THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 1, Issue of January 2, pp. 188–196, 2004
Printed in U.S.A.

Structural Control of Endoplasmic Reticulum-associated Degradation

EFFECT OF CHEMICAL CHAPERONES ON 3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE*

Received for publication, July 17, 2003, and in revised form, October 10, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M307734200

Alexander G. Shearer‡ and Randolph Y. Hampton§

From the University of California San Diego Division of Biological Sciences, Section of Cell and Molecular Biology, La Jolla, California 92093

The endoplasmic reticulum (ER) quality control pathway destroys misfolded and unassembled proteins in the ER. Most substrates of this ER-associated degradation (ERAD) pathway are constitutively targeted for destruction through recognition of poorly understood structural hallmarks of misfolding. However, the normal yeast ER membrane protein 3-hydroxy-3-methylglutaryl-CoA reductase (Hmg2p) undergoes ERAD that is physiologically regulated by sterol pathway signals. We have proposed that Hmg2p ERAD occurs by a regulated transition to an ERAD quality control substrate. Consistent with this, we had previously shown that Hmg2p is strongly stabilized by chemical chaperones such as glycerol, which stabilize misfolded proteins. To understand the features of Hmg2p that permit regulated ERAD, we have thoroughly characterized the effects of chemical chaperones on Hmg2p. These agents caused a reversible, immediate, direct change in Hmg2p degradation consistent with an effect on Hmg2p structure. We devised an *in vitro* limited proteolysis assay of Hmg2p in its native membranes. *In vitro*, chemical chaperones caused a dramatic, rapid change in Hmg2p structure to a less accessible form. As in the living cell, the *in vitro* action of chemical chaperones was highly specific for Hmg2p and completely reversible. To evaluate the physiological relevance of this model behavior, we used the limited proteolysis assay to examine the effects of changing *in vivo* degradation signals on Hmg2p structure. We found that changes similar to those observed with chemical chaperones were brought about by alteration of natural degradation signal. Thus, Hmg2p can undergo significant, reversible structural changes that are relevant to the physiological control of Hmg2p ERAD. These findings support the idea that Hmg2p regulation is brought about by regulated alteration of folding state. Considering the ubiquitous nature of quality control pathways in biology, it may be that this strategy of regulation is widespread.

Protein degradation has two principal functions in the living cell: regulation and quality control. Regulation generally involves the precise targeting of normal, functional proteins as a means of controlling specific cellular processes. Examples of proteins regulated by degradation include IκB (1), HIF1a (2), and numerous proteins required for cell cycle progression (3–5). Quality control entails destruction of proteins that are misfolded or that have failed to correctly assemble into multimeric complexes as a means of limiting these potentially hazardous species.

In both modes of degradation, targeting is highly specific but the criteria for recognition are nearly orthogonal. In regulation, unique features of a normally folded protein must be recognized by the degradation machinery to guarantee specific control of the appropriate cellular process. In contrast, quality control requires recognition of structural hallmarks that distinguish members of the broad set of abnormal proteins from their correctly folded counterparts in a manner that transcends protein sequence and identity. As a consequence, quality control and regulation are traditionally envisioned as distinct processes. It is possible, however, that the mechanisms that recognize misfolded proteins could be harnessed for regulation via controlled entry of the target protein into a constitutively acting quality control pathway. Our work on the regulated degradation of HMG-CoA reductase (HMGR),[1] the rate-limiting enzyme of sterol synthesis, has revealed that such a mechanism operates to control its degradation.

Much of eukaryotic protein degradation occurs through the action of the ubiquitin-proteasome pathway, by which targeted proteins are covalently tagged with multiple copies of the 8 kDa protein ubiquitin, allowing recognition and proteolysis by the 26S proteasome. Specific transfer of ubiquitin to substrate requires an E3, or ubiquitin ligase, of which a large and growing collection has been identified and characterized. E3s are key determinants of specificity in ubiquitination pathways, as they mediate the recognition of target proteins.

