysis buffer, as described in Fig. 1, and centrifuged at 16,000 × g for 30 min. The pellets were resuspended and sonicated in lysis buffer, which also contained 1% SDS. Aliquots (15 µg out of 150 µg of total protein) of the supernatant fraction and the entire pellet (15 µg of protein) fractions were resolved by 5–15% SDS-PAGE. Proteins were transferred to nitrocellulose and probed with A9 anti-HMGR monoclonal antibody. Note the presence of higher molecular weight species of HMGR, especially in lanes 5 and 6 (indicated by the square bracket).
extent even in cells that were continuously maintained in lovastatin (Fig. 4A; lane 1). As early as 30 min after lovastatin removal, the level of ubiquitinated HMGR species rose 2-fold (Fig. 4A; lane 2), they peaked to nearly 3-fold by 1 h (lane 3) and persisted at elevated levels during the following 4 h. During this time course, the amount of HMGR protein decreased with a half-life of 2 h (Fig. 4B). The decline in the ubiquitinated HMGR from the 6-h time point and on (Fig. 4A) was a reflection of the actual loss of the protein (Fig. 4B). In Fig. 4C, we calculated the ratio between the intensity of the HMGR-associated ubiquitinated material and the intensity of HMGR band. This value of “specific ubiquitination” rapidly increased ~5-fold within the first hour after lovastatin removal, suggesting that, at this early stage, the rate of HMGR polyubiquitination is faster than its degradation and consistent with the notion that polyubiquitination is a prerequisite for reductase proteolysis. Specific ubiquitination remained relatively constant for up to 5–6 h and then rose again, peaking at nearly 17-fold by 8.5 h, after which time it dropped precipitously. This indicates that attachment of multiquitin chains to HMGR proceeds efficiently, with a rate approximating its proteolytic elimination, even when only residual HMGR remains in the cell.

Second, we analyzed whether HMGal was also ubiquitinated in a sterol-enhanced manner. HMGal is an excellent model protein to study the regulated turnover of HMGR, because the intracellular localization and fate of both proteins are essentially indistinguishable (15, 18, 19). In Fig. 5, HMGal-expressing HEK-293 cells were treated for up to 5 h with sterols, with MG-132, or with both. HMGal was immunoprecipitated with anti-β-galactosidase antibodies and blotted with anti-ubiquitin antibody. Clearly, within 30 min after addition of sterols, there was a noticeable increase in the levels of ubiquitinated material (Fig. 5A, lanes 2–4). These levels remained relatively constant for up to 5 h. At the same time, the levels of HMGal protein decreased by about 50% (Fig. 5B, lanes 1–4). Because HMGal is constitutively expressed from a sterol-insensitive viral promoter, its levels, as detected by immunoblotting, decrease more slowly than endogenous HMGR (Fig. 3B). Blockage of proteasomal activity with MG-132 resulted in an impressive rise in ubiquitinated HMGal (Fig. 5A, lanes 5–7), and the combination of sterols and MG-132 caused accumulation of even greater amounts of ubiquitinated species than with either agents alone (Fig. 5A, lanes 8–10). Again, because HMGal is constitutively expressed, during the 5 h of proteasome inhibition there was 50–75% increase in the amounts of HMGal (Fig. 5B, lanes 7 and 10). As for HMGR, the ubiquitinated material on the blot was demarcated with HMGal at its leading edge (Fig. 5A, lane 11). Interestingly, at longer times of exposure to MG-132, there was a buildup of ubiquitinated material of heterogeneous size that migrated faster than HMGR (see Fig. 3A, lanes 6, 7 and 9, 10) or HMGal.

**Fig. 4.** Ubiquitination of HMGR in lovastatin-relieved LP-90 cells. LP-90 cells, maintained in MEM/5% LPDS/90 μM lovastatin, were washed three times in phosphate-buffered saline and re-fed with medium lacking lovastatin. At the indicated time points, the cells were lysed and HMGR was immunoprecipitated with anti-membrane domain antiserum. The immune complexes were resolved by 5–15% SDS-PAGE and blotted onto nitrocellulose membrane. A, ubiquitin conjugates were detected with P4D1 anti-ubiquitin monoclonal antibody (αUb). B, the membrane was stripped and reprobed with A9 anti-HMGR monoclonal antibody (αHMGR). HMGR is indicated by the arrow. C, the densitometric intensity of the ubiquitin reactive material in A was divided by the intensity of the 97-kDa HMGR band in B to yield specific ubiquitination, and this ratio was arbitrarily set as 100% at time zero. Results are the mean of three independent experiments.

