The steady-state level of the resident endoplasmic reticulum protein, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), is regulated, in part, by accelerated degradation in response to excess sterols or mevalonate. Previous studies of a chimeric protein (HM-Gal) composed of the membrane domain of HMGR fused to Escherichia coli β-galactosidase, as a replacement of the normal HMGR cytosolic domain, have shown that the regulated degradation of this chimeric protein, HM-Gal, is identical to that of HMGR (Chun, K. T., Bar-Nun, S., and Simoni, R. D. (1990) J. Biol. Chem. 265, 22004–22010; Skalnik, D. G., Narita, H., Kent, C., and Simoni, R. D. (1988) J. Biol. Chem. 263, 6836–6841). Since the cytosolic domain can be replaced with β-galactosidase without effect on regulated degradation, it has been assumed that the cytosolic domain was not important to this process and also that the membrane domain of HMGR was both necessary and sufficient for regulated degradation. In contrast to our previous results with HM-Gal, we observed in this study that replacement of the cytosolic domain of HMGR with various heterologous proteins can have an effect on the regulated degradation, and the effect correlates with the oligomeric state of the replacement cytosolic protein. Chimeric proteins that are oligomeric in structure are relatively stable, and those that are monomeric are unstable. To test the hypothesis that the oligomeric state of the cytosolic domain of HMGR influences degradation, we use an “inducible” system for altering the oligomeric state of a protein in vivo. Using a chimeric protein that contains the membrane domain of HMGR fused to three copies of FK506-binding protein 12, we were able to induce oligomerization by addition of a “double-headed” FK506-like “dimerizer” drug (AP1510) and to monitor the degradation rate of both the monomeric form and the drug-induced oligomeric form of the protein. We show that this chimeric protein, HM-3FKBP, is unstable in the monomeric state and is stabilized by AP1510-induced oligomerization. We also examined the degradation rate of HMGR as a function of concentrations within the cell. HMGR is a functional dimer; therefore, its oligomeric state and, we predict, its degradation rate should be concentration-dependent. We observed that it is degraded more rapidly at lower concentrations.

The endoplasmic reticulum resident membrane protein, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (EC 1.1.1.34; GenBank™ accession number M12705), is the rate-limiting enzyme in the cholesterol biosynthetic pathway, catalyzing the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate, which is further metabolized to many downstream isoprenoid products as well as cholesterol (1). These downstream metabolites participate in feedback regulation of HMGR to reduce its protein levels when sufficient cholesterol is obtained through exogenous sources, and conversely, flow through the mevalonate pathway is increased by up-regulating synthesis and extending the protein half-life of HMGR when intracellular sterol levels are low (2–5). This phenomenon of regulated HMGR degradation has been a focus of study for many years; however, the mechanism of regulation and the identity of the proteolytic components responsible for degradation remain largely unknown.

The topology of HMGR, which is a 97-kDa glycoprotein, consists of two major domains: a transmembrane domain that spans the endoplasmic reticulum membrane eight times and the C-terminal catalytic domain, which contains the active site and resides in the cytosol (6–8). Structural studies of the Pseudomonas mevalonii HMGR counterpart have revealed that the catalytic domain, in which key residues involved in substrate recognition and catalysis are conserved between bacteria and mammals, requires dimerization of two HMGR molecules through this domain to form the substrate-binding and active site (9). In addition, radiation inactivation studies of HMGR from rat liver have demonstrated that the enzymatically active form of HMGR is a dimer (26, 27).

The membrane domain of HMGR has been of interest due to work that has shown that determinants for regulated degradation reside in this region (8, 10). Experiments in which the cytosolic domain of HMGR was replaced with the soluble Escherichia coli β-galactosidase enzyme showed that the resulting chimera (termed HM-Gal), when stably transfected into Chinese hamster ovary (CHO) cells, exhibits a half-life that mirrors that of HMGR and is similarly subject to sterol-accelerated degradation (11, 12, 31). Based on this observation, we have assumed that the cytosolic domain was not important and that the membrane domain was both necessary and sufficient to confer regulated degradation.

