Inhibition of Degradation of 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase in Vivo by Cysteine Protease Inhibitors*

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3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is a key regulatory enzyme of cholesterol biosynthesis and is located in the endoplasmic reticulum (ER). A fusion protein, HMGal, consisting of the membrane domain of HMG-CoA reductase fused to Escherichia coli β-galactosidase and expressed in Chinese hamster ovary (CHO) cells from the SV40 promoter, was previously constructed and was found to respond to regulatory signals for degradation in a similar fashion to the intact HMG-CoA reductase. Degradation of both HMG-CoA reductase and HMGal in CHO cells was enhanced by addition of mevalonate or low density lipoprotein (LDL). In this report we show that 2 cysteine protease inhibitors, N-acetyl-leucyl-leucyl-norleucinal (ALLN) and N-acetyl-leucyl-leucyl-methioninal (ALLM), completely inhibit the mevalonate- or LDL-accelerated degradation of HMG-CoA reductase and HMGal and also block the basal degradation of these enzymes. It has been shown that in vitro these protease inhibitors inhibit the activities of Ca2+-dependent neutral proteases as well as lysosomal proteases, including cathepsin L, cathepsin B, and cathepsin D. However, the mevalonate-accelerated degradation of HMG-CoA reductase and HMGal is not affected by lysosomotropic agents, suggesting that the site of action of these inhibitor peptides in preventing the degradation is not the cathepsins. In brefeldin A-treated cells, where protein export from the ER is blocked, ALLN is still effective in inhibiting the degradation of HMG-CoA reductase and HMGal. These results indicate the involvement of non-lysosomal Ca2+-dependent proteases in the basal and the accelerated degradation of HMG-CoA reductase and HMGal. Enzymatic assays in vitro and immunoblot analyses have revealed calpain- and calpastatin-like proteins in CHO cells. The activities and the amount of these proteins do not change under conditions of enhanced degradation, indicating that the levels of these proteins are not subject to mevalonate regulation.

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A

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‡ This work was done while on leave from the Biochemistry Department, Tel Aviv University, Israel, which is her current address.

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The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGal, a fusion protein consisting of the membrane domain of HMG-CoA reductase and β-galactosidase; LDL, low density lipoprotein; CHO, Chinese hamster ovary; LPS, lipid-poor serum; ALLM, N-acetyl-leucyl-leucyl-methioninal; ALLN, N-acetyl-leucyl-leucyl-norleucinal; PMSE, phenylmethylsulfonyl fluoride; APMSF, (4-amidinophenyl)methanesulfonyl fluoride; EGTA, ethylenbis(oxyethylenthlenenitrilo)tetraacetate acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; PBS, phosphate-buffered saline.

The native HMG-CoA reductase is a 97-kDa membrane glycoprotein located in the endoplasmic reticulum (ER). Previous biochemical studies and analyses of the primary structure, as derived from the nucleotide sequence of the mammalian genes, indicate that the protein can be topologically divided to a catalytic domain, a membrane-embedded domain, and a linker region that connects these domains (4–6).

We have previously constructed a hybrid gene consisting of the coding sequence for the membrane domain of HMG-CoA reductase fused to the coding sequence for the soluble β-galactosidase of Escherichia coli. When this chimeric protein (HMGal) is expressed in CHO cells under the control of the SV40 early promoter (7), it is found to reside in the ER, and its degradation is enhanced by mevalonate and low density lipoprotein (LDL), as observed for the native HMG-CoA reductase (7, 8). This fusion protein allows us to study the degradation of HMG-CoA reductase by measuring the β-galactosidase activity, irrespective of the effects on transcriptional or translational controls of the endogenous reductase.

The mechanisms by which HMG-CoA reductase is degraded are not well understood. Using a mouse mammary cell line, Tanaka et al. (9) found that NH₂Cl inhibits the basal degradation of HMG-CoA reductase but not the accelerated degradation induced by 25-hydroxycholesterol. Parker et al. (10) reported that lysosomotropic agents such as methylamine and propylamine, as well as monensin, dampen both basal and mevalonate-induced HMG-CoA reductase degradation in hepatocytes. However, these agents only partially diminish the enhanced degradation caused by 25-hydroxycholesterol. Parker et al. (11) also demonstrated that phosphorylation of HMG-CoA reductase enhances its susceptibility to proteolysis in vitro. We have shown recently that the mevalonate-regulated degradation may occur in the ER by using brefeldin A, which inhibits protein export from the ER (8). In vitro, calpain II (a Ca²⁺-dependent cysteine protease) purified from