**Fig. 5.** Sterols enhance ubiquitination of HMGal expressed in HEK-293 cells. HEK-293 cells stably transfected with pHMGal-Neo plasmid and expressing high HMGal activity were isolated by limited dilution, as described previously (38). A, cells were prepared for the experiment and processed, exactly as described in Fig. 3. HMGal was immunoprecipitated with anti-β-galactosidase polyclonal antibodies, and ubiquitin conjugates were detected by immunoblotting with anti-ubiquitin monoclonal antibody (αUb). Lane 11 and panel B were obtained by reprobing the membrane with anti-β-galactosidase monoclonal antibody (αβGal). The migration of molecular mass standards (in kDa) is indicated. HMGal is denoted by the arrow.
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The degradation of Hmg2p in yeast is insensitive to inhibitors of protein translation such as cycloheximide (25, 35, 36). In contrast, the regulated turnover of HMGR in CHO cells requires continuous protein synthesis, suggesting the involvement of short-lived protein(s) in relaying the metabolic degradation signal(s) evoked by MVA/sterols and/or in tagging HMGR for proteolysis (20, 21). Thus, if conjugation of polyubiquitin chain(s) to HMGR earmarks it for proteolysis by the 26S proteasome, then it would be interesting to examine whether inhibition of protein synthesis significantly affected this process. As previously shown, arresting protein synthesis with cycloheximide markedly inhibited the sterol-accelerated degradation of HMGR in CHO cells (Fig. 6A and Refs. 20, 21). Cycloheximide also decreased 5-fold the levels of ubiquitin immunoreactive material that was precipitated with anti-HMGR antibodies (Fig. 6B, e.g. compare lane 1 to 5), despite the fact that equal amounts of enzyme were present in these cells (Fig. 6C). These results suggest that the putative short-lived protein(s) involved in the controlled turnover acts upstream of tagging HMGR with ubiquitin and, again, couple HMGR degradation with ubiquitination.

Because the levels of polyubiquitin chains on a target protein are determined by the relative rates of ubiquitination and de-ubiquitination (40), it was important to examine whether the lower amounts of HMGR-ubiquitin conjugates in cycloheximide-treated cells resulted from attenuated ubiquitination or and increased rate of de-ubiquitination. Ubiquitin conjugates were first allowed to accumulate by incubating CHO cells for 3 h with sterols and MG-132. The cells were then thoroughly washed and chased for additional 6 h in the absence or presence of cycloheximide, MG-132, or both, but without sterols, and the immunoprecipitated HMGR was blotted with anti-ubiquitin antibodies (Fig. 7). Clearly, high levels of ubiquitin conjugates were present in the cells after a 3-h exposure to both sterols and MG-132 (Fig. 7A, compare lane 1 to 2). Under these conditions of proteasome inhibition, the amounts of HMGR remained constant even though sterols were present during this preincubation period (Fig. 7B, lane 1 and 2). When the cells were chased for 6 h without MG-132, the level of the preformed ubiquitin conjugates was markedly reduced (Fig. 7A, lane 3), concomitantly with the disappearance of HMGR protein (Fig. 7B, lane 3), despite the absence of sterols during the chase period. This result shows that MG-132 inhibits HMGR degradation in a reversible fashion. The decline, both in HMGR and in its attached polyubiquitin chains, was blocked...
by MG-132 (Fig. 7, B and A, respectively, lane 5), demonstrating that ubiquitinated HMGR is readily eliminated by the proteasome. When the cells were chased in the presence of cycloheximide, HMGR-ubiquitin conjugates were almost completely depleted (Fig. 7A, lane 4), indicating that, once formed, cycloheximide no longer interferes with their proteolysis. These results illustrate that the polyubiquitination of HMGR and the actual proteasomal degradation of ubiquitinated enzyme can be temporarily uncoupled. That is, the sterol-enhanced ubiquitinated HMGR that accumulates when the proteasome is blocked may be rapidly degraded once proteasomal inhibition is released, and sterols are no longer required for this proteolysis. The lower levels of HMGR-ubiquitin conjugates in lane 4 are expected, because, in addition to their disposal, no new HMGR molecules were synthesized in the cycloheximide-treated cells. This is also indicated by the near absence of HMGR in the extracts of such cells (Fig. 7B, lane 4). Moreover, no sterols were present during this incubation period to evoke ubiquitination of the residual HMGR molecules. Remarkably, when exposed to both cycloheximide and MG-132, very high levels of ubiquitinated HMGR species persisted in the cells during the 6-h chase (Fig. 7A, lane 6), even though the cells contained very little HMGR protein (Fig. 7B, lane 6). That is, HMGR was labeled to a much greater “specific ubiquitination.” These results demonstrate that cycloheximide does not stimulate the de-ubiquitination of HMGR. Rather, the fewer polyubiquitin chains on HMGR under conditions of inhibited protein synthesis are due to an attenuated rate of ubiquitination.