Most ubiquitin ligases studied to date function in regulatory targeting of specific proteins (6–8). A smaller group are known to participate in protein quality control, promoting the ubiquitination of misfolded proteins (9–11). In *Saccharomyces cerevisiae*, the HRD complex, consisting of Hrd1p and Hrd3p, is an integral membrane E3 that mediates ER-associated degradation (ERAD) of a diverse group of misfolded and unassembled proteins, including luminal proteins such as CPY* and integral membrane proteins (10, 12, 13). Thus, the HRD E3 specifically targets unrelated proteins that share only the still-unidentified hallmarks of misfolding. As predicted from these studies on model ERAD substrates, the yeast HRD complex has a general

* This work was supported by NIDDK, National Institutes of Health Grant GM51996-06 and an American Heart Association Established Investigator Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Health Training Grant 3 T32 GM07240-25S1.

§ To whom correspondence should be addressed. Tel.: 858-822-0511; Fax: 858-534-0555; E-mail: rhampton@ucsd.edu.

[1] The abbreviations used are: HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; CFTR, cystic fibrosis transmembrane conductance regulator; ERAD, endoplasmic reticulum-associated degradation; FPP, farnesyl pyrophosphate; GFP, green fluorescent protein; HERG, human ether-a-go-go related gene; HRD, HMG-CoA reductase degradation; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; TAME, *p*-toluene sulfonyl-L-arginine methyl ester.

role in cellular quality control: *hrd1*Δ-null strains have elevated levels of unfolded proteins in the ER, indicating that the HRD pathway functions in limiting the abundance of spontaneously occurring misfolded proteins (14). Along with this clear role in quality control, the HRD complex also functions in regulation of a normal, essential metabolic enzyme, HMGR.

HMGR catalyzes a key step in sterol biosynthesis and is the primary drug target for treatment of high cholesterol. Both mammalian HMGR and the yeast isozyme Hmg2p undergo feedback-regulated, ubiquitin-mediated degradation in response to signals from the sterol pathway, in addition to more conventional regulation at the transcriptional level (15). Increases in pathway signals lead to increased degradation and a concomitant reduction in the Hmg2p steady-state level. Conversely, decreased signals lead to decreased degradation and a rise in steady-state levels. This degradation leads to rapid, fine control of Hmg2p levels in response to the physiological requirements of the cell.

In our genetic analysis of Hmg2p degradation, we identified the HRD complex as the ubiquitin ligase that mediates Hmg2p-regulated degradation (10, 16). Thus, the controlled destruction of this enzyme is an intersection between regulation and quality control, being an instance in which a constitutively operating quality control pathway participates in the physiologically regulated targeting of a specific normal protein, Hmg2p. It is our goal to understand this mechanism of regulation, both to allow clear understanding of HMGR regulation, and as an exemplar for the harnessing of cellular quality control for regulatory purposes in nature and the clinic.

The signal for Hmg2p degradation is the sterol pathway intermediate farnesyl pyrophosphate (FPP) or a molecule derived from it (17). When FPP levels are high, the Hmg2p degradation rate is high, and when FPP levels are low, the Hmg2p degradation rate is low. In this way, the half-life of Hmg2p can vary from about 10 min to over 4 h. Experiments in which we rapidly manipulate FPP levels with drugs indicate that the extant pool of mature Hmg2p is subject to this stability control and appears to be poised to undergo changes in degradative status that happen rapidly and reversibly in response to the FPP-derived signal.

How does FPP specifically regulate Hmg2p entry into the constitutively active HRD quality control pathway? We have previously proposed the most direct model: that Hmg2p acquires structural features of a quality control substrate in response to the FPP-derived signal (15, 18, 19). This regulated structural transition would occur in mature protein and be reversed rapidly when signal decreases. In other words, the Hmg2p status as a quality control substrate could be switched, and during circumstances when the FPP-derived signal is high, for example in strains with high flux through the sterol synthesis pathway, Hmg2p should have properties or behaviors of a misfolded protein.