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rat liver can cleave the native HMG-CoA reductase and produce fragments containing the catalytic domain (10-12). In this study, we demonstrate that, in vitro, cysteine protease inhibitors completely inhibit both the basal degradation and the mevalonate- or LDL-accelerated degradation of HMG-CoA reductase as well as HMGal. However, lysosomal, cytosolic, and nuclear extracts have no effect on the mevalonate-accelerated degradation of HMGal. We also show a Ca\(^{2+}\)-dependent protease activity in CHO cells and the presence of calpain II-like protein and its endogenous inhibitor calpastatin-like protein by immunoblot analysis. We were unable to show any differences in the in vitro protease activity or the protein amounts after treatment of cells with mevalonate or LDL. These results suggest that a non-lysosomal calpain-like protease is involved in the degradation of HMG-CoA reductase but that this protease may not represent the mevalonate- or LDL-dependent pathway.

EXPERIMENTAL PROCEDURES

Materials—All materials, unless specified, were obtained from commercial sources. Proteinase inhibitors were purchased from Calbiochem Mannheim and Sigma, and dissolved in water, ethanol, or dimethyl sulfoxide (Me2SO). Casein, labeled with N-(resorufin-4-carboxyl)piperidine-4-carboxylic acid, was obtained from Boehringer Mannheim. L-\(^{35}\)S)Methionine (>1,000 Ci/mmol, >37 TBq/mmol) was from Amersham Corp. Human LDL was from Organon Teknika. Horseradish peroxidase-labeled anti-calpain and anti-calpastatin blotting detection kit were purchased from Amersham Corp. Phenylnsulfure Cl-4B was from Pharmacia LKB Biotechnology Inc. Nitricellulose membrane was from Schleicher & Schuell. Anti-\(\beta\)-galactosidase monoclonal antibody was purchased from Promega. Anti-HMG-CoA reductase polyclonal antibodies were a generous gift of Dr. Peter A. Edwards (University of California in Los Angeles) and Hans-Stefan Jenke (Institute for Zellchemie, Munich, Germany). Anti-calpain II antibody, anti-calpastatin antibody, and a purified calpastatin D3 fragment were kindly provided by Dr. Yoshifumi Adachi (Kyoto University, Japan). Brefeldin A was a generous gift of Dr. Richard Klausner (National Institute of Child Health and Human Development, Bethesda, MD).

Cell Culture—Cells used in these studies were Chinese hamster ovary (CHO-K1) cells transfected with the plasmid pSV2-HMGal (7) (CHO-HMGal cells). Cells were grown as monolayers in minimal essential medium supplemented with 250 pg/ml active geneticin, 1 \(\mu\)M compactin, and 0.1 mM mevalonate. This pretreatment was sufficient to maximize \(\beta\)-galactosidase activity of HMGal and increase the protein amounts of HMGal and HMG-CoA reductase about 4- to 5-fold (8).

\(\beta\)-Galactosidase Assay—\(\beta\)-Galactosidase activity was measured as previously described (7). Protein concentrations were determined by the method of Lowry et al. (14). Specific activity was expressed as n mole/hr/mg of protein.

Pulse-Chase Experiments—Pulse-chase experiments were done as described previously (8). Briefly, cells grown in 100-mm plates were washed with phosphate-buffered saline (PBS) and incubated for 1 h in 2 ml of methionine-free minimal essential medium supplemented with 5% LPS, 220 \(\mu\)g/ml geneticin, 1 \(\mu\)M compactin, and 0.1 mM mevalonate. Cells were then pooled for 4 h with 250 \(\mu\)Ci of \[^{35}\]S methionine and chased for various periods of time in complete minimal essential medium (containing 2 mM methionine) without or with 20 mM mevalonate or 125 \(\mu\)g/ml LDL and/or 100 \(\mu\)g/ml ALLN. After 1 h incubation, the cells were homogenized in 1 ml of buffer A (1% deoxycholate, 1% Nonidet P-40, 2 mM phosphymethanesulfonyl fluoride (PMSF), 0.1 \(\mu\)M leupeptin, 5 \(\mu\)M ALLN, 5 \(\mu\)M EDTA, and 5 mM EGTA in PBS). The cleared lysates were used for immunoprecipitation with anti-\(\beta\)-galactosidase or anti-reductase antibody, as described previously (8). The immunoprecipitates were separated by 5-15% gradient SDS-PAGE (15), transferred to nitrocellulose membrane, and probed with the first antibodies followed by horseradish peroxidase-conjugated secondary antibody. The blots were developed according to the manufacturer’s directions as described in enhanced chemiluminescent (ECL) Western blotting system. ECL was produced by exposure to XAR-5 film, the bands intensities were measured using a Pharmacia LKB Biotechnology Inc. Ultrascan XL laser densitometer. The areas of the peaks corresponding to HMG-CoA reductase and HMGal were normalized to the total counts/min of \[^{35}\]S methionine incorporated into proteins in cells. Where indicated, brefeldin A (5 \(\mu\)g/ml) was added in the incubation with the methionine-free medium and fresh brefeldin A (5 \(\mu\)g/ml) was present during the pulse and the chase period.

