Impaired Regulation of 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase Degradation in Lovastatin-resistant Cells*

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L-90 cells were selected to grow in the presence of serum lipoproteins and 90 \( \mu \)M lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR). L-90 cells massively accumulate HMGR, a result of >10-fold amplification of the gene and 40-fold rise in mRNA, and also overexpress other enzymes of the mevalonate pathway. Western blot and promoter-luciferase analyses indicate that transcriptional regulation of sterol-responsive genes by 25-hydroxycholesterol or mevalonate is normal. Yet, none of these genes is regulated by lipoproteins, a result of severe impairment in the low density lipoprotein receptor pathway. Moreover, L-90 cells do not accelerate the degradation of HMGR or transfected HMGal chimera in response to 25-hydroxycholesterol or mevalonate. This aberrant phenotype persists when cells are grown without lovastatin for up to 37 days. The inability to regulate HMGR degradation is not due to its overproduction since in L-90 cells, which were selected for lovastatin resistance in lipoprotein-deficient serum, HMGR is overexpressed, yet its turnover is regulated normally. Also, the rapid degradation of transfected \( \alpha \) subunit of T cell receptor is markedly retarded in L-90 cells. These results show that in addition to gene amplification and overexpression of cholesterogenic enzymes, statin resistance can follow loss of regulated HMGR degradation.

Mevalonate (MVA) is the first committed precursor for biosynthesis of cholesterol and a variety of essential nonsterol isoprenoids such as ubiquinone, dolichol, isopentenyl-modified tRNAs, side chain of heme A, and the farnesyl and geranylglycerol moieties of many cellular proteins (1–4). Cells must constantly synthesize MVA yet avoid accumulation of these products to toxic levels. This problem is especially acute in mammalian cells since cholesterol, the bulk end product of the MVA pathway, can be obtained also by receptor-mediated endocytosis of low density lipoproteins (LDL). Classical experiments by Brown and Goldstein (5) and their co-workers have demonstrated that the increase in intracellular levels of LDL-derived cholesterol triggers a feedback response that down-regulates the expression of the LDL receptor (LDLR) and represses endogenous cholesterol synthesis. Conversely, depletion of cholesterol leads to increased surface expression of LDLR and enhanced rate of cholesterol synthesis (6).

The major regulatory step in the MVA pathway is the conversion of HMG-CoA to MVA by the enzyme HMGR. Early experiments have shown that cultured mammalian cells maintain low rate of endogenous MVA production even in the presence of saturating amounts of sterols. HMGR activity can be fully repressed only if the synthesis of MVA-derived nonsterol is allowed, suggesting that HMGR activity (hence MVA synthesis) is controlled through multivalent feedback regulation by sterol and nonsterol MVA-derived metabolite(s) (7). These studies were greatly advanced by the discovery of statins, potent HMGR inhibitors, such as compactin and lovastatin (8, 9). When naive cells are exposed to statins in their growth medium, they respond by drastically increasing the amounts of HMGR to overcome the acute depletion in endogenously synthesized sterol and nonsterol compounds (6, 10). This metabolic control of HMGR is achieved through changes in the rate of HMGR gene transcription (11–13), differential translational efficiency, and stability of mRNA (14–18) and post-translationally by altering the stability of the reductase protein (15, 19–22). Thus, when cells are starved for sterols/MVA, the enzyme is stabilized and its half-life is prolonged, whereas abundance of sterols/MVA leads to its rapid degradation.

The mechanisms underlying the regulated degradation of HMGR are not yet fully understood. Unlike other early enzymes in the MVA pathway, HMGR is a resident glycoprotein of the endoplasmic reticulum (ER) with its active site, contained within the C-terminal portion of the protein, facing the cytosol (23). A highly conserved N-terminal hydrophobic domain, which spans the membrane eight times, anchors the enzyme in the ER (24). Deletion analyses have demonstrated that the membrane domain is dispensable for catalytic activity, protein; ER, endoplasmic reticulum; TCR\( \alpha \), T cell receptor \( \alpha \) subunit; kb, kilobase pair.
yet it is essential for the regulated turnover of the enzyme (22, 25). Moreover, when fused to the N termini of heterologous proteins, e.g. β-galactosidase of E. coli, the membrane domain confers ER localization and sterol/MVA-regulated degradation onto this chimeric HMGal protein (26–28). Although the precise structural determinants that alter enzyme stability have not been fully elucidated (27, 29), the membrane domain is believed to function as a sensor that monitors the levels of sterols and/or MVA-derived nonsterols in the ER membrane (29, 30). Other key questions relate to the intracellular site(s) of degradation, the role of potential trans-acting factor(s) that recognize HMG and tag it for rapid elimination when sterols/MVA are abundant, and the protease(s) that actually destroys the HMG protein. Current evidence indicates that HMG is degraded in the ER (31) in a process that involves a yet unidentified short-lived protein(s) (31, 32). Furthermore, HMG degradation is severely inhibited by ALLN (33, 34) and lactacystin (35) suggesting the participation of the 26 S proteasome. Indeed, a mutation in the 26 S proteasome has been shown to affect HMG degradation in yeast (36). Although conjugation of multi-ubiquitin chains to HMG was demonstrated in yeast (37), it remains to be established whether the ubiquitin pathway is involved also in the degradation of mammalian HMG.

To gain further insight into the regulated degradation of HMG, we sought to isolate cells that are defective in this process. In this work, we describe a CHO-derived cell line, designated L-90, that was selected for growth in the presence of serum lipoproteins and increasing concentrations of lovastatin. Our results demonstrate that L-90 cells survived the lovastatin selection by increasing the levels of HMG enzyme, in part by losing their ability to degrade HMG in a regulated manner. Analysis of L-90 cells should provide information on cellular factors that are involved in HMG turnover.