We have now generated several different HMGR fusion proteins with the cytosolic domain replaced with various heterologous proteins. We have discovered that there is an apparent correlation between the oligomeric state of the heterologous
cytosolic protein and resulting stability of the fusion protein, with monomeric fusion proteins being degraded relatively rapidly and oligomeric fusion proteins degraded slowly. Based on these findings, we have developed a hypothesis that the oligomeric state of HMGR, determined at least in part by interactions through the cytosolic catalytic domain, plays a role in determination of the degradation rate.

In this paper, we have tested this hypothesis using an "inducible oligomerization" system that utilizes FK506-binding protein 12 (FKBP) and the synthetic dimeric drug AP1510 (13, 14). The drug FK506 is a well characterized immunosuppressant that binds to FKBPs, mimicking an unknown endogenous ligand and activating the calcineurin signaling pathway (15, 16). AP1510 is a nontoxic, cell-permeable compound that consists essentially of two FK506 derivatives fused together through a linker region and thus has the capability of dimerizing two FKBP-containing proteins in vivo (14). We have constructed a fusion protein called HM-3FKBP, which consists of three tandem repeats of FKBP fused to the C terminus of the membrane domain of HMGR. Using the dimeric AP1510 drug, we manipulated the oligomeric state of HM-3FKBP in vivo and studied the degradation phenotype both as a monomer and as an oligomer. Additionally, we have also fused three FKBP sequences to the C terminus of one of our "fast" monomeric constructs, HM-Hyg, and we tested whether AP1510 can stabilize its fast degradation through oligomerization.

If the oligomeric state of HMGR is important for stability and degradation, we predict that the level of HMGR expression should influence the relative proportion of monomers and dimers and thus should also influence the degradation rate. We have varied the in vivo expression levels of HMGR by expression from an inducible promoter system, and the results show that the protein is more rapidly degraded at lower concentrations. These findings further support our hypothesis that oligomerization of HMGR molecules through the cytosolic domain stabilizes the protein and that the monomeric state results in faster degradation.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—DNA purification kits were obtained from QIAGEN Inc. Restriction enzymes and molecular biology reagents were purchased from Life Technologies, Inc. and New England Biolabs Inc. Transfection and cell culture reagents were obtained from Life Technologies, Inc. The Tet-OFF Expression System was purchased from CLONTECH. All chemicals unless otherwise noted were obtained from Sigma. FK506 was obtained from Gerald Crabtree (Stanford University) and Fujisawa, Inc. AP1510 was generously provided by Ariad Pharmaceuticals. Compactin was the kind gift from Akira Endo (Tokyo University) and Fujisawa, Inc. Anti-beta-galactosidase monoclonal antibody was purchased from Roche Molecular Biochemicals. Anti-beta-galactosidase monoclonal antibody was purchased from Promega. Sodium mevalonate was purchased from mevalonolactone as described by Brown and co-workers.

**Cell Culture**—Stably transfected CHO-K1 cell lines were maintained in minimal essential medium supplemented with nonessential amino acids, 5% fetal calf serum (MEM-FCS medium), and 0.25 mg/ml active G418 in a humidified 5% CO2 incubator at 37 °C and passed every 3 days. Stable Tet-Off UT-2 cells were maintained under the same conditions, except that MEM-FCS medium was replaced with MEM-PBS medium, which contained 5% Tet System approved fetal bovine serum (CLONTECH) instead of 5% fetal calf serum and also 1 mM sodium mevalonate and 0.1 mg/ml active G418. Double-stable Tet-Off UT-2 cells were maintained under the same conditions as described above, except that the medium did not contain sodium mevalonate. The double-stable Tet-Off CHO-AAS cell line was maintained under the same conditions as described above, except that the medium also contained 0.1 mg/ml hygromycin B.