Partial Purification of Calpains—Preparation of soluble fraction from cells and partial purification of calpains were done at 4 °C, according to Oshima et al. (16). Cells were trypsinized, collected in cold PBS by centrifugation, washed twice with PBS, and homogenized in 5 volumes of a buffer A (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mM PMSF and 10 mM \(\beta\)-mercaptoethanol) in a Dounce homogenizer. The homogenate was centrifuged at 30,000 \(\times \)g for 30 min, and the supernatant was collected (crude extract). The supernatant (1 ml) was brought to 2 \(\mu\)M leupeptin and 0.25 M NaCl and mixed with 0.5 ml of buffer B (20 mM Tris-HCl, pH 7.4, 0.1 mM CaCl\(_2\), 10 mM \(\beta\)-mercaptoethanol, and 20 \(\mu\)M leupeptin) containing 0.25 M NaCl. The suspension was shaken for 5 min at 4 °C, and 50 \(\mu\)l of 100 mM CaCl\(_2\) was added. The mixture was shaken for an additional 10 min and then poured into an 8-mm column. The packed column was washed successively with 0.5 ml of buffer B containing 0.25 M NaCl, 0.5 ml of buffer B and 0.5 ml buffer B without leupeptin. The calpains, bound and not bound to the column, were eluted with 0.5 ml of buffer C (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM \(\beta\)-mercaptoethanol, and 0.1 M NaCl) and then with 0.5 ml of buffer C without NaCl. Two fractions were combined and used in the calpain assay.

Calpain Assay—\(\beta\)-dependent protease activity was determined with resorufin-labeled casein as a substrate (17). Each incubation mixture (final volume of 100 \(\mu\)l) contained sample fraction, 0.02% (w/v) casein, 100 mM Tris-HCl, pH 7.5, 5 mM \(\beta\)-mercaptoethanol, and either 5 mM EDTA, 4 mM CaCl\(_2\), or 0.7 mM CaCl\(_2\). After 1 h incubation at 37 °C, the reaction was terminated by adding 125 \(\mu\)l of 5% (w/v) trichloroacetic acid. Acid-soluble products were mixed with 2 ml of 0.5 M Tris-HCl, pH 8.8, and the fluorescence emission was determined at 584 nm (excitation at 574 nm) (\(\epsilon = 66 \text{nm}^{-1} \cdot \text{cm}^{-1}\)). Reactions carried out in the presence of 5 mM EDTA were taken as background. Protein concentrations were determined by the method of Bradford (18). Where indicated, 15 \(\mu\)M ALLN was added to the incubation mixture.

Immunoblot and Dot-blot Analyses—Cells were trypsinized, collected by centrifugation, washed twice with PBS, and lysed in 5 volumes of lysis buffer, as described above. The nuclei were removed by centrifugation at 1000 \(\times \)g for 10 min at 4 °C. The protein concentration of the supernatant was determined by the method of Bradford. The protein fraction from each sample was resolved by 5-15% gradient SDS-PAGE (15), transferred to nitrocellulose membrane, and probed with the first antibodies followed by horseradish peroxidase-conjugated secondary antibody. The blots were developed according to the manufacturer’s directions as described in enhanced chemiluminescent (ECL) Western blotting system. ECL was produced by exposure to XAR-5 film, the intensities of the dots on x-ray film were quantified by densitometry.