Studies in mammalian cells and in yeast have strongly implicated FPP, or its derived free alcohol farnesol, as the MVA-derived nonsterol metabolite that signals for the accelerated degradation of HMGR (41–46). Increasing the levels of FPP in S. cerevisiae, either pharmacologically or genetically, accelerated the rate of Hmg2p degradation. Conversely, decreasing the rate of FPP synthesis stabilized the enzyme. These variations in FPP concentrations were accompanied by the respective increase or decrease in Hmg2p ubiquitination (32, 33). To study the possible role of FPP in signaling for ubiquitination of mammalian HMGR, we first examined the effect of ZA on the regulated turnover of the enzyme in a radioactive pulse-chase experiment (Fig. 8). In the absence of MVA or sterols, inclusion of 150 μM ZA in the chase increased the half-life of HMGR from 12 h to ~30 h (Fig. 8, lanes 1–5). Addition of MVA increased the rate of HMGR turnover 4-fold ($t_{1/2} = 3$ h), and ZA blocked this accelerated degradation effectively ($t_{1/2} = 18$ h; Fig. 8, lanes 5–9). This result concurs with an earlier report by Correll and Edwards (43) in which the effect of ZA on the degradation of HMGR was examined in the CHO variant met-18-b2 cells. However, in contrast to their findings, we found that ZA did not block the sterol-induced elimination of the enzyme ($t_{1/2} = 2.5$ h and 2 h in the absence or presence of ZA, respectively; Fig. 8, lanes 10–14). Similarly, ZA did not inhibit the degradation of HMGR in cells chased with sterols plus MVA ($t_{1/2} = 1.5$ h regardless of the presence of ZA; Fig. 8, lanes 14–18).

Next, the effect of ZA on HMGR ubiquitination was assessed in cells treated with MVA, sterols, or MVA plus sterols (Fig. 9). Clearly, ZA nearly completely abolished the MVA-dependent, but not sterol-enhanced, conjugation of ubiquitin to HMGR (Fig. 9A; compare lanes 6 and 7 to lanes 2 and 3, respectively). ZA had only a negligible inhibitory effect on HMGR-ubiquitin levels in cells challenged with both sterols and MVA (Fig. 9A; compare lane 8 with lane 4). Correspondingly, ZA did not inhibit the decrease in the amounts of HMGR protein upon addition of sterols (Fig. 9B; compare lane 7 to lane 3), and only marginally attenuated the drop in enzyme levels in cells treated with MVA plus sterols (Fig. 9; compare lane 8 to lane 4). Taken together, these results indicate that the inhibition of the MVA-accelerated HMGR degradation by ZA is mainly, if not exclusively, the result of blockage of sterol synthesis. Thus, the putative accumulation of FPP in ZA-treated CHO cells does not appear to lead to rapid elimination of HMGR.

The results presented in this work are the first direct demonstration that the ubiquitin-proteasome pathway operates in the metabolically regulated degradation of HMGR in mammalian cells. Our conclusions are at variance with previous reports, utilizing temperature-sensitive mammalian cell lines, which showed that inactivation of the thermolabile ubiquitin-activating enzyme E1 did not hamper sterol-accelerated degradation of HMGR (24, 47). Although these mutant cell lines are a valuable tool to study the role of the ubiquitin pathway in proteolysis, failure to demonstrate the necessity for E1 in protein ubiquitination or/and degradation can be attributed to many factors (48). We show that mammalian HMGR and HM-Gal are modified with mult ubiquitin chains and are subse-
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...ently eliminated by the 26 S proteasome. Furthermore, the extent of HMGR/HMGal polyubiquitination is greatly enhanced by sterols or by flooding the cells with high concentrations of MVA, either form exogenous (Fig. 9) or endogenous (Fig. 4) sources. These metabolic conditions have been shown to accelerate the rate of degradation in vivo. It should also be noted that, even without proteasome inhibitors, appreciable amounts of ubiquitinated HMGR/HMGal could be detected in the absence or very shortly (within 30 min) after challenging the cells with degradation stimuli, much before the disappearance of these proteins could be actually observed (in many cases, at times > 2 h; e.g. Figs. 4 and 5). Moreover, we show (Fig. 7) that ubiquitination of HMGR can be uncoupled from proteasomal degradation. Thus, it is most likely that the attachment of mult ubiquitin chains to HMGR/HMGal destined them for degradation.