**Plasmid Construction**—pMKIT HM-Hyg was constructed by PCR of the E. coli hygromycin phosphotransferase (hph) gene from a plasmid template, with restriction sites introduced in the 5' and 3' PCR primers, followed by restriction enzyme digestion and ligation into the pMKIT plasmid vector containing the HMGR membrane domain sequence. The maximum amount of PCR sequence was replaced with native sequence using naturally occurring restriction sites to reduce errors introduced by PCR, and the remaining unexpanded sequence was subjected to DNA sequencing to verify accuracy. pMKIT HM-Gal deletion mutants were generated by PCR of the 3' sequence of the lacZ gene, using PCR primers to create deletions and primer pairs to fragmentize the C-terminal 10 or 20 amino acids from the beta-galactosidase protein. The PCR products were ligated into pMKIT HM-Gal and verified by DNA sequencing. pCMV HM-eGFP was created by ligation of the HMGR membrane domain sequence (EcorRI/BstEII fragment) into the commercially purchased pCMV-N1 eGFP vector (CLONTECH) in frame and upstream of the eGFP coding sequence. FKBP-containing constructs were generated by PCR of triple copies of FKBP and ligation of the PCR product into pMKIT HM or pMKIT HM-Hyg to create pMKIT HM-3FKBP and pMKIT HM-Hyg-3FKBP, respectively. The stop codon was removed from the end of the coding sequence of Hyg by PCR prior to FKBP insertion. All constructs were verified by DNA sequencing. pTRE HMGR was produced by ligation of full-length HMGR cDNA sequences (EcoRI/BstXI fragment) into the commercially purchased pTRE-Off vector (CLONTECH). pTRE HM-Gal was generated by insertion of full-length HM-Gal cDNA sequences (EcoRI/XbaI fragment) into the pTRE-Off vector.

**Generation of Stable CHO-K1 Cell Lines**—CHO-K1 cells were plated in 60-mm dishes at 60–80% confluence and transfected by lipofection using LipofectAMINE P/L (Life Technologies, Inc.) according to the manufacturer's instructions. Cells were split the following day onto multiple 10-cm dishes and fed selection medium containing 1–2 mg/ml active G418 for 9–14 days. Resistant colonies were either pooled or cylinder-cloned and analyzed by radiolabeling to identify high expressing populations. The HM-eGFP stable cell line was selected for high expressers by fluorescence-activated cell sorting.

**Generation of Double-stable Tet-Off UT-2 Cell Line**—The pTet-Off regulator plasmid DNA (CLONTECH) was transfected into UT-2 cells with the same method as described above. The stable Tet-Off cells were selected with 1 mg/ml G418 for 14 days, and the resistant colonies were pooled. After the stable Tet-Off cells were obtained, the pooled stable Tet-Off cells were transfected with pTRE HMGR recombinant plasmid DNA with the same method as described above. The stable Tet-Off cells were selected with minimal essential medium supplemented with 5% lipid poor serum (MEM-LPS medium) prepared by the method of Rothblat et al. (18) for 14 days. The resistant cells were cylinder-cloned and analyzed by radiolabeling to identify high expressing populations.

**Generation of Double-stable Tet-Off CHO-AAS Cell Line**—pTRE HM-Gal DNA and pTK Hyg DNA (CLONTECH) were cotransfected into cellularly purchased Stable Tet-Off CHO-AAS cells (CLONTECH) in a ratio of 20:1 with the same method as described above. Transfectants were selected with 0.6 mg/ml hygromycin B for 14 days, and resistant populations with the highest beta-galactosidase activity were isolated using fluorescence-activated cell sorting (10).