EXPERIMENTAL PROCEDURES

Materials—DNA restriction and modification enzymes were purchased from New England Biolabs (Beverly, MA) and MBI-Fermentas (Vilnius, Lithuania). HighPrime random priming DNA labeling kit was obtained from Roche Molecular Biochemicals (Mannheim, Germany). HighPrime random priming DNA labeling kit was purchased from Ambion (Austin, TX). Similarly, 32P-labeled antisense probe was stripped (45), and the membrane was successively rehybridized at 60 °C with 3 × 10^6 cpm/ml of an ~1.4-kb 32P-labeled EcoRI-PstI cDNA fragment of hamster HMG membrane-spanning region in 6× SSC, 5× Denhardt’s solution, 0.5% SDS, 100 μg/ml sheared denatured salmon sperm DNA. The membrane was washed in 2× SSC, 0.1% SDS (30 min; 42 °C), 1× SSC, 0.1% SDS (60 min; 42 °C), 0.1× SSC, 0.1% SDS (40 min; 42 °C), and an additional 30 min wash at 50 °C in the same solution. The membrane was dried and exposed to x-ray film at ~80 °C with intensifying screens. The radioactive probe was stripped (45), and the membrane was successively reprobed with 32P-labeled human LDLR and rat glyceroldehyde-3-phosphate dehydrogenase cDNAs.

DNA Transfections—CHO and L-90 cells were transfected by LipofectAMINE with 2 μg of each of the above-described promoter-Luc plasmids together with 0.2 μg of βGal-Neo, according to the manufacturer’s instructions. Stable transfectants were selected in a medium containing 750 μg/ml genetin, and 300–500 resistant colonies were then isolated by limiting dilution. The clones were then used to create a cDNA library in the E. coli vector pBluescript. The resulting library was hybridized overnight at 60 °C with 3 × 10^6 cpm/ml of an ~1.4-kb 32P-labeled EcoRI-PstI cDNA fragment of hamster HMG membrane-spanning region in 6× SSC, 5× Denhardt’s solution, 0.5% SDS, 100 μg/ml sheared denatured salmon sperm DNA. The membrane was washed in 2× SSC, 0.1% SDS (30 min; 42 °C), 1× SSC, 0.1% SDS (60 min; 42 °C), 0.1× SSC, 0.1% SDS (40 min; 42 °C), and an additional 30 min wash at 50 °C in the same solution. The membrane was dried and exposed to x-ray film at ~80 °C with intensifying screens. The radioactive probe was stripped (45), and the membrane was successively reprobed with 32P-labeled human LDLR and rat glyceroldehyde-3-phosphate dehydrogenase cDNAs. 

RNAase Protection Assay—To prepare an antisense RNA probe for HMG, we amplified a 206-base pair DNA fragment from the cytoplasmic domain of Chinese hamster HMG using pDG6 plasmid as the template and oligonucleotides 5′-CGGATTCTGGTCCGAGGTA- AACAGG-3′ (46 positions 1629–1648, including an EcoRI site), and 5′-CCGCTACGGGTTCAAGCAGGC-3′ (46 positions 1833–1814 plus a KpnI site) as the forward and reverse primers, respectively. The PCR product was digested with EcoRI and KpnI and ligated into Bluescript. The resulting DNA was linearized with KpnI and transcribed with T7 RNA polymerase in the presence of [α-32P]UTP using MaxiScript™ kit obtained from Ambion (Austin, TX). Similarly, 32P-labeled antisense RNA probe for the S17 ribosomal protein was transcribed using the AviII-digested pRS17 plasmid (17). Total cellular RNA was extracted with RNAzol B reagent (Biorex Laboratories, Houston, TX) and hybridized overnight at 50 °C with get-purified antisense probes, according
ing to the instructions provided by the manufacturer (RPAlT™ kit; Ambion). Following RNase digestion, the protected fragments were analyzed by 8 M urea/PAGE and autoradiography. After exposure, the protected bands were cut out of the gel and quantified by liquid scintillation.

**Metabolic Labeling, Immunoprecipitation, and Immunoblotting—** On Day 0, CHO (8 × 10^5) or L-90 (1.2 × 10^5) cells were plated in FCS medium in 60-mm dishes. On Day 1, the cells were refed with LPDS medium containing 2 μM compactin plus 100 μM MVA (CHO) or 90 μM lovastatin (L-90). On Day 2, the medium was aspirated, and the cells were starved for 1 h in 1 ml of starvation medium (methionine- and cysteine-free MEM supplemented with 5% LPDS and 2 μM compactin, 100 μM MVA (CHO) or 90 μM lovastatin (L-90 and LP-90). The cells were pulse-labeled for 30 min in 0.5 ml of starvation medium containing 100–150 μCi of Expres35S35S protein labeling mix and chased in LPDS medium supplemented with 2 mM methionine. Unless otherwise noted (e.g. Fig. 11), L-90 and LP-90 cells were chased in LPDS medium containing 90 μM lovastatin. CHO cells were chased in LPDS medium containing 2 μM compactin plus 100 μM MVA. Where indicated, sterols (0.25 g/ml hydroxysterol plus 20 μg/ml cholesterol) or MVA (20 μM) were added to the chase medium. The cells were lysed, processed for immunoprecipitation with the indicated antibodies, and samples were resolved by 5–15% gradient SDS-PAGE, as described previously (32). Because L-90 and LP-90 cells contain high levels of enzyme, only 10–13% of lysate volume was used for immunoprecipitating HMGR from these samples. This allowed comparable studies with CHO cells and showed that HMGR was precipitated quantitatively (data not shown). For immunoblot analyses, cells were plated as described above. Twenty four h prior to analysis, the cells received fresh FCS, LPDS, LPDS + ST, or LPDS + MVA media. After lysis and estimation of protein content, samples containing equal amounts of protein were separated by SDS-PAGE and transferred to Optitran BAS-83 reinforced nitrocellulose membranes (Schleicher & Schuell). The membranes were probed with the appropriate primary antibodies, followed by hors eradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reaction (32).

**Electron Microscopy—** Cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at room temperature. Fixed cells were scraped off the dish and post-fixed in 2% OsO4 in cacodylate buffer for 2 h. After dehydration in graded ethanol solutions, the cells were embedded in Epon. Ultra-thin sections were contrasted with uranyl acetate and lead citrate and examined with a Joel-1200 EX II electron microscope.

**Degradation of 125I-LDL—** On Day 0, 1 × 10^5 CHO cells or 1.5 × 10^5 L-90 cells were plated in a 24-well dish in FCS medium. On Day 1, the cells were refed with LPDS medium containing 2 μM compactin plus 100 μM MVA (CHO) or 90 μM lovastatin (L-90), in the absence or presence of 2 μg/ml 25-hydroxycholesterol and 20 μg/ml cholesterol. On Day 2, quadruplicate wells received 125I-LDL (250–650 Ci/ml) in the absence or presence of 400 μg/ml unlabeled LDL. Following incubation at 37 °C for 5 h, the amount of trichloroacetic acid-soluble 125I radioactivity was determined, as described (38).