RESULTS

Degradation of HMGal and HMG-CoA Reductase Is Inhibited by ALLN and ALLN—We have previously demonstrated that \(\beta\)-galactosidase activity in CHO cells transfected with the HMGal gene (CHO-HMGal cells) reflects the degradation of HMGal protein, and that this degradation is enhanced by mevalonate or LDL similar to the accelerated degradation of the endogenous HMG-CoA reductase (7, 8). To further characterize this process, we examined the effect of several protease inhibitors on the mevalonate-accelerated degradation of HMGal (Fig. 1). Cells were treated with various protease inhibitors for 2 h prior to addition of mevalonate, and after 20 h incubation with both the inhibitors and mevalonate, the specific activity of \(\beta\)-galactosidase was determined. In mevalonate-treated cells, HMGal activity decreases to about 20% of that in control cells. Among the 14 different proteases

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
Inhibition of HMG-CoA Reductase Degradation

Figure 1: Effect of various protease inhibitors on the mevalonate-accelerated degradation of HMGal. Cells were grown for 24 h in medium supplement with 5% FBS, 1 µM compactin, and 0.1 mM mevalonate. Protease inhibitors (200 µg/mL, EDTA (2 mg/mL), or solvent (1%) were added to the medium and cells were incubated for 2 h before receiving 20 mM mevalonate (hatched bars). After a 20-h incubation, the specific activity of β-galactosidase was determined as described under "Experimental Procedures" and is expressed as percentage of the activity in control cells receiving no addition (solid bar). Each value is the mean of quadruplicate determinations.

Inhibitors tested, only the cysteine protease inhibitors, ALLN and ALLM, completely block the mevalonate-accelerated degradation of HMGal (Fig. 1). ALLM and ALLN are synthetic peptides which in vitro inhibit the activity of cathepsin L, cathepsin B, and calpains (19). No inhibition of HMGal degradation is observed with the serine protease inhibitors, APMSF, aprotinin, or PMSF; the amino peptidase inhibitor, bestatin; the aspartic protease inhibitor, pepstatin; the metalloprotease inhibitors, phosphoramidon or EDTA; or with the chymotrypsin inhibitor, chymostatin (Fig. 1). E-64 (N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine) is also considered a specific inhibitor of cysteine proteases; however, it did not prevent the effect of mevalonate, most likely because it is not membrane-permeant (20). Chicken egg white cathepsin, which is also a potent in vitro inhibitor of cathepsin L, cathepsin B, and cathepsin H, but not of the calpains (21), had no effect on the mevalonate-accelerated degradation of HMGal. Since the molecular weight of cystatin is about 13,000, it probably does not enter the cells. Finally, leupeptin and antipain, which inhibit both serine and cysteine proteases, were also ineffective. Structurally, leupeptin has an arginal residue at the carboxy terminus, whereas ALLM and ALLN have methioninal or norleucinal residues, respectively. It is suggested that ALLM and ALLN are hydrophobic and therefore might penetrate the membrane more easily than the water-soluble leupeptin and antipain.

The concentration and the time course of ALLN required to inhibit the degradation of HMGal are presented in Fig. 2. In the absence of mevalonate, the specific activity of β-galactosidase increases about 3-fold after treatment with 10 µg/mL ALLN for 16 h (Fig. 2A). In the presence of mevalonate, 40 µg/mL of ALLN completely prevents the mevalonate-accelerated degradation of HMGal. These results indicate that ALLN inhibits both the basal and the mevalonate-accelerated degradation of HMGal. The enhanced degradation of HMGal in the presence of LDL (7) is also inhibited by ALLN (Fig. 2B). The effect of ALLN is apparent after 5 h and reaches a plateau at 10 h. The same results are obtained when 25-hydroxycholesterol is added to the growth medium (data not shown). In the CHO cells transfected with the gene coding for the soluble β-galactosidase (CHO-Gal cells), β-galactosidase activity neither decreased in the presence of mevalonate (8) nor increased with ALLN treatment (data not shown). Thus, the inhibition of degradation of HMGal by ALLN was specific for the membrane-bound HMGal and not for the soluble β-galactosidase.