**Pulse-Chase Analysis**—Cells were grown in 60-mm dishes to 70–80% confluency in MEM-FCS or MEM-PBS medium. The following day, the cells were washed with MEM-LPS medium containing 10 mM compactin and 100 μM sodium mevalonate. After 20 h, cells were starved for 1 h in methionine/cysteine-free minimal essential medium supplemented with 10 μM compactin and 100 μM sodium mevalonate and labeled for 0.5 h in methionine/cysteine-free minimal essential medium containing 10 μM compactin, 100 μM sodium mevalonate, and 100 μCi of Tran35S-Ser per plate. Cells were chased in MEM-LPS medium supplemented with 10 μM compactin, 100 μM sodium mevalonate, 2 mM methionine, and 2 mM cysteine out to various time points. At each chase time point, cells were collected by washing three times in ice-cold phosphate buffered saline, followed by lysis in ice-cold solubilization buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 2 μg/ml calpain inhibitor I, and 100 mM dithiothreitol). Lysates were centrifuged at 16,000 × g for 15 min at 4 °C to remove insoluble materials, and supernatants were collected. 10-μl aliquots were reserved for boiling trichloroacetic acid precipitation and scintillation counting. The remaining supernatants were processed for immunoprecipitation with the appropriate antisera. Antigen-antibody complexes were precipitated with protein A-Sepharose (Amersham Pharmacia Biotech) and washed twice in ice-cold 2×-polyacrylamide gels in 1× Tris-HCl (pH 7.5) and 0.1% Nonidet P-40, and then solubilized in sample buffer (62.5 mM Tris-HCl (pH 6.8), 8% urea, 15% SDS, 20% glycerol, 0.25% bromphenol blue, and 25 mg/ml dithiothreitol) for 30 min at 37 °C. Samples normalized for equal trichloroacetic acid-precipitable counts were loaded on 5–15% SDS-polyacrylamide gradient gels for electrophoresis. The gels were then fixed in a solution containing...
24-well dishes were permeabilized with 500 μM sodium mevalonate and 10 μM compactin for 20 h prior to pulse-chase analysis as described above. Drug concentrations were maintained throughout the experiment in starvation, labeling, and chase media.

**Enzyme Activity Assays**—β-Galactosidase activity assays were performed as described previously (12). Briefly, cells seeded in triplicate in 24-well dishes were permeabilized with 50 μg/ml digitonin and incubated at 37 °C with a 1 mg/ml concentration of the colorimetric enzyme substrate o-nitrophenyl-β-d-galactopyranoside until color change was visually detectable. Reactions were stopped by addition of 1 M Na2CO3. Reactions were terminated by addition of 1 M Na2CO3. Specific activity values were calculated by normalization to time of incubation and total protein, which was determined on duplicate plates by the method of Lowry et al. (19).

**RESULTS**

**Monomeric HMGR Membrane Domain Fusion Proteins Are Subject to Rapid Degradation, whereas Oligomeric Fusion Proteins Are More Stable**—We have now generated several chimeric proteins consisting of the membrane domain of HMGR fused to various heterologous proteins. In the course of analyzing these fusion proteins, we observed that some chimeric proteins are degraded rapidly, whereas others are stable and exhibit normal regulated degradation characteristic of HMGR despite the fact that all constructs have the identical membrane domain from HMGR. We have found that the degradation phenotype correlates with the oligomeric state of the cytosolic domain, with monomeric proteins being degraded rapidly and dimeric and higher order proteins degraded normally. In addition to HM-Gal (HM designates the HMGR membrane domain and is followed by a designation for the heterologous cytosolic protein), another construct we have made is HM-Hyg, which is composed of the HMGR membrane domain fused to the hygromycin phosphotransferase protein, which confers resistance to the antibiotic hygromycin B. We have also constructed HM-XGPRT, which consists of the membrane domain fused to the bacterial xanthine guanosylphosphoribosyltransferase, which enables cells to utilize xanthine as an alternate source for purines. Additionally, we have created HM-eGFP (with the membrane domain fused to enhanced green fluorescent protein) and HM-3HA (with three HA epitope tags attached to the end of the membrane domain). The results of degradation studies by pulse-chase analysis of these proteins in stably transfected cells are shown in Table I.

**Fusion Proteins with Normal Degradation Phenotypes** include HM-Gal and HM-XGPRT, whereas HM-Hyg, HM-3HA, and HM-eGFP are degraded relatively rapidly (Table I). Consideration of the oligomeric state of these heterologous proteins fused to the membrane domain reveals that β-galactosidase is enzymatically active as a tetramer; xanthine guanosylphosphoribosyltransferase is a trimer (15, 20); and hygromycin phosphotransferase (21), green fluorescent protein, and the HA epitope tag are monomers, suggesting that the oligomeric state of the cytosolic domain may influence degradation of the membrane domain.