**Measurements of Lipid Synthesis and Content—** CHO and L-90 cells were set on Day 0 in their respective FCS medium. On Day 1, the cells were washed extensively to remove lovastatin, and LPDS medium (without lovastatin) was added to both cell types. On Day 2, all dishes were washed, and duplicate plates received FCS medium with or without 90 μM lovastatin, as indicated in Table I, and the cells were pulse-labeled with 50 μCi of [3H]acetate. Cells were harvested, and [3H]-labeled lipids were separated by thin layer chromatography and quantified, as described (47). Cellular cholesterol content was quantified by gas-liquid chromatography.

**Enzymatic and Other Assays—** On Day 0, 1 × 10^5 CHO cells or 1.5 × 10^5 L-90 cells were plated in a 24-well dish in their respective FCS media. On Day 1, replicate wells were washed with PBS, and the cells were refed either with fresh FCS, LPDS, LPDS + ST, or LPDS + MVA media (see above). On Day 2, the cells were washed with PBS and processed for measurements of luciferase activity, according to the manufacturer’s instructions (Promega). The hydrolysis of o-nitrophenyl β-d-galactopyranoside by β-galactosidase was measured in digitonin-permeabilized cells, as described previously (26). Protein was measured according to Lowry et al. (48) or with the protein microassay reagent (Bio-Rad). Luciferase-specific activity was calculated as light units/mg of cell protein. β-Galactosidase-specific activity was calculated as A420/mg cell protein/h. In both cases, the results are expressed as percent of the specific activity in LPDS-treated cells (100%) in the same experiment. X-ray films were scanned and quantified using a

![Fig. 1. Effect of lovastatin on growth of CHO and L-90 cells.](image-url)
shown). Furthermore, electron microscopy (Fig. 2B) revealed the presence of extensive proliferation of smooth membranes and formation of crystallloid ER (49) to accommodate the large amounts of HMGR in these cells. The increased levels of HMGR in L-90 cells were, in part, the result of HMGR gene amplification, as determined by slot blot hybridization of genomic DNA with a 32P-labeled cDNA probe for HMGR (Fig. 2C). We estimated that L-90 cells contain 10–30-fold more copies of the gene for HMGR than CHO cells (Fig. 2C). Neither the gene for LDLR (Fig. 2C) nor for HMGS (data not shown) was amplified.

We also determined whether enzymes of cholesterol biosynthesis other than HMGR were elevated and properly regulated in L-90 cells (Fig. 3). Cells were incubated for 24 h with (FCS) or without (LPDS) serum lipoproteins or in LPDS medium supplemented with sterols or MVA, and equal amounts of cell lysates were analyzed by immunoblotting using antibodies specific to HMGS, HMGR, FPPS, and SQS. Clearly, relative to CHO cells in FCS, the levels of all examined proteins were highly elevated in L-90 cells (Fig. 3, e.g. compare lanes 1 and 5). However, unlike CHO cells, the amounts of these enzymes did not increase further when L-90 cells were switched to LPDS medium (Fig. 3, compare lanes 1 and 2 with lanes 5 and 6). Moreover, their steady state levels in L-90 cells decreased only moderately when sterols or MVA were also included in the LPDS medium (Fig. 3, compare lanes 3 and 4 with lanes 7 and 8). This is in a sharp contrast to the response of CHO cells where such additions prevented the LPDS-induced up-regulation of the enzymes. Quantitatively, if a value of 100% was assigned to the level of a protein in LPDS-treated cells (Fig. 3, lanes 2 and 6), then addition of sterols to CHO and L-90 cells, respectively (lanes 3 and 7), reduced the levels of HMGS to 5 and 60%; HMGR declined to 10 and 50% (see also Fig. 9A), and FPPS decreased to 15 and 80%.

Since cholesterol biosynthetic enzymes are known to be coordinately regulated at the transcriptional level, we examined whether the blunted response of these enzymes in L-90 cells might be the consequence of defect(s) in common transcriptional regulatory factor(s), such as SREBPs (50, 51), or merely might be the consequence of defect(s) in common transcriptional regulation of sterol-sensitive genes nor any hindrance in the accessibility of exogenously added sterols or MVA to the intracellular pool(s) that are involved in this regulation. Hence, the attenuated response to sterols or MVA in the steady levels of endogenous HMGS, FPPS, and SQS enzymes, observed in Fig. 3, may be attributed to their relatively slow and unregulated turnover rates (52). Moreover, under such conditions HMGR appeared as a short-lived protein in CHO cells but not in L-90 cells (see below).

The aberrant regulation of L-90 cells by serum lipoproteins could result from defects in receptor-mediated uptake of LDL. In Table II we quantified the output of the LDLR pathway by measuring the rate of 125I-LDL degradation at 37 °C. The results clearly show that, when cultured under induced conditions, L-90 cells degraded 125I-LDL at only 35% the rate measured in CHO cells. Moreover, whereas exogenous sterols down-regulated LDL degradation by >80% in CHO cells, the lower activity of the LDLR pathway in L-90 cells appeared resistant to suppression by saturating concentrations of sterols and decreased by less than 40% (Table II). Thus, the inability of L-90 cells to activate the transcription of sterol-responsive genes upon removal of serum lipoproteins stemmed from the attenuated activity of their LDLR pathway.