To evaluate this idea, we used studies on the clinically important ΔF508 variant of CFTR as a conceptual guide. CFTR is a multispanning membrane protein that folds in the ER and is then transported to the plasma membrane, where it functions in ion transport. The ΔF508 variant is especially slow in folding to the proper native conformation, with ~95% of the protein undergoing ERAD, resulting in insufficient CFTR at the cell surface and the consequent clinical manifestations of cystic fibrosis (20, 21). This defect of folding can be partially corrected in living mammalian cells by growth in medium containing chemical chaperones, small molecules that promote the folding of proteins both *in vivo* and *in vitro* (22, 23). Similarly, defective trafficking of a mutant version of the HERG potassium channel is corrected *in vivo* following treatment with glycerol (24).

These and other studies indicate that the effect of chemical chaperones can be used as a criterion for poor folding of a protein under study. Specifically, our model predicts that in conditions that promote degradation, Hmg2p would be stabilized by chemical chaperones in the manner of a misfolded protein. Indeed, we have previously shown that glycerol stabilizes Hmg2p in living yeast cells at concentrations appropriate for chemical chaperone activity, indicating that Hmg2p undergoing regulated degradation has features similar to known quality control substrates (18).

In this work we have thoroughly evaluated this action of chemical chaperones on Hmg2p. We show that the *in vivo* action of glycerol is rapid, reversible and highly specific for Hmg2p. To directly examine the structural effects of chemical chaperones, we have developed an *in vitro* limited proteolysis assay for Hmg2p in isolated ER microsomes. *In vitro*, Hmg2p shows a rapid and reversible structural change in response to chemical chaperones, consistent with the idea that Hmg2p is capable of undergoing relevant structural transitions. Furthermore, we show that a similar structural change in Hmg2p occurs when physiological degradation signals are altered *in vivo*. Thus it appears that Hmg2p has unique features that allow a regulated transition between states that are more and less susceptible to quality control degradation. By this novel mechanism Hmg2p can be modulated by a cellular housekeeping pathway to serve the regulatory needs of the cell.

MATERIALS AND METHODS

Restriction enzymes, Vent DNA polymerase and T4 DNA ligase were obtained from New England Biolabs. Lovastatin and zaragozic acid were generously provided by Dr. James Bergstrom (Merck, Rahway, NJ). 9E10 cell culture supernatant was produced in our laboratory from cells (CRL 1729, American Type Culture Collection) grown in RPMI 1640 culture medium (Invitrogen) with 10% fetal calf serum and supplements. Anti-HA antibodies were purchased as ascites fluid from Covance. Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibodies were purchased from Jackson ImmunoResearch. HRP-conjugated goat anti-rabbit antibodies were purchased from Zymed Laboratories Inc. Chemiluminscence immunodetection reagents were obtained from PerkinElmer Life Sciences. All other chemical reagents were obtained from Sigma or Fisher.

Plasmid Construction and DNA Manipulation—Plasmid pRH1581 (integrating) expressed Hmg2p-GFP with the c-Myc epitope tag (EQKLISEEDL) in place of a similar portion of sequence in the first luminal loop (TYLSIKPDEL) from a *TDH3* promoter (myc_L-Hmg2p-GFP). pRH1581 was constructed using the PCR-mediated overlap extension method as described previously (25), with pRH469 (Hmg2p-GFP behind a *TDH3* promoter for constitutive expression) (25), as template (oligos available on request). pRH469 was partially digested with PstI then cut with SpeI and treated with shrimp alkaline phosphatase. The PCR product was digested with PstI and SpeI, then ligated into pRH469, and product evaluated by restriction digestion.

Yeast Culture and Strains—Yeast strains (Table I) were grown in minimal media (Difco Yeast Nitrogen Base) with glucose and the appropriate supplements as described previously, unless otherwise noted (27). Experiments were performed at 30 °C unless otherwise noted. Yeast was transformed with plasmid DNA by the LiaOAc method. All transformants at the *ura3-52* locus were selected based on acquisition of Ura+ prototrophy.