Pulse-chase experiments confirmed that the increase in β-galactosidase activity of HMGal caused by ALLN is due to a decrease in the rate of enzyme degradation (Fig. 3). When mevalonate is present in the medium during the chase, HMGal is degraded faster than in cells receiving no mevalonate. The half-life of HMGal under the various assay conditions are presented in Table I. As can be seen, mevalonate treatment increases the rate of degradation about 1.5-fold (8). When the cells are chased in the presence of ALLN with or without mevalonate, slower degradation is observed and the half-life of HMGal is extended to more than 20 h. Essentially, a similar phenomenon is observed when HMG-CoA reductase was monitored (Table I). Experiments in which ALLN was added during the pulse showed that ALLN (100 µg/mL) did not affect the rate of synthesis of either HMGal or HMG-CoA reductase (data not shown). These results demonstrate that ALLN inhibits the basal as well as the mevalonate-accelerated degradation of both HMGal and HMG-CoA reductase.

Inhibition of Degradation of HMGal by ALLN Is Not Affected by Lysosomotropic Agents—In addition to inhibition of calpains, ALLN inhibits the activity of the lysosomal proteases, cathepsin L and cathepsin B, in vitro (19). It is, thus, possible that the action of ALLN on lysosomal proteases may
Cells were incubated with tropic agents for degradations (control). The values presented are the mean ± S.D. of four independent experiments. ND, not determined.

Table II
Specific activity of HMGal upon treatment with lysosomotropic agents, with or without ALLN

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>94 ± 16</td>
</tr>
<tr>
<td>Chloroquine + ALLN</td>
<td>241 ± 25</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>NH4Cl + ALLN</td>
<td>336 ± 40</td>
</tr>
<tr>
<td>ALLN</td>
<td>357 ± 48</td>
</tr>
</tbody>
</table>

Table III
Half-lives of HMGal and HMG-CoA reductase in brefeldin A-treated cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HMGal</th>
<th>HMG-CoA reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFA</td>
<td>9.94 ± 0.21</td>
<td>9.10 ± 0.62</td>
</tr>
<tr>
<td>BFA + MVA</td>
<td>7.08 ± 0.56</td>
<td>6.87 ± 1.53</td>
</tr>
<tr>
<td>BFA + ALLN</td>
<td>20.3 ± 4.2</td>
<td>20.7 ± 5.1</td>
</tr>
<tr>
<td>BFA + MVA + ALLN</td>
<td>21.5 ± 5.0</td>
<td>17.3 ± 6.5</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of ALLN on the degradation rate of HMGal and HMG-CoA reductase. Cells were grown for 24 h and then pulse-labeled, as described under "Experimental Procedures." At the end of the pulse, cells were collected (t = 0) or chased for 2-20 h in medium supplemented with no addition (C), with 20 mM mevalonate (●) or 100 μg/ml ALLN (□), or both mevalonate and ALLN (△). At the indicated timepoints, cells were collected, lysed, and the HMGal (A) and HMG-CoA reductase (B) were immunoprecipitated and subjected to 7.5% SDS-PAGE. The bands corresponding to HMGal and HMG-CoA reductase (inset) were quantified by densitometric scanning. The area of the peaks were normalized to the total counts/ min of [35S]methionine incorporated into cellular proteins. 100% is the amount of the respective proteins at the end of the pulse (t = 0).

Table I
Half-lives of HMGal and HMG-CoA reductase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HMGal</th>
<th>HMG-CoA reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.53 ± 2.19</td>
<td>7.69 ± 2.15</td>
</tr>
<tr>
<td>MVA</td>
<td>4.85 ± 1.45</td>
<td>5.21 ± 1.05</td>
</tr>
<tr>
<td>ALLN</td>
<td>25.70 ± 5.70</td>
<td>17.65 ± 4.54</td>
</tr>
<tr>
<td>MVA + ALLN</td>
<td>22.00 ± 6.70</td>
<td>17.60 ± 4.41</td>
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</tbody>
</table>

account for its inhibition of HMGal and HMG-CoA reductase degradation. To investigate this possibility, the specific activity of HMGal was measured in cells treated with lysosomotropic agents for 20 h. As can be seen in Table II, chloroquine neither affects the activity in control cells nor does it inhibit the mevalonate-accelerated degradation. Furthermore, when cells are treated with both chloroquine and ALLN, HMGal activity increases in both control and mevalonate-treated cells to a similar extent as in ALLN-treated cells (Table II). The same results were obtained with NH4Cl. When cells were treated with LDL, both lysosomotropic agents inhibit the LDL-accelerated degradation of HMGal (Table II). These results demonstrate that chloroquine and NH4Cl inhibit the lysosomal function in the cells, since cholesterol release from LDL requires hydrolysis in the lysosomes (22, 23). However, the mevalonate-accelerated degradation is not inhibited, and ALLN still inhibits the degradation. These results indicate that a non-lysosomal protease(s), which is inhibited by ALLN, is necessary for the degradation of HMGal.