We assayed the heterologous cytosolic domains of the chimeras for functional activity as evidence of oligomeric assembly in vivo while fused to the HMGR membrane domain. We detected enzyme activity for HM-Gal (12) and HM-XGPRT (data not shown), indicating they are present as tetramers and trimers, respectively. HM-Hyg is capable of conferring resistance to hygromycin B in transfected CHO cells (data not shown), suggesting that HM-Hyg is capable of folding correctly and that, most likely, the rapid degradation observed in this construct is not due to misfolding. Similarly, HM-eGFP is also correctly folded as evidenced by detectable GFP fluorescence in transfected cells (data not shown). Based on the correlation between the oligomeric state and degradation phenotype, we hypothesize that interaction between the membrane domains induced through the cytosolic domain is required for normal stability and sterol-regulated degradation to occur.

**Monomeric HM-Gal Deletion Mutants Are Rapidly Degraded**—As a first test of our hypothesis, we constructed deletion mutants in which the C-terminal 10–20 amino acids of the β-galactosidase enzyme in HM-Gal were removed since it has been shown that these residues are critical for tetramerization of β-galactosidase and formation of the active enzyme (22, 23). Removal of these amino acid residues should result in the monomerization of the cytosolic region of HM-Gal. Transfected CHO cell lines expressing either HM-GalA10 (missing the C-terminal 10 amino acids) or HM-GalA20 (missing the C-terminal 20 amino acids) were analyzed by pulse-chase analysis to measure half-lives. As shown in Fig. 1, both deletion mutants, in contrast to full-length HM-Gal, were degraded abnormally fast (Fig. 1, compare A and B with C). The half-life of HM-Gal was ~9 h, and the presence of sterols reduced it to ~5 h. For both HM-GalA10 and HM-GalA20, the half-lives were <2 h, and there was no acceleration by sterols. To demonstrate that our deletion mutants were no longer assembled into tetramers, we measured β-galactosidase activity in these cell lines, as β-galactosidase activity is known to be dependent upon tetra-
ramerization of the enzyme. The results in Fig. 2 support the conclusion that tetramerization of β-galactosidase in the deletion mutants has been abolished since HM-GalD10 and HM-GalD20 cell lines have lost β-galactosidase activity. In these experiments, the HM-Gal/HM-GalD10/HM-GalD20 β-galactosidase protein ratio was 1:0.8:0.2 (data not shown).

**Induced Oligomerization Results in Stabilization of HMGR Membrane Domain Fusion Proteins**—In the experiments described above, it is possible the deletions in HM-Gal protein could result in the proteins being recognized as misfolded and degraded by a process unrelated to normal HMGR degradation even though the mutant β-galactosidase is attached to the membrane domain of HMGR. A better test of our hypothesis would be to study the same HM-X chimeric protein both as a monomer and a dimer in the same cell line and to compare the degradation phenotypes in each of these states. This was made possible by taking advantage of the previously described inducible dimerization system utilizing FKBP and synthetic “dimerizer” drugs based on FK506 (14, 16, 24). Treatment of cells expressing chimeric proteins including FKBP with the dimerizer drug AP1510, which is composed of two FK506 derivatives,...
fused together, resulted in dimerization of the FKBP-containing fusion proteins. A schematic of this system is shown in Fig. 3.

We constructed a new fusion protein with three tandem copies of FKBP fused to the HMG2 membrane domain, called HM-3FKBP. Initial experiments with chimeras containing only one FKBP gave ambiguous results, and more striking results were obtained with three copies of FKBP as reported previously (14). However, with three copies of the ligand-binding protein, there can be higher order structures formed in addition to dimers. We stably expressed HM-3FKBP in CHO-K1 cells and subjected the monomeric fusion construct to the same drug regimen as described for Fig. 6. Cells were chased in the absence (A) or presence (B) of 2.5 μM 25-hydroxycholesterol. Cells were lysed, immunoprecipitated with anti-HA antibodies, separated by SDS-polyacrylamide gel electrophoresis, and subjected to autoradiography.

which is near the normal half-life of HMG2 in the absence of sterols. The control testing the effects of FK506 is critical since it is often the case that ligand binding can stabilize a protein. As shown in Fig. 4A, FK506 did appear to have a small effect, but could not stabilize HM-3FKBP to the same extent as AP1510. In three repetitions of this experiment, the greatest effect of FK506 was observed in the experiment shown here, and in one experiment, there was no effect.