Ubiquitin is covalently attached in an isopeptide bond via its C-terminal carboxyl to ε-amino group of specific lysine(s) in the substrate protein (48). Inasmuch as both HMGR and HMGal share the same sequence in their membrane-spanning and linker domains (up to residue 449 in the hamster protein (18)), either the ubiquitin-modified lysines are located in this common portion of both proteins or the membrane domain specifies ubiquitin attachment to lysine(s) in the proteins’ unique cytoplasmic domains. A recent extensive analysis by site-directed mutagenesis of Hmg2p revealed that the MVA-regulated degradation of the yeast enzyme requires structural information that is distributed over its entire transmembrane domain (“distributed degron”), with critical dependence on Lys6 and Lys357 (36). Whether these lysines also serve as acceptor sites for polyubiquitin conjugation to Hmg2p is not known. Although the overall secondary structure of the membrane domain of mammalian and yeast HMGR appears similar (49) and consists of eight transmembrane spans (15), there is little sequence conservation in this region between the two enzymes. The lysine(s) that serve as acceptor(s) for the polyubiquitin moieties in mammalian HMGR are yet to be identified.

The selective disposal of a protein substrate in the ubiquitin pathway in response to appropriate cues requires the attachment of polyubiquitin chains in a process distal to the signals. This is executed by distinct sets of ubiquitin-conjugating (E2) and ubiquitin-protein ligating (E3) enzymes, which display a rather high degree of substrate specificity (50). Thus, the key questions still remaining relate to what constitutes the metabolic signal(s) that earmark HMGR for destruction, the identity of the E2 and E3 enzymes that attach polyubiquitin onto HMGR, and how is the metabolic signal relayed to these enzymes. It has been shown that steroid-accelerated degradation of HMGR does not proceed in cells that are acutely deprived of MVA (7, 21). Whether MVA-derived nonsterols can stimulate HMGR degradation when endogenous sterol synthesis is specifically inhibited is still controversial (21, 43). Nevertheless, the notion that rapid degradation of mammalian HMGR requires distinct sterol and nonsterol signals is further substantiated by their differential sensitivity to perturbation of cellular Ca2+ stores (21). Although our results with ZA (Figs. 8 and 9) indicate that sterols, rather than FPP, evoke the ubiquitination and subsequent degradation of HMGR in CHO cells, they may reflect quantitative differences between the sterol and nonsterol signals and not necessarily distinguish between their qualitative roles in reductase elimination. Yet, both signals are mediated by a cycloheximide-sensitive component(s) (Figs. 6, 7) (20, 21). Interestingly, regulation by sterols of ubiquitination and proteasomal degradation of translocation-arrested apolipoprotein B55 in CHO cells was also recently proposed by Du et al. (51). This is in contrast to the situation in yeast where ubiquitination/degradation of Hmg2p is not affected by cycloheximide and appears to be signaled exclusively by the intracellular levels of the nonsterol FPP (32, 33). These differences exemplify the additional complexity of regulated HMGR degradation in mammalian cells, which normally derive their bulk cholesterol through endocytosis of plasma lipoproteins, on top of endogenous synthesis of nonsterols in the MVA pathway. This contrast between yeast and mammalian cells may also reflect their distinct composition of sterol metabolites with ergosterol, rather than cholesterol, being the principal bulk end product of the MVA pathway in yeast.

Finally, it has been recently shown that inhibition of proteasomal activities or overexpression of some ER-retained membrane proteins lead to their intracellular accumulation as polyubiquitinated species in detergent-insoluble aggregates (52, 53). It was proposed that these “aggresomes” are normally formed when the capacity of the proteasome to degrade misfolded and/or aggregation-prone proteins is exceed (52). Although reminiscent of these studies, our results with LP-90 cells (Figs. 1 and 2) cannot be simply explained by proteasome inhibition, because the closely related ALLN and MG-132 exert disparate effects on HMGR detergent solubility at concentrations higher than those required for full blockade of proteasomal activity. Moreover, HMGR is highly overexpressed in LP-90 cells, yet these cells rapidly degrade the massive amounts of this polytopic membrane protein with half-life of ≤2 h (Fig. 1 and Ref. 38). If HMGR is indeed ubiquitinated and degraded by the proteasome, as our data here clearly demonstrate, then at this level of reductase overexpression the capacity of the proteasome to degrade it is hardly exceeded. Considered together, our results indicate that, even at high levels of protein expression, HMGR is being efficiently folded and maintained such that aggregation-prone hydrophobic surfaces are rarely exposed, even when the enzyme is polyubiquitinated and fed into the proteasome for final proteolysis.

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