Brefeldin A Has No Effect on the ALLN-sensitive Degradation of HMGal and HMG-CoA Reductase—In order to further establish the cellular localization of the ALLN-sensitive degradation of HMGal and HMG-CoA reductase, their half-lives were measured in brefeldin A-treated cells (Table III). It has been shown that brefeldin A blocks the exit of proteins from the ER (24, 25), and we have shown that in CHO cells, 5 μg/ml of brefeldin A prevents the export of vesicular stomatitis virus G protein from the ER (26). As we have demonstrated previously, the mevalonate-accelerated degradation of HMGal is not affected by brefeldin A, indicating that this degradation may occur in a pre-Golgi compartment (8). As can be seen in Table III, the half-lives of HMGal and HMG-CoA reductase are extended in the presence of ALLN even in brefeldin A-treated cells and regardless of mevalonate addition. The results presented here suggest that the pre-Golgi mevalonate-accelerated degradation of HMGal and HMG-CoA reductase is inhibited by ALLN.

Ca2+-dependent ALLN-sensitive Protease in CHO Cells—Calpains are Ca2+-dependent neutral cysteine proteases which are found in many mammalian and avian tissues (27, 28). There are two major types of calpains, one with a high affinity for calcium (calpain I or μCANP, active at micromolar concentrations of calcium), and one with low affinity for calcium (calpain II or mCANP, active at millimolar concentrations of calcium), and both are located mainly in the cytoplasm of cells (27, 28). Both calpains are composed of two subunits;
their catalytic 80-kDa subunits are similar but clearly different, whereas their 30-kDa regulatory subunits are identical. Calpain activities are regulated in vitro by an endogenous inhibitor, calpastatin, and by Ca2+ (28, 29). ALLM and ALLN have been shown to be potent inhibitors of calpains in vitro (21, 30). This suggest that calpain(s) may participate in the degradation of HMG-CoA reductase and HMGal. Since there is no evidence for the presence of calpains in CHO cells, we have measured the Ca2+-dependent proteolytic activity in vitro (Table IV). The extracts of CHO-HMGal cells show a Ca2+-dependent protease activity, which is inhibited about 70% by 13 μM ALLN. After partial purification on phenyl-Sepharose, the specific activity increases about 60-fold and this Ca2+-dependent activity is still efficiently inhibited by ALLN. In addition to inhibition by ALLN, this Ca2+-dependent activity was inhibited by leupeptin (serine and cysteine protease inhibitor) and E-64 (cysteine protease inhibitor), but not by APMSF (serine protease inhibitor) or cystatin (inhibits the activity of cathepsins but not of calpains) (data not shown).

When CHO-HMGal cells were treated with mevalonate for 20 h, no significant change was observed in the Ca2+-dependent protease activity in either the crude extracts or the phenyl-Sepharose-purified fractions. The data presented in Fig. 4 demonstrate the presence of calpain- and calpastatin-like proteins in CHO-HMGal cells. Post-nuclear extracts from cells treated with mevalonate, LDL, or ALLN were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies. When anti-HMG-CoA reductase antibody was used, a band with a Mr, 96,000 corresponding to HMG-CoA reductase is detected. The intensity of the reductase band decreases in cells treated with mevalonate or LDL, and increases after treatment with ALLN (Fig. 4A). When anti-human calpain II antibody was used, a band with a Mr, 80,000, representing the catalytic subunit, is observed. A band with the same size was detected using two other antibodies raised against bovine and rabbit calpain II (data not shown). However, the intensity of this band did not change after treatment with mevalonate, LDL or ALLN (Fig. 4A). A band with a Mr, 30,000 corresponding to the regulatory subunit of calpains was also detected, and its intensity was also unchanged under conditions of enhanced or blocked degradation of HMGal (data not shown). Dot-blot analysis was performed to quantify the amounts of calpain II in cells. The amount of HMG-CoA reductase decreases to about 30% of control level after treatment with mevalonate and increases about 3-fold upon treatment with ALLN. The amount of calpain II does not change under the same conditions. To explore the possibility that the amount of calpastatin may be regulated by these treatments, we blotted CHO-HMGal extracts with anti-calpastatin antibody. Fig. 4A shows that this antibody detects a band of Mr, 120,000, which is specifically competed by purified calpastatin D3 fragment (31). Again, the amounts of calpastatin do not change in cells treated with mevalonate, LDL, or ALLN. The size of calpastatin on SDS-PAGE is larger than the Mr, 70,000 for this protein as calculated from the cDNA sequence (29).