We also examined the effect of AP1510 on the degradation rate of HM-3FKBP in the presence of a regulatory sterol, 2.5 μM 25-hydroxycholesterol. In the presence of sterols, we detected only a minor increase in degradation rate (Fig. 4A, and B, compare No Drug lanes) since the half-life was short even in the absence of sterol treatment. However, AP1510 treatment was capable of moderately stabilizing sterol-accelerated degradation as well (Fig. 4B). We feel that it is striking that the half-life of HM-3FKBP when measured in the presence of AP1510 is very similar to that of HMG2 in both the absence and presence of sterols, suggesting that this artificial system is able to mimic the normal physiological situation.

To be sure that the effect of AP1510 we observed is due to oligomerization through the FKBP domain, we tested the protein HM-3HA, which is identical to HM-3FKBP except that it is missing the three FKBP domains. When we subjected HM-3HA-transfected cells to the same drug regimen as described above for HM-3FKBP, we saw that AP1510 treatment no longer had any effect on degradation (Fig. 5, A and B). These results indicate that stabilization of HM-3FKBP by AP1510 is mediated through the FKBP domains.

If the stabilization we observed in HM-3FKBP is due specifically to the dimerizer AP1510 and not to other artifactual reasons involved in the drug treatment, we would predict that addition of an excess of the monomeric form of the drug (FK506) should be able to competitively abolish the stabilization through competition for FKBP-binding sites. Fig. 6 shows that treatment of cells with AP1510 plus a 5-fold molar excess of FK506 resulted in the loss of stabilization of HM-3FKBP induced by AP1510. These results also support our conclusion that AP1510 is able to oligomerize HM-3FKBP and that the resulting oligomers stabilize degradation of the membrane domain.

AP1510 Can Also Stabilize HM-Hyg-3FKBP—In an effort to determine whether our other previously studied monomeric constructs were degraded rapidly due to the monomeric state of the membrane domain, we fused three copies of FKBP to the C-terminus of one of them, HM-Hyg, and studied its degradation using the AP1510 system. Cells stably transfected with this HM-Hyg-3FKBP construct were treated with no drug or with

**Fig. 4. Oligomerization of the membrane domain through three FKBP domains stabilizes HM-3FKBP degradation in the presence of a dimerizer.** Cells stably transfected with HM-3FKBP were pretreated with no dimerizer (No Drug lanes) or with 200 nM AP1510 (+ AP1510 lanes) or 400 nM FK506 (+ FK506 lanes) for 20 h in MEM-LPS medium prior to pulse-chase analysis. AP1510 and FK506 were maintained throughout the starvation, labeling, and chase periods as described under “Experimental Procedures.” Cells were chased in the absence (A) or presence (B) of 2.5 μM 25-hydroxycholesterol. Cells were lysed, immunoprecipitated with anti-HA antibodies, separated by SDS-polyacrylamide gel electrophoresis, and subjected to autoradiography.

**Fig. 5. Control HM-3HA cells, which lack FKBP domains, are not stabilized by a dimerizer.** Control HM-3HA cells (no FKBP domains) were treated identically with drugs and subjected to pulse-chase analysis as described for Fig. 6. Cells were chased in the absence (A) or presence (B) of 2.5 μM 25-hydroxycholesterol prior to harvesting, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis.
200 nM AP1510 or 400 nM FK506 and subjected to pulse-chase analysis in the absence or presence of 2.5 μM 25-hydroxycholesterol. Our results again show a significant stabilization of HM-Hyg-3FKBP in both the presence and absence of sterols when the cytosolic domains are oligomerized by AP1510 (Fig. 7A).

As a control, we also subjected cells stably transfected with HM-Hyg, which lacks the FKBP domains, to the same drug regimen and pulse-chase analysis. As shown in Fig. 7B, AP1510 treatment had no effect on HM-Hyg degradation.