RHY2723 and RHY2842 were made by targeted integration of the plasmids pRH1581 and pRH1692, respectively, at the StuI site of the ura3-52 locus in RHY519 (25). RHY2803 and RHY2866 were derived from RHY2723 and RHY2842 by transformation with the *hrd1*Δ::KanMX deletion cassette excised from pRH1122 (17), then selection on YPD with G418. RHY2853 was made by targeted integration of pRH1581 at the StuI site of the *ura3-52* locus in the *cod1*Δ strain RHY1954.

RHY2853 was made by targeted integration of plasmid pRH1581 at the StuI site of the *ura3-52* locus in a strain in which the *COD1* locus was completely deleted via a KanMX cassette.

Flow Cytometry of Yeast Cells—Flow cytometry was carried out as described previously (28). Briefly, cells were grown to early log phase in minimal medium, then incubated with drugs or chemical chaperones

TABLE I
Yeast strains

Strain	Genotype
RHY522	*MATa ade2–101 met2 lys2–801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3–52::URA3::HMG2cd::HMG2-GFP*
RHY832	*MATa ade2–101 met2 lys2–801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3–52::URA3::HMG2cd::6MYC-HMG2-GFP*
RHY2723	*MATa ade2–101 met2 lys2–801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3–52::URA3::HMG2cd::myc$_L$-Hmg2p-GFP*
RHY2485	*MATa ade2–101 met2 lys2–801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3–52::URA3::1MYC-HMG2 ARS/CEN HA-CPY**
RHY2803	*MATa ade2–101 met2 lys2–801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3–52::URA3::HMG2cd::myc$_L$-Hmg2p-GFP hrd1Δ::KanMX*
RHY2843	*MATa ade2–101 met2 lys2–801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3–52::URA3::HMG2cd::3MYC-OLE1*
RHY2853	*MATalpha ade2–101 met2 lys2–801 his3200 ura3–52::URA3::myc$_L$-Hmg2p-GFP cod1Δ::KanMX*

for the times indicated and analyzed for individual cell fluorescence with a BD Biosciences FACSCalibur flow cytometer. All histograms in this paper represent results from 10,000 cells.

In Vivo Reversibility of Chemical Chaperone Treatment—Cells were grown overnight in minimal medium containing 10% glycerol, then aliquoted into 2-ml samples for each time point. At each time point, cells were pelleted in a clinical centrifuge, medium was removed by aspiration and cells were resuspended into new medium with or without glycerol, as indicated. Cycloheximide was added to 50 μg/ml, then the cultures were returned to incubation for the indicated times. Hmg2p-GFP levels were evaluated by flow cytometry, as indicated above. Reported Hmg2p-GFP levels were determined based on the mean fluorescence of 10,000 cells per time point.

Assays for Protein Degradation—Cycloheximide chase was used to measure protein stability as described previously (25). Briefly, cells were grown to early log phase then spun down, medium removed by aspiration, and replaced with new medium with cycloheximide (50 μg/ml) and with or without glycerol, as indicated. Cultures were then incubated for the indicated times. Samples were collected, lysed, and evaluated by SDS-PAGE on 8% gels, and immunoblotted for the substrate protein under study.