**DISCUSSION**

The degradation of HMG-CoA reductase is enhanced by addition of mevalonate, LDL, or 25-hydroxycholesterol to the medium (1, 2, 22, 23). We have previously provided evidence that the degradation of HMG-CoA reductase occurs in ER and that the membrane domain of the reductase is necessary and sufficient to confer this regulated degradation (7, 8). In this study we demonstrate that both the basal and the mevalonate- or LDL-accelerated degradations of HMG-CoA reductase are inhibited by two of the cysteine protease inhibitors, ALLM and ALLN, but not by lysosomotropic agents. Moreover, the basal and the mevalonate-accelerated degradations are also inhibited by ALLN under conditions in which protein exit from ER is blocked by brefeldin A.

It has been reported that the basal degradation of reductase in hepatocytes or in a mouse mammary cell line was blocked by several inhibitors of lysosomal function, such as propylene, methylamine (10), and NHCl (9). We could not detect any increase in the activity of HMGal in cells treated with NHCl or chloroquine. This apparent discrepancy may be attributed to the different cell lines tested, or the different susceptibility of the cells to these agents, since NHCl and chloroquine were reported to have side effects on protein synthesis (9). Concerning the accelerated degradation, it has been shown that propylene and monensin fully inhibit the mevalonate-accelerated degradation of HMG-CoA reductase but only partially blocked the effect of 25-hydroxycholesterol on the enhanced degradation, suggesting that an acidic compartment is required for the mevalonate-stimulated reductase degradation (10). Here, we show that ALLN inhibits the enhanced degradation of reductase caused not only by mevalonate but also by LDL and 25-hydroxycholesterol. Furthermore, the treatment with NHCl or chloroquine does not prevent the mevalonate-accelerated degradation. This observation is consistent with the results of Tanaka et al. (9) that the accelerated degradation of HMG-CoA reductase induced by 25-hydroxycholesterol was not inhibited by NHCl. Our results suggest that the target of ALLN is involved in both the basal as well as the degradation accelerated by mevalonate, LDL, or 25-hydroxycholesterol.

Apolliprotein B is another component of lipid metabolism that has been shown to be degraded in the ER in a regulated fashion (33). Preincubation of human hepatoblastoma cells with LDL induced a 20-32% increase in the degradation of apoprotein B-100. However, this degradation was not en-

<table>
<thead>
<tr>
<th>Ca2+</th>
<th>ALLN</th>
<th>Specific activity</th>
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<tr>
<td>mM</td>
<td>μM</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.7</td>
<td>0</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>0.7</td>
<td>13</td>
<td>29 ± 2</td>
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<td>0</td>
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<td>150 ± 37</td>
</tr>
<tr>
<td>0.7</td>
<td>13</td>
<td>24 ± 2</td>
</tr>
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</table>

**TABLE IV**

Ca2+-dependent ALLN-sensitive protease activity in CHO-HMGal cells

Cells were grown for 20 h in the absence (control) or presence of 20 mM mevalonate (MVA) and then homogenized. Crude extract is the 150,000 × g supernatant. Purification by phenyl-Sepharose chromatography was done as described under "Experimental Procedures." Specific activity was measured as described under "Experimental Procedures" and is expressed as percentage of the activity in the crude extract or in the phenyl-Sepharose fraction of control cells in the presence of 4 mM Ca2+. Each value is the mean ± S.D. of three independent experiments. The specific activities of the crude extract and the phenyl-Sepharose fraction were 1.25 and 74.8 arbitrary units/h/mg of protein, respectively. ND, not determined.
Inhibition of HMG-CoA Reductase Degradation

A

HMG-CoA reductase Calpain II Calpastatin

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B

HMG-CoA Reductase

- BSA
- Control
- MVA
- ALLN

Graph showing absorbance (arbitrary units) vs. protein (μg/dot). Values are 0.25, 0.5, 1, 2.5, and 5 μg of protein.

Calpain II

- BSA
- Control
- MVA
- ALLN

Graph showing protein (μg/dot) vs. protein (μg/dot). Values are 0.25, 0.5, 1, 2.5, and 5 μg of protein.