The decreased steady state levels of HMGR in sterols- or MVA-treated L-90 cells could have resulted from slower protein translation. Therefore, we measured the rate of HMGR synthesis in cells maintained under different metabolic conditions (FCS, LPDS, LPDS plus sterols, or LPDS plus MVA) (Fig. 5). The incorporation of radiolabeled amino acids was linear for up to 40 min (data not shown), and HMGR was immunoprecipitated from equal amounts of labeled trichloroacetic acid-precipitable material. The amounts of labeled HMGR rose ~4-fold when CHO cells were transferred from FCS- to LPDS-containing medium, and this increase was prevented when cells also received sterols or MVA (Fig. 5, lanes 1–4). When incubated in LPDS medium, the rate of HMGR synthesis was nearly 30-fold higher in L-90 cells compared with CHO cells (Fig. 5, compare lanes 2 and 6). This was expected in light of the multiple copies of HMGR gene in these cells (see Fig. 2C). However, there was again no significant change (~20% increase) in HMGR synthesis when L-90 cells were switched from FCS to LPDS (compare lanes 5 and 6). Nevertheless, in the presence of MVA or sterols

| Table I | Lipid metabolism in CHO and L-90 cells |
|--------------|----------|
| Cellular lipid synthesis and content were determined as described under “Experimental Procedures.” |
| CHO* | L-90* |
| **[^H]acetate incorporation** | | |
| Sterols | 10 | <0.05 |
| Fatty acids | 115 | 156 |
| Total | 34 | 35 |
| Free | 26 | 30 |
| Esters | 8 | 5 |

* Values are µg/mg protein.

| Cholesterol content | |
| Total | 24 | 19 |
| Free | 21 | 19 |
| Esters | 3 | 0 |

* With and without lovastatin.

a Values are µg/mg protein.

b Values are µg/mg protein.

Subsequently, we calculated the promoter activities of HMGS, FPPS, or SQS genes. When transcribed from the HMGR or LDLR promoters, the removal of lipoproteins resulted in approximately 2-fold increase in luciferase activity and only in a 40% rise when the enzyme was driven by the FAS promoter (Fig. 4). As expected, addition of sterols completely abolished this up-regulation, resulting in 4–5-fold differences in luciferase activities between the LPDS-induced and sterol-repressed states. This magnitude was comparable to the degree of repression reported in previous studies (12, 40–42). Addition of excess MVA only partially prevented the induction of luciferase upon transfer to LPDS medium (approximately 2-fold difference; Fig. 4). Remarkably, in L-90 cells all tested reporter constructs were completely refractory to the up-regulation induced by the omission of serum lipoproteins, but the regulation by sterols added in ethanol or by MVA appeared normal (Fig. 4). Yet, we noted some small but consistent differences between CHO and L-90 cells with respect to the degree of luciferase repression by sterols (e.g. HMGS, SQS, LDLR; Fig. 4). These differences may be related to the genomic integration site(s) and/or subtleties in sterol accessibility to the regulatory machinery of these promoters. In contrast to the lipoprotein-dependent response, we concluded that the non-lipoprotein-mediated transcriptional regulation of transfected genes was intact in L-90 cells. This indicates that there was neither a defect in general transcriptional regulation of sterol-sensitive genes nor any hindrance in the accessibility of exogenously added sterols or MVA to the intracellular pool(s) that are involved in this regulation. Hence, the attenuated response to sterols or MVA in the steady levels of endogenous HMGS, FPPS, and SQS enzymes, observed in Fig. 3, may be attributed to their relatively slow and unregulated turnover rates (52). Moreover, under such conditions HMGR appeared as a short-lived protein in CHO cells but not in L-90 cells (see below).

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cells were fed with the same fresh medium (designated with 2 LPDS medium that was supplemented or L-90 cells were incubated for 24 h in A, L-90 cells. On Day 0, CHO or L-90 cells were plated in FCS MVA pathway.

Gene for HMGR, but not for LDLR, is am-

VMA. The media for L-90 cells included 90 m

L-90 cells maintained in FCS medium

crystalloid ER is present in L-90 cells. In-

set, cross-section of crystalloid ER. No such structures were observed in CHO cells (data not shown). Bar = 1 μm. C, the gene for HMGR, but not for LDLR, is ampli-

fied in L-90 cells. Genomic DNA was isolated from CHO and L-90 cells, and the indicated amounts were adsorbed onto a GeneScreen Plus membrane using a slot blot filtration manifold. The membrane was successively hybridized with 32P-la-

droled cDNA probes for HMGR, LDLR, and glyceraldehyde-3-phosphate dehy-

drogenase, as described under “Experimental Procedures.”

there was an −1.5- and −2-fold decrease in HMGR synthetic rate, respectively (Fig. 5, lanes 7 and 8).

The rates of HMGR synthesis in CHO and L-90 cells directly correlated with the steady state levels of reductase mRNA, as determined by RNase protection assays (Fig. 6). 32P-Labeled antisense RNA probes for Chinese hamster HMGR and S17 ribosomal protein were hybridized with total RNA from CHO and L-90 cells grown under different metabolic conditions, digested with RNase, and analyzed by PAGE. As shown, L-90 cells contained −40-fold more HMGR mRNA compared with CHO cells (Fig. 6B), a result consistent with multiple copies of HMGR gene in L-90 cells. Fig. 6 also demonstrates that in both cell lines HMGR mRNA levels declined to 40–50 and −65% upon addition of sterols and MVA, respectively, when compared with cells maintained in LPDS medium (Fig. 6A, lanes 5 and 6 and 10 and 11). However, contrary to CHO cells where FCS caused >50% decline, in L-90 cells HMGR mRNA levels were hardly affected by serum lipoproteins (Fig. 6A, compare lanes 3 and 4 with lanes 8 and 5, respectively). These results were in full accordance with the data shown in Fig. 4 (HMGR panel) and demonstrated that the transcription of the multiple copies of the endogenous HMGR gene is regulated normally in L-90 cells. A major post-translational mode for controlling HMGR levels is achieved through regulated degradation of the enzyme. Therefore, we next examined whether the turnover of HMGR was regulated in the same manner in CHO and L-90 cells. Cells, maintained in LPDS medium, were pulsed with [35S]me-

thionine/cysteine and chased in the absence or presence of excess MVA or sterols, and at various time points HMGR was immunoprecipitated (Fig. 7 and Table III). In LPDS medium HMGR turned over with a half-life of 12.5 h in CHO cells. Upon addition of MVA or sterols the rate of reductase degradation was accelerated more than 4-fold, decreasing its t1⁄2 to <3 h (Fig. 7B and Table III). Strikingly, neither MVA nor sterols had any significant effect on the rate of HMGR degradation in L-90 cells (Fig. 7, Table III L-90 “on”). In multiple experiments the half-life of HMGR in L-90 cells averaged 12 h, with only a marginal (15–30%) effect by MVA (t1⁄2 = 11.3 h) or sterols (t1⁄2 = 11 h) (Fig. 7B and Table III L-90 “on”). Importantly, we have verified that the amount of antibody used was sufficient for the quantitative and complete precipitation of HMGR from L-90 cell lysates (see “Experimental Procedures”). Moreover, the presence of lovastatin in the chase medium did not alter the immunogenic properties of the enzyme (data not shown).