Microsome Preparation—Microsomes for limited proteolysis were prepared as follows: Cells were grown in YPD to an OD_{600} of 0.5–1.0, and 100 ODs pelleted. The pellet was washed once by resuspension in XL buffer (1.2 M sorbitol, 5 mM EDTA, 0.1 M KH_2PO_4/K_2HPO_4, pH 7.5), then resuspended in 5 ml of lysis buffer (0.5 ml XL, 2 ml H_2O, 260 nM AEBSF, 100 nM TPCK) per 100 ODs, aliquoted into one 2 ml microfuge tube per 10 ODs, mixed with an equal volume 0.5 mm acid-washed glass beads and chilled 2 min on ice. Cells were lysed by vortexing 6 × 1 min, with 1 min on ice between each vortexing. Debris were pelleted by 5-s pulses at 16,000 × *g* in 1.5-ml microcentrifuge tubes until no pellet was formed (typically three pulses required). From the resulting clarified supernatant, microsomes were pelleted by 30 min at 21,000 × *g* at 4 °C. The supernatant was aspirated, and the pellet overlaid twice with fresh XL buffer, with a 5-min centrifugation following each wash to ensure pellet cohesion. The resulting microsomal pellet was then resuspended in XL buffer with or without glycerol as described under "Results," for use in the limited proteolysis assay.

Limited Proteolysis Assay—This assay was performed on microsomes as follows: trypsin (porcine IX-S, EC 3.4.21.4) was prepared by resuspension of lyophilized powder in SED buffer (20 mM Tris, pH 7.5, 500 μg/ml bovine serum albumin, 10 mM β-mercaptoethanol), followed by a 5-min spin at 16,000 × *g* to pellet any debris. Supernatant was diluted in additional SED to the desired 20× trypsin stock concentration, then added to the final concentration described. Samples were incubated at 30 °C, with 30-μl aliquots removed at the time points indicated, and the reaction halted by addition of 30 μl of 2× USB (75 mM MOPS, pH 6.8; 4% SDS; 200 mM dithiothreitol; 0.2 mg/ml bromphenol blue; 8 M urea) followed by solubilization for 10 min at 55 °C. Samples were resolved on 14% SDS-PAGE, then transferred with 15% methanol to nitrocellulose, and immunoblotted for the Myc epitope as described (27).

TAME Chromogenic Protease Activity Assay—35 μl of 15 μg/ml trypsin in SED buffer was added to a cuvette containing a mixture of 865 μl of test buffer (XL buffer with glycerol as indicated) and 100 μl of 10 mM *p*-toluene sulfonyl-L-arginine methyl ester (TAME), then absorbance at 247 nm measured at the indicated time points.

RESULTS

The sensitivity of Hmg2p degradation to chemical chaperones is an important indicator of the mechanism of its regulated degradation. In this work we have characterized this response, both *in vivo* and *in vitro*. We have taken advantage of Hmg2p-GFP, a normally regulated version of Hmg2p in which the catalytic domain that is nonessential for regulation has been replaced with GFP. The use of this protein allows precise quantitation of regulation, and obviates concerns about possible interactions of drugs or pathway molecules with the catalytic domain, which might conceivably affect Hmg2p structure in a manner unrelated to regulated degradation, a process we know to be mediated solely by the N-terminal transmembrane region (26, 27)

Glycerol stabilizes Hmg2p undergoing normal, physiologically regulated degradation. This effect is most evident in strains with high FPP levels, in which the degradation rate of Hmg2p is high. Rapid degradation is promoted by co-expression of a free, soluble HMGR catalytic domain, providing high HMGR activity and thus a high level of FPP-derived degradation signal. The degraded Hmg2p-GFP is expressed from the constitutive *TDH3* promoter so that any effects on its steady-state level are due solely to changes in degradation rate. Hmg2p-GFP stability is thus directly indicated by the steady-state cellular fluorescence as measured by flow cytometry, a technique we routinely employ (10, 12, 16–18, 25, 26, 28, 29).

When the strain expressing rapidly degraded Hmg2p-GFP was treated with glycerol, the steady-state levels of Hmg2p-GFP rose rapidly due to stabilization by the chemical chaperone. Glycerol stabilization of Hmg2p-GFP was dose-dependent, saturable, and occurred at concentrations appropriate for its action as a chemical chaperone (Fig. 1*A*). The extent of the stabilizing effect was similar to that caused by lowering the FPP-derived signal with lovastatin, and the stabilizing effects of glycerol and lovastatin were not additive, as measured by flow cytometry and biochemically (18). Hmg2p-GFP was also stabilized by similar concentrations of other typical chemical chaperones, including sorbitol and mannitol (data not shown). Two others, trimethylamine *N*-oxide (TMAO) and 4-phenylbutyric acid, had untoward effects on the cells that precluded *in vivo* analysis.