HMGR Concentration Can Affect Its Rate of Degradation—Our work described above presents the following: 1) the correlation of degradation rates with the oligomeric structure of the cytosolic domain of various HM-X proteins, 2) mutations in the oligomerization domain of HM-Gal resulting in monomer formation and rapid degradation, and 3) the AP1510-induced oligomerization of HM-3FKBP and HM-Hyg-3FKBP. These findings all suggest that oligomerization of HMGR is required for its stability. If this hypothesis is correct and since oligomerization is a concentration-dependent process, we predict that changes in HMGR concentration inside cells should change the ratio of oligomers to monomers and, as a result, also the HMGR degradation rate.

To test this prediction, the HMGR cDNA was cloned into the Tet-Off Expression System (see “Experimental Procedures”) and transfected into UT-2 cells, which lack endogenous HMGR. Using the Tet-Off system, the level of expression can be modulated by various concentrations of doxycycline. The half-life of HMGR at the various HMGR concentrations was determined with pulse-chase assays. As shown in Fig. 8, the half-life of HMGR was dramatically decreased in cells treated with doxycycline to reduce the steady-state levels of HMGR. When cells were treated with 0.25 ng/ml doxycycline, the half-life of HMGR was reduced to ~2.5 h compared with the normal half-life of ~7.5 h in untreated cells. When the expression level of HMGR was further suppressed with a higher concentration of doxycycline (0.50 ng/ml), the basal half-life of HMGR declined further to ~1.9 h. These results also support our hypothesis that the rates of degradation of HMGR degradation are determined, in part at least, by the equilibrium between oligomeric and monomeric states.

**DISCUSSION**

We have considered the membrane domain of HMGR necessary and sufficient for regulated degradation based on initial studies with HM-Gal, a model protein that mirrors full-length HMGR in all aspects of degradation while lacking the native cytosolic catalytic domain entirely (12, 25). Our current studies of various chimeras of HMGR with the cytosolic domain replaced with different heterologous proteins suggest that this assumption may not be entirely correct, and with HM-Gal, we had studied a chimera that retained an additional aspect of HMGR important for degradation. We now propose that, in

**FIG. 6.** Excess FK506 treatment can abolish stabilization induced by the dimerizer AP1510 in HM-3FKBP cells. Cells were pretreated with 200 nM AP1510, 200 nM AP1510 + 2 μM FK506, or 2 μM FK506 for 20 h prior to the pulse-chase experiments. Drugs were maintained throughout the entire experiment as described under “Experimental Procedures.” Degradation was measured in the absence (A) and presence (B) of 2.5 μM 25-hydroxycholesterol.

**FIG. 7.** HM-Hyg-3FKBP degradation is also stabilized by the dimerizer AP1510. A, cells stably transfected with HM-Hyg-3FKBP were pretreated with no drug or with 200 nM AP1510 or 400 nM FK506 in MEM-LPS medium for 20 h prior to pulse-chase experiments carried out as described under “Experimental Procedures.” Drugs were maintained throughout the experiment, and cells were chased either in the absence (top panel) or presence (bottom panel) of 2.5 μM 25-hydroxycholesterol. B, control HM-Hyg cells were subjected to the same drug treatment and experimental conditions as described for A.

**FIG. 8.** HMGR concentration impacts on the degradation rate, with a faster degradation rate correlated with a lower expression level. Double-stable Tet-Off UT-2 cells were pretreated with no doxycycline (Dox) or with 0.25 or 0.50 ng/ml doxycycline for 20 h in MEM-LPS medium prior to pulse-chase analysis. Doxycycline was maintained throughout the entire pulse-chase periods as described under “Experimental Procedures.” At various chase time points, cells were collected, lysed, immunoprecipitated with anti-HMGR antibodies against the HMGR membrane domain, separated by SDS-polyacrylamide gel electrophoresis, and subject to autoradiography.
addition to the membrane domain that is clearly required, interaction through the cytosolic domain of HMGR is important for normal regulated degradation to occur, most likely by bringing key elements of the membrane domain into close proximity. We believe that dimerization of the membrane domain, or at least close apposition, that is promoted through interactions in the cytosolic domain is an additional requirement for HMGR stability.

To test our hypothesis, we used three approaches. 1) We mutated a known oligomeric, chimeric protein (HM-Gal) that exhibits normal regulated degradation in order to convert it into a monomeric protein. 2) We used an inducible oligomerization system to generate oligomeric proteins with drugs in vivo. 3) We examined the effect of altering the levels of HMGR with a regulated expression system to determine the concentration dependence of the degradation rate. All three approaches support the hypothesis that oligomerization of HMGR through the catalytic cytosolic domain determines the half-life of the protein.