The obvious lack of regulated HMGR degradation in L-90 cells could have been the consequence of alterations in either the enzyme molecule or the cellular machinery that operates in its degradation. To distinguish between these possibilities,
CHO and L-90 cells were transfected with the plasmid pHMGal-Neo that encodes the chimeric protein HMGal (26) under the control of the constitutive immediate-early promoter of human cytomegalovirus. Stable G418-resistant colonies were pooled and assayed for expression and regulation of human cytomegalovirus. Stable G418-resistant colonies were stably transfected with the indicated promoter-luciferase DNA constructs and 300–500 resistant colonies of each were pooled. Cells were initially plated in FCS medium and then switched to the indicated media for additional 24 h incubation, as described in Fig. 3. The LPDS media for CHO cells contained 2 μM compactin and 100 μM MVA, and all media for L-90 cells included 90 μM lovastatin. Luciferase-specific activities (light units/mg cell protein) were determined in triplicate wells and normalized relative to cells incubated in LPDS (100%). The results are mean ± S.E. of five to nine independent experiments.

**Table II**

*Impaired 125I-LDL degradation in L-90 cells*

Degradation of 125I-LDL at 37 °C was determined as described under “Experimental Procedures.” The results, corrected for nonspecific LDL degradation, are the mean ± S.E. of 10 separate experiments.

| Addition | CHO | L-90 | L-90/L-CHO |
|----------|-----|------|-----------|
| None     | 829.2 ± 121.1 | 100 | 290.1 ± 52.5 | 100 | 0.35 |
| Sterols  | 162.7 ± 52.8 | 19.6 ± 4.8 | 185.4 ± 45.8 | 67.7 ± 5.6 |

* For each cell line, “None” was set as “100%.”

b Ratio of activity between L-90 and CHO cells.

CHO and L-90 cells were transfected with the plasmid pHMGal-Neo that encodes the chimeric protein HMGal (26) under the control of the constitutive immediate-early promoter of human cytomegalovirus. Stable G418-resistant colonies were pooled and assayed for expression and regulation of β-galactosidase activity. The results, normalized to the activity in LPDS medium (100%), are shown in Fig. 8A. Consistent with numerous previous reports (see Ref. 32 and references therein), HMGal activity in CHO cells dropped to 13–30% of control values when the cells were incubated without lipoproteins (FCS), MVA, or sterols in ethanol. This is due to a 3–6-fold acceleration in the rate of HMGal turnover (32). None of these additions had any appreciable effect on HMGal activity in L-90 cells (Fig. 8A). That this persistent activity resulted from a highly stabilized HMGal protein was directly demonstrated in a pulse-chase experiment where HMGal and HMGm were sequentially immunoprecipitated from the same labeled samples (Fig. 8B). In CHO cells the degradation of both HMGal and HMGm was accelerated to the same extent following the addition of MVA or sterols, whereas neither agents had any significant effect on the half-life of HMGm or HMGal in L-90 cells (Fig. 8B).

The loss of regulated HMGm degradation in L-90 cells could have been the outcome of selecting cells for lovastatin resistance in the presence of serum lipoproteins (see below). Alternatively, it was conceivable that this aberrant phenotype resulted from the massive overproduction of HMGm protein that could have saturated a rate-limiting step and/or depleted critical trans-acting factor(s) that operates in reductase degradation. The latter possibility was directly tested through analyzing yet another HMGm overexpressing cell line, designated LP-90. Unlike L-90, LP-90 cells were gradually adapted to grow in 90 μM lovastatin in a medium that lacks lipoproteins (LPDS medium; see “Experimental Procedures”). Western blot analysis (Fig. 9A) demonstrated that LP-90 cells overproduce HMGm to the same extent as L-90 cells (Fig. 9A, compare lanes 6 and 10). However, unlike L-90 cells, it was evident that the levels of HMGm in LP-90 cells were tightly regulated by serum lipoproteins, MVA, or sterols, in a similar fashion as in CHO cells (Fig. 9A).

A direct examination of degradation rate by pulse-chase analysis (Fig. 9B) demonstrated that the half-life of HMGm in LP-90 cells decreased from 12 to 3 h or to 1.5 h upon addition of MVA or sterols, respectively (mean results of three experiments). These changes in reductase turnover were very similar to the effects observed in parental CHO cells and in clear contrast to L-90 cells (Fig. 9B). Thus, the finding that LP-90 cells were capable of degrading large amounts of HMGm with t1/2 of less than 2 h demonstrated that overexpression of HMGm itself is not likely to cause impaired regulation of HMGm degradation.

Based on the results presented in Fig. 8, we concluded that the loss of regulated degradation of HMGm in L-90 cells is due to changes in trans-acting factor(s) required for accelerated turnover of the enzyme. This factor(s) might be involved in metabolic cues that signal for HMGm destabilization and/or in tagging the enzyme for degradation and/or in the terminal proteolytic elimination of the protein. We reasoned that metabolic cues might be specific for degradation of HMGm, whereas tagging mechanisms and/or cellular proteolytic systems could be generally involved and possibly utilized in the ER degrada-
proteolysis (43, 56). The intracellular fate of TCR reverse translocation to the cytoplasm for deglycosylation and translationally inserted and glycosylated in the ER before its variety of cell lines that a singly expressed TCR was extracted, and 5 L-90 cells were set for the experiment, as described in Fig. 3. Total RNA was isolated from CHO and L-90 cells incubated in FCS (F), LPDS (L), LPDS + MVA (M), and LPDS + sterols (S) media, respectively, as specified in Fig. 3. In lane 7, both probes were hybridized to yeast tRNA. To assess properly the degree of regulation, the HMGR probe used for hybridizing L-90 RNA was synthesized in the presence of a 30-fold molar excess of unlabeled UTP. Following autoradiography, the protected bands were cut out of the gel and counted.