FIG. 4. Identification and quantification of calpain II- and calpastatin-like proteins. Cells were grown for 20 h in medium supplemented with either 20 mM mevalonate, 125 μg/ml LDL, or 50 μg/ml ALLN. Cell lysates were prepared and subjected to immunoblot (A) or dot-blot (B) analyses, as described under “Experimental Procedures.” A, proteins resolved by SDS-PAGE were transferred onto nitrocellulose. The membranes were probed with anti-HMG-CoA reductase antibody (lanes 1-4), with anti-calpain II antibody (lanes 5-8), with anti-calpastatin antibody (lanes 9-12), or with anti-calpastatin antibody preincubated with calpastatin peptide D3 fragment (lanes 13-16). Followed by peroxidase-conjugated secondary antibody, the enhanced chemiluminescence detection system was used and the membranes were exposed to x-ray film. The extracts were prepared from cells treated with no addition (lanes 1, 5, 9, and 13), with mevalonate (lanes 2, 6, 10, and 14), with LDL (lanes 3, 7, 11, and 15), or with ALLN (lanes 4, 8, 12, and 16). Marker proteins used were: phosphorylase b (97.4 kDa), bovine serum albumin (67 kDa), and β-galactosidase (116 kDa). B, lysates were diluted with lysis buffer and spotted onto nitrocellulose. The membrane were probed with anti-HMG-CoA reductase (left panel) or anti-calpain II antibody (right panel), and the dots were detected by the same method described above. The intensity of the dots on the film (inset) were quantified by densitometric scanning. Lysates were prepared from cells treated with no addition (control, ○), with mevalonate (MVA, □), or with ALLN (ALLN, ▲). Bovine serum albumin was used as a control for nonspecific binding (C).

HMG-CoA reductase degraded by purified calpain II to both membrane-bound 62-kDa and the soluble 52-56-kDa fragments (11, 12). Phosphorylation of the 97-kDa reductase was also reported to enhance the in vitro proteolysis by calpain II. However, there has been no evidence that calpain II is the protease responsible for the degradation of HMG-CoA reductase in vivo. The results presented in this paper, although indirect, suggest that calpain or calpain-like protease is involved in the regulated degradation of HMG-CoA reductase in vivo.

Our studies show the presence of Ca2+-dependent protease as well as calpain-like protein in CHO cells. However, under the condition of enhanced degradation of HMG-CoA reductase, no change was observed in the in vitro activity or the protein amounts of calpain II or the levels of a calpastatin-like protein. However, it is possible that in these cells calpain(s) is activated under the condition of enhanced degradation, because most of the calpain(s) is believed to be in an inactive form in vivo (27–29) and in the presence of Ca2+ both calpains are autolytically activated (38, 39). We have recently...
shown that perturbation of cellular Ca\textsuperscript{2+} impairs the mevalonate-dependent accelerated degradation of HMG-CoA reductase. These observations suggest that mevalonate or a mevalonate-derived metabolite(s) might influence the concentration or distribution of Ca\textsuperscript{2+} and activate a Ca\textsuperscript{2+}-dependent protease(s) to degrade HMG-CoA reductase.

Since we do not know the events connecting the signal, mevalonate, LDL, or 25-hydroxycholesterol, and the target, HMG-CoA reductase degradation, we can only speculate about the role of calpain inhibitors. ALLN might work directly by inhibiting the protease that degrades HMG-CoA reductase or indirectly by inhibiting the pathway between the signal and the target. However, since it appears that a calpain-like protease is involved in HMG-CoA reductase degradation in vitro, there is an important apparent paradox to be resolved: in vitro proteolysis of membrane-bound HMG-CoA reductase with purified calpain II releases the catalytically active cytoplasmic 52-kDa fragment; in vivo expression of a cDNA for this soluble catalytic domain yields a catalytically active stable protein, which does not respond to the regulatory molecules which enhance the degradation of the membrane-bound, intact reductase (40). Thus, if in vivo calpain releases this cytoplasmic domain as has been shown in vitro, there must be an additional mechanism for the rapid degradation of the catalytic domain. Our results, nevertheless, strongly suggest the involvement of a calpain-like protease in the degradation mechanism of HMG-CoA reductase.

Acknowledgments—We are grateful to Dr. P. A. Edwards, Dr. H.-S. Jenke, Dr. Y. Adachi, Dr. D. E. Croall, and Dr. S. Kawashima for generously providing antibodies. We thank R. Morales for the preparation of the illustrations.

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