We next examined the specificity of this effect for Hmg2p. We have previously shown that HRD-dependent degradation of 6myc-Hmg2p, a severely misfolded version of Hmg2p with a transmembrane span replaced by six tandem Myc epitopes, is unaffected by concentrations of glycerol that stabilize Hmg2p (18). To further evaluate the selectivity of this effect we tested glycerol action on several other ERAD substrates, including CPY*, a non-folding mutant of carboxypeptidase Y (30), Ole1p (31), a natural multispanning membrane protein that undergoes ERAD that is not dependent on either of two known ER ligases, Hrd1p or Doa10p, and Hrd1p, which undergoes self-ubiquitination and degradation in the absence of Hrd3p (16). In all cases, glycerol had minimal effect on ER degradation of these various test substrates (Fig. 1*B* and data not shown). Thus, the strong stabilizing effect of glycerol and other chemical chaperones was not due to any alteration in general ERAD, but rather due to specific features of Hmg2p that render it susceptible to the action of chemical chaperones.

The correct sequence and structure of Hmg2p are important

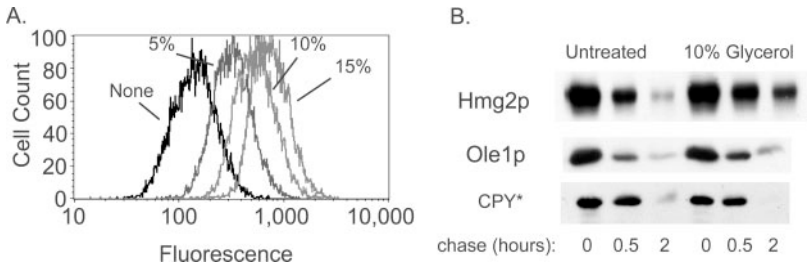

FIG. 1. **Glycerol specifically stabilized Hmg2p and Hmg2p-GFP *in vivo*.** *A*, early log phase cultures were grown for 4 h in the presence of an increasing percentage glycerol as indicated and analyzed for GFP fluorescence via flow cytometry. Each histogram represents 10,000 cells. *B*, cells expressing the indicated ERAD substrates were grown in selective medium, harvested, and resuspended in medium with 50 μg/ml cycloheximide in the presence or absence of glycerol, as indicated. At the indicated times, cells were lysed and subjected to immunoblotting for the indicated substrate to evaluate its degradation.

in glycerol sensitivity, as indicated by the lack of glycerol effect on 6myc-Hmg2p. However, the response of Hmg2p to the FPP-derived degradation signal is also very specific for correct Hmg2p sequence and structure (26). Thus, an alternate model for the specificity of glycerol effect on Hmg2p is that somehow the FPP-derived signal is made ineffective by chemical chaperones. We have previously shown that flux through the mevalonate pathway is unaffected by glycerol treatment, indicating that there is likely to be normal production of FPP (18). However, any effect of glycerol on the response to an FPP-derived molecule would not be addressed by that control.