Because HMGR probes were of different specific radioactivity, percent regulation was determined separately for each cell type by calculating the ratio of cpm in HMGR band/cpm in S17 band and normalizing it to the value obtained in LPDS-treated cells. B, RNase protection assay was performed on RNA isolated from LPDS-treated CHO (lane 1) and L-90 (lane 2) cells, as described above, with the exception that the HMGR probe was of the same specific radioactivity. “Fold” difference was calculated from the ratio of cpm in HMGR band/cpm in S17 band for each sample, setting the value in CHO cells as 1. In both panels, the quantitative results are the mean of seven experiments done on five separate RNA preparations.

tioned of other membrane proteins. To address directly this question and further focus on the mechanism(s) altered in L-90 cells, CHO and L-90 cells were stably transfected with the plasmid pTCRα-Neo that encodes the α subunit of the T cell receptor (TCRα; Ref. 43). It has been previously shown in a variety of cell lines that a singly expressed TCRα is degraded rapidly and constitutively. Indeed, for many years it served as a prime model for protein degradation in the ER (53–55). Recent studies, however, have demonstrated that TCRα is actually degraded by the proteasome in the cytosol or at the cytoplasmic face of the ER (43, 56). Nevertheless, TCRα is cotranslationally inserted and glycosylated in the ER before its reverse translocation to the cytoplasm for deglycosylation and proteolysis (43, 56). The intracellular fate of TCRα was monitored in CHO and in L-90 cells by pulse-chase and immunoprecipitation with specific monoclonal antibodies (Fig. 10). TCRα was synthesized in both cell types as a ~40-kDa peptide NGlycosidase F-sensitive protein (Fig. 10A), indicating that TCRα is initially inserted and core-glycosylated in the ER. We consistently observed higher levels of expression in L-90 cells (Fig. 10A; compare lanes 1 and 2 with lanes 3 and 4, respectively). As shown in Fig. 10B, TCRα was rapidly degraded in CHO cells with a half-life of ~1/2 h, a value consistent with previous reports (55). Remarkably, degradation of TCRα was significantly retarded in L-90 cells, and high levels of the protein persisted for over 2 h after synthesis. Appreciable amounts could also be observed by 5 h of chase (Fig. 10B). From this and similar experiments, the half-life of TCRα in L-90 cells was determined to be 2.5–3 h, over 5-fold longer than in CHO cells. Addition of sterols during the chase had no effect on the half-life of TCRα in either cell lines (data not shown).

The failure of L-90 cells to accelerate the turnover of HMGR in response to exogenous MVA or sterols was manifested in cells that were continuously maintained at 90 μM lovastatin. Therefore, it was of interest to investigate whether this aberrant phenotype is stable and whether it depended on the pres-
Degradation rates of HMGR and HMGal were determined in CHO-HMGal cells, in L-90-HMGal cells that were continuously maintained in 90 μM lovastatin (L-90 “on”), and in L-90 cells that were grown without lovastatin for 2, 5, 9, 16, 23, and 37 days (L-90 “off”). Pulse-chase experiments were performed as described under "Experimental Procedures" and in Figs. 7 and 11. L-90 “off” cells were assayed without lovastatin. The half-lives of HMGR and HMGal in L-90 “off” cells were very similar between 2 and 37 days (see text) and thus were averaged. In each experiment, "fold acceleration by sterols" was calculated by dividing the protein’s t½ in the absence of sterols (−) by its t½ in the presence of sterols (+). The results are the mean ± S.E. of 11–16 separate experiments.

### Table III

|                     | HMGal half-life | HMGR half-life | Fold acceleration by sterols |
|---------------------|-----------------|----------------|-------------------------------|
|                     | −  | +  | −  | +  | −  | +  |                |
| CHO                 | 12.5 ± 0.7      | 2.7 ± 0.1      | 11.0 ± 0.7                   | 2.5 ± 0.2      | 4.7 ± 0.4      | 4.5 ± 0.5      |
| L-90 “on”           | 12.1 ± 0.9      | 11.0 ± 1.1     | 12.8 ± 1.6                   | 9.2 ± 0.5      | 1.2 ± 0.1      | 1.3 ± 0.1      |
| L-90 “off”          | 4.6 ± 0.3       | 3.6 ± 0.3      | 5.1 ± 0.2                    | 4.1 ± 0.3      | 1.3 ± 0.1      | 1.2 ± 0.1      |

**Fig. 8.** Degradation of HMGal is not regulated in L-90 cells. A, on Day 0, pools of HMGal-transfected CHO (open bars) and L-90 (closed bars) cells were plated in FCS medium without or with 90 μM lovastatin, respectively. On Day 1, the cells were fed with FCS, LPDS, LPDS + MVA, or LPDS + sterols, as detailed in Fig. 3. After 24 h, the cells were permeabilized with digitonin and β-galactosidase-specific activity was measured, as described under "Experimental Procedures." The results (A405/nl/mg protein) were normalized relative to cells incubated in LPDS and are the mean ± S.E. of four experiments, each done on quadruplicate wells. B, transfected CHO and L-90 cells, expressing highest HMGal activity, were cloned by limited dilution. The cells were pulse-labeled and chased in the indicated media, as described in Fig. 7. HMGal and HMGR were sequentially immunoprecipitated with anti-β-galactosidase and anti-HMGR antibodies, respectively. Similar results were obtained with three independent clones. Half-life values are also given in Table III.

**Fig. 9.** LP-90 cells overexpress HMGR yet normally regulate its degradation. A, CHO or L-90 cells were plated in FCS medium without or with 90 μM lovastatin, respectively. LP-90 cells were plated in LPDS medium with 90 μM lovastatin. On the following day, the cells were fed with FCS (L-90 and LP-90 cells, lanes 1–12) or LPDS (L-90 and LP-90 cells, lanes 13–18), and all media of L-90 and LP-90 cells contain 90 μM lovastatin. Following 24 h incubation, the cells were lysed, and 75 μg (CHO cells, lanes 1–4) or 15 μg (L-90 and LP-90 cells, lanes 5–12) of post-nuclear cell extracts were analyzed by immunoblotting using anti-HMGR antibodies. B, on Day 0, CHO, L-90, or LP-90 cells were plated in FCS (CHO, FCS + 90 μM lovastatin (L-90), or LPDS + 90 μM lovastatin (LP-90) media, respectively. On Day 1, all cells were refed with LPDS medium, as described under "Experimental Procedures" and in Fig. 7. On Day 2, the cells were pulse-labeled and chase in LPDS medium supplemented with the indicated additions, as detailed in Fig. 7. HMGR was immunoprecipitated with anti-HMGR antibodies and analyzed by SDS-PAGE.
“off” cells ($t_{1/2} = 2$ h in cells that were off lovastatin for 19 days as compared with 2.5 h in L-90 “on” cells and 0.5 h in CHO cells). Moreover, within 24 h after re-challenging L-90 “off” cells with 90 $\mu M$ lovastatin, HMGR re-acquired its extended half-life (as in L-90 “on” cells), again without any appreciable effect of sterols on reductase degradation (data not shown). These results demonstrated that the loss of regulation of HMGR degradation is a stable phenotype of L-90 cells that does not depend on the presence of lovastatin.