It is of note that in many of our monomeric constructs, although the basal half-life is fast, addition of sterols in some cases is still capable of further accelerating degradation. There are a few possibilities to explain this. The first is that sterols induce an additional effect either directly or indirectly on the membrane domain to facilitate recognition of HMGR as a substrate for proteolysis. This effect could be independent of the oligomeric state of the membrane domain and would be less striking in proteins that have an extremely fast half-life, as they are already being rapidly degraded. Another possibility is that the membrane domains are capable of weak interactions without the cytosolic domain, so there are some weak dimers present even between the presumed monomeric constructs, and addition of sterols results in a more complete dissociation of these dimers to monomers. This may explain the variable half-lives observed in the different fast monomeric proteins. It is possible that differences in the extent of interaction in the membrane domain permitted by the cytosolic component (perhaps due to varying degrees of steric hindrance) are reflected in the differences in the resulting equilibrium between monomers and dimers. Certainly these possibilities are not mutually exclusive, and the explanation could be some combination of the above. However, the simplest model would be to propose that sterols bind directly to the membrane domain of HMGR and that this binding can induce dissociation of dimers within the membrane domain and/or a conformational change that exposes a degradation signal or cleavage site to target HMGR for proteolysis.

HMGR exists as a dimer, as indicated by structural studies of bacterial HMGR (9) and also by radiation inactivation studies that have identified the functionally active form of HMGR as a mass that is equivalent to two HMGR molecules (26, 27). We speculate that dimerization of the cytosolic domain is important in promoting dimerization of the membrane domain and that the oligomeric state of the membrane domain affects its degradation such that monomers are more susceptible to degradation and dimers are more stable. Dimerization of the membrane domain may slow or prevent recognition of degradation signals, resulting in a stable complex, whereas conformational changes and/or dissociation into monomers normally induced by sterols may serve to expose these signals and to promote recognition by the proteolytic machinery.

There is also correlative evidence that the membrane domain of HMGR has a direct sterol-sensing/binding function based primarily on mutagenesis studies demonstrating that mutations within the membrane domain can render HMGR insensitive to sterols (25). In addition, other proteins involved in cholesterol sensing (sterol regulatory element binding protein (SREBP) cleavage-activating protein (28)) and transport (NPC1 gene product (29, 30)) share sequence homology in the putative sterol-binding region of the HMGR membrane domain.

There are examples in other systems that suggest that the oligomerization state of membrane proteins may change in response to changes in the membrane lipid composition. One example is the UDP-GlcNAc:dolichol-P GlcNAc-1-phosphotransferase in the endoplasmic reticulum, which is responsible for the committed step of dolichol-linked oligosaccharide synthesis. UDP-GlcNAc:dolichol-P GlcNAc-1-phosphotransferase is detectable as both monomers and dimers, and it has been reported that the activity of this enzyme varies depending upon local phospholipid composition (32, 33). Dan and Lehman (34) have suggested that different phospholipid concentrations may alter the ratio of monomers to dimers and, in this manner, regulate activity. Another example of membrane lipids regulating oligomerization has been reported in work with transcobalamin receptor II (35, 36), which is normally present in the plasma membrane. Bose et al. (37) have suggested that transcobalamin receptor II can transition between monomeric and dimeric forms depending on the membrane cholesterol content. The authors were able to show that cholesterol depletion in native intestinal plasma membranes or its enrichment in microsomal membranes resulted in the in situ conversion of the dimeric to the monomeric form or of the monomeric to the dimeric form, respectively.

In summary, the model we currently favor in light of our work is shown in Fig. 9. The simplest hypothesis would involve a direct sterol-sensing function by the membrane domain that would initiate dissociation of the membrane domains and not necessarily of the cytosolic domains. This sterol-bound monomeric form of the membrane domain would then be the prime substrate for proteolysis. Our current work is aimed at demonstrating a sterol-induced change in the interaction between membrane domains using cross-linking experiments.

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