**DISCUSSION**

Mevalonate is an essential molecule for life of all eukaryotic cells. Therefore, exposing naive cells to inhibitory concentrations of statins causes elevation in HMGR levels in an effort to overcome the blockage in endogenous MVA production. Unless MVA is added to the growth medium, prolonged incubations with these drugs lead to cell death. This homeostatic response has been exploited to select several cell lines that are resistant to the cytotoxic effects of these potent HMGR inhibitors (10, 49, 57, 58). In most cases, selection for statin resistance has been achieved with cells that were initially adapted to grow in a cholesterol-deficient medium. Under such conditions, the sole source of the cell for cholesterol was through its endogenous synthesis in the MVA pathway. Upon addition of statins, first at very low concentrations, only cells that could develop higher HMGR levels have survived, and resistant lines have been established by iterating this step at increasing drug concentrations. The best studied of these lines is UT-1, a variant of CHO cells selected to grow in 40 $\mu M$ compactin (49). UT-1 cells survive at such high compactin concentrations due to over-expression of HMGR (−2% of total cell protein) resulting from an ∼15-fold amplification of the gene (49, 59). Importantly, transcriptional control of HMGS, HMGR, and LDLR genes, as well as sterol-regulated turnover of HMGR protein, is normal in UT-1 cells (49, 59, 60).

Although gene amplification is frequently encountered in acquired drug resistance (61), it is not the sole mechanism that allows cell growth at high statin concentrations. Skalnik et al. (57) have shown that in ML-100 cells, which grow in 100 $\mu M$ compactin, neither the gene for HMGR is amplified nor are HMGR mRNA levels elevated. Sinensky et al. (62) reported that lovastatin resistance of the MX1 cell line appears to be the result of a mutation in HMGR that lowers its $K_v$ value toward HMG-CoA. Finally, a point mutation in the promoter of the HMGR gene confers lovastatin resistance to the archaeabacterium *Haloferax vulcanii* (63).

In the current study, we show that L-90 cells withstand lovastatin toxicity by elevating the levels of HMGS and by the massive accumulation of HMGR. Concomitant with the over-expression of HMGR, a membrane-bound enzyme, there is an impressive proliferation of smooth ER and formation of crystallloid ER, similar to the situation observed in UT-1 cells (49). However, L-90 cells are unique in that they achieve accumulation of HMGR not only by gene amplification and rise in mRNA levels but also by loss of regulated turnover of the protein. In addition, compared with parental CHO cells, L-90 cells have reduced content of cholesterol. This may account for the highly elevated levels of FPPS and SQS in L-90 cells, presumably in an effort to compensate for their chronic deprivation in MVA-derived isoprenoids and sterols. Inasmuch as the transcription of many, if not all, genes of the MVA pathway is regulated by SREBP (30), the finding that L-90 cells simultaneously over-express HMGS, HMGR, FPPS and SQS suggests that the proteolytic cleavage/activation of the ER precursors for these transcription factors occurs in these cells at a much higher rate, and/or that the mature SREBP resides in the nuclei of L-90 cell for a longer time. This is corroborated by the finding that fatty
acids synthesis is also accelerated in L-90 cells, in agreement with the involvement of SREBPs in the transcription of acetyl-CoA carboxylase, FAS, and stearoyl-CoA desaturase (30). The slow and stepwise selection of cells for drug resistance may cause the accumulation of multiple genetic lesions, leading to a complex phenotype. Indeed, L-90 cells stand out from similar statin-resistant cells because of two important features that make them especially interesting. First, L-90 cells have lost their ability to degrade HMGR in a regulated fashion. Hence, in these cells reductase appears to be regulated solely at the transcriptional level, by controlling amounts of mRNA. Second, L-90 cells exhibit a severe impairment in their capacity to respond to changes in the availability of cholesterol carried in serum lipoproteins. We show that this attenuated response is the result of reduced activity of the LDLR pathway, which resembles the defective LDL-mediated regulation of cholesterogenic enzymes in cells from individuals afflicted with familial hypercholesterolemia (5). In experiments to be presented elsewhere, we demonstrate that the reduced LDLR activity is due to fewer surface receptors rather than lower LDL binding affinity. Decreased activity and abnormal regulation of LDLR have been also reported in the compactin-resistant CR200 cells (58).

Cells have evolved a highly complex mechanism to get rid of excess HMGR molecules when products of the MVA pathway are in abundance. Although the molecular details of this mechanism are still vastly unknown, it is firmly established that the membrane domain of HMGR is the cis-acting element in the regulated degradation of the enzyme. The results presented here clearly demonstrate that, similar to endogenous reductase, the degradation of transfected HMGal in L-90 cells is not accelerated by addition of exogenous sterols or MVA. Thus, the fault in the regulation of degradation is a trans-acting property of the cells and not a cis defect in HMGR protein. This loss of regulated turnover is a stable phenotype since after 37 days in culture without lovastatin (~30 generations) such L-90 “off” cells could not accelerate the degradation HMGR or HMGal when challenged with excess sterols (see below). It is unlikely that sterols or MVA fail to reach their target intracellular compartments/factors since L-90 cells manifest sterol-sensitive transcriptional regulation of HMGR and other responsive genes. Also, L-90 cells display normal sterol stimulation of acyl-CoA cholesterol acyltransferase activity as well as [14C]-MVA-dependent synthesis of cholesterol and prenylated proteins. These results indicate that in L-90 cells sterols reach the ER and are capable of regulating ER events such as the proteolytic activation of SREBPs and cholesterol esterification. Yet, in L-90 cells sterols fail to accelerate HMGR degradation, another ER-associated process (31). It is equally unlikely that the apparent loss of regulated HMGR degradation in L-90 cells is due to the overproduction of HMGR protein that might have overwhelmed this complex degradative process. Indeed, we show that comparably massive amounts of HMGR do not impede the capacity of LP-90 cells to accelerate the turnover of the enzyme when challenged with MVA or sterols. This is in full agreement with observations in other statin-resistant lines, such as UT-1, C100, or CR200 cells, which overproduce HMGR to a similar or even greater extent, yet regulate normally the turnover of the enzyme (19, 35, 58).

What might have caused the loss of regulation of HMGR turnover in L-90 cells? Unlike other statin-resistant lines (LP-90, UT-1, and C100), L-90 cells were gradually adapted to grow in increasing lovastatin concentrations in the presence of serum lipoproteins. Studies by Nakanishi et al. (15) and Rottelman and Simoni (32) demonstrated that sterol-accelerated degradation of HMGR will not ensue unless MVA-derived nonsterols are synthesized in the cells. Yet, MVA can stimulate HMGR degradation even when endogenous sterol synthesis is specifically inhibited (32). The notion that rapid HMGR degradation requires both sterol and nonsterol metabolic signals is also supported by the differential sensitivity of these signals to perturbation of cellular Ca2+ stores (32). Therefore, under normal growth conditions, when LDL-cholesterol is abundant and endogenous MVA production is not inhibited (i.e. in FCS and with no statins added), HMGR is unstable and is degraded at a fast rate. Combined with sterol-mediated transcriptional repression of the gene, the steady state levels of HMGR protein in such cells are very low. It is under these conditions that L-90 cells were gradually selected forLovastatin resistance. At early stages of statin selection, the cells have an ample supply of cholesterol through the LDLR pathway, but they are starved for MVA-derived nonsterol isoprenoids. Thus, to allow endogenous MVA production, the cells could have elevated the steady state levels of HMGR either by increasing the synthesis rate of additional enzyme molecules and/or by stabilizing pre-existing ones. However, if the “regulatory circuit” that operates in HMGR degradation has responded normally, the combined signals from incoming LDL-cholesterol and endogenously synthesized MVA-derived nonsterols would have caused the rapid elimination of HMGR and decreased its steady state below the levels necessary for survival. Therefore, in order to survive in the presence of lovastatin, the cells should have modified the putative regulatory circuit so that it no longer responded to the signals for rapid HMGR degradation. In addition, if such a hypothetical regulatory circuit is also responsive to the intracellular pool of sterols, it is conceivable that lowering the amounts of cholesterol intake through the LDLR pathway would prevent this pool from reaching the size that otherwise would trigger the accelerated degradation of HMGR. Indeed, compared with CHO cells, L-90 cells have reduced cholesterol content and lower activity of the LDLR pathway.

Although this model is probably oversimplified, it may be correct insofar as it is compatible with the isolation by Hampton et al. (36) of yeast mutants that are defective in the degradation of HMGR. Similar to the mammalian enzyme, the rate of degradation of one of the two yeast HMGR isozymes, Hmg2p, is regulated by the flux of metabolites through the MVA pathway. Again, this regulation is conferred onto the enzyme by its membrane-spanning domain (64). Hampton et al. (36) utilized a yeast strain in which a constitutively expressed Myc-tagged version of Hmg2p (6myc-Hmg2p) supplies the cells with MVA. Relying on the fact that this modified HMGR protein is extremely unstable (thus its steady state levels are low), Hampton et al. (36) selected for cells that are resistant toLovastatin without prior mutagenesis. This situation is analogous to the selection scheme we have employed. In both cases, the cells were forced to maintain high steady state levels of HMGR, which is inherently unstable due to either metabolic conditions (this report) or destabilizing modification of the protein (36). By this protocol, Hampton et al. (36) have identified defects in three genes, designated HRD1, HRD2, and HRD3, that operate in the degradation of HMGR. In all three complementation groups, both Hmg2p and 6myc-Hmg2p were stabilized (36), indicating that these gene products operate in unregulated, presumably distal, steps in the HMGR degradation pathway. Indeed, Hrd1p (also known as Der3p) was shown to be involved in the ER degradation of other proteins (65), and Hrd2p was identified a subunit of the 26 S proteasome (36). Although L-90 cells were obtained under seemingly analogous conditions as the yeast hrd mutants, they clearly do not display a hrd-like phenotype. This conclusion is based on our finding that L-90

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cells are capable of degrading the vast amounts of HMGCR rather rapidly when no longer exposed to lovastatin (in L-90 "off" cells). This capacity is evident only in cells that are off lovastatin for more than 24 h and is fully reversed 24 h after re-challenge with the drug. Since hrd mutations affect distal steps in the ER degradation of HMGCR, the enzyme should have remained stable also in L-90 "off" cells, which is clearly not the case. Thus, it appears that L-90 cells are affected in sensing/integrating the sterol and nonsterol signals, and/or in relaying these signals to the membrane domain-dependent regulated degradation of HMGCR.

The lovastatin-dependent reversible stabilization of HMGCR is particularly intriguing. To the best of our knowledge, the sole degradation of HMGCR.

The notion that several proteolytic steps in the ER degradation of HMGCR, the enzyme should have further accelerated by sterols, it is evident that the half-life of further HMGR degradation in L-90 "off" cells. Although it may be argued that in L-90 "off" cells reductase degradation proceeds at its maximal velocity ($t_{1/2} \approx 4$ h), hence cannot be further accelerated by sterols, it is evident that the half-life of massive amounts of HMGCR in sterol-treated LP-90 cells is shorter than 2 h. Therefore, it is tempting to propose that the sterol-accelerated degradation of HMGR in responsive cells (e.g. LP-90, UT-1, C-100, CHO) is signaled and/or executed by a different mechanism(s) than the rapid turnover of the enzyme in L-90 "off" cells. The notion that several proteolytic pathways may operate in the ER is supported by our observation that the degradation of TCRα is retarded to a similar extent both in L-90 "on" and L-90 "off" cells. Elucidating the basis for the aberrations in L-90 cells will shed light on cellular components that are involved in the ER degradation of proteins in general and in the metabolically regulated degradation of HMGCR in particular.

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While this manuscript was under review, Rao et al. (66) reported that lovastatin inhibits proteasome activity. If HMGR and HMGα are indeed degraded by the 26 S proteasome, these results are compatible with our observations of the reversible stabilization of these proteins by lovastatin, which is independent of its activity as an inhibitor of MVA synthesis.