In order to rule out the involvement of the FPP-derived signal in the stabilizing action of glycerol, we employed the *cod1*Δ mutant characterized in our laboratory (28). In a *cod1*Δ-null strain, normal Hmg2p (or Hmg2p-GFP) is constitutively degraded in a manner that is totally independent of FPP levels: pharmacological inhibition of sterol pathway enzymes to induce a buildup or decrease in FPP levels results in no change in Hmg2p degradation or steady-state level in this mutant. Thus, we directly compared the effect of glycerol on Hmg2p in wild-type and *cod1*Δ strains. Incubation of wild-type cells in 10% glycerol resulted in stabilization of Hmg2p-GFP to an extent equal to that caused by down-regulation of the FPP-derived signal with the pathway inhibitor lovastatin (Fig. 2*A*). In a *cod1*Δ strain, lovastatin had essentially no effect on Hmg2p-GFP levels but treatment with 10% glycerol still strongly stabilized the reporter protein (Fig. 2*B*). Indeed, Hmg2p-GFP was stabilized to the same steady-state levels in both strains (Fig. 2*C*). Thus, glycerol action was independent of the degradation signal, since it continued to be effective in a *cod1*Δ strain where the FPP-derived signal does not function.

We next tested the reversibility of glycerol stabilization of Hmg2p. Because the onset of glycerol stabilization was immediate and it acted on mature protein (18), we imagined that the chemical chaperone caused a rapid structural change in the mature Hmg2p protein, and we wondered if this effect would be reversible. As shown above, growth in glycerol of cells expressing rapidly degraded Hmg2p-GFP results in stabilization of the reporter and a concomitant increase in its steady-state level. To test the reversibility of the stabilizing effect, cells were grown overnight in 10% glycerol, causing the expected increase in Hmg2p-GFP steady-state levels as measured by fluorescence. These cells were then washed by centrifugation and resuspensed in medium with or without added glycerol in the presence of cycloheximide (to halt protein synthesis), incubated, and analyzed by flow cytometry at successive time points to determine the rate of Hmg2p-GFP degradation. Resuspension of glycerol-grown cells with stabilized Hmg2p-GFP into medium lacking glycerol allowed the immediate resumption of normal Hmg2p-GFP degradation as measured by time-dependent loss of steady-state fluorescence, with a half-life appropriate for normal degradation. If the washed cells were instead resuspended in glycerol-containing medium, the Hmg2p-GFP remained stable, with an appropriately slowed degradation rate (Fig. 3). This restored Hmg2p-GFP degradation was normally regulated: addition of lovastatin to cells resuspended in glycerol-free medium caused increased stability, indicating that regulation and thus protein structure was intact following removal of glycerol (data not shown). Thus, the effect of glycerol on Hmg2p was rapid, and rapidly reversible.

Taken together, the above *in vivo* studies indicated that the Hmg2p transmembrane region has structural features that render it susceptible to the action of glycerol and other chemical chaperones. This property of Hmg2p is dependent on its correct sequence and is rapidly reversible. These *in vivo* studies imply that Hmg2p can undergo structural changes that alter its susceptibility to HRD-dependent degradation. In order to better understand these structural features, we developed a limited proteolysis assay to directly study the structure of Hmg2p *in vitro*. We were particularly interested in the following questions: does glycerol directly affect the structure of Hmg2p, and do these structural effects pertain to the physiological control of Hmg2p ERAD.

We and other groups have developed methods to produce ER-derived microsomes that are cytosol side out (16, 18, 32). Thus, luminal epitopes of ER-resident membrane proteins in such microsomes are spared from proteolytic digestion, allowing observation of changes in proteolysis rate or site number by immunoblotting without loss of signal. Accordingly, we added an epitope to a luminal region of Hmg2p to let us carry out limited proteolysis in microsomes. In making this reporter, it was imperative to not perturb regulated degradation of Hmg2p by the addition of the tag, since we ultimately want to understand regulation with this assay.

We used our previous analysis of over three hundred Hmg2p mutants to guide epitope placement (26). These studies revealed that while many luminal regions of Hmg2p are critical for correct regulation, a sequence "cold spot" that can be altered without regulatory consequence exists in the predicted first luminal loop. We produced a version of the Hmg2p-GFP reporter with a small region in this first loop replaced with a single Myc epitope tag (myc_L-Hmg2p-GFP). Flow cytometry was then used to verify that the resulting luminally tagged Hmg2p-GFP was normally regulated (Fig. 4*A*). As shown, the steady-state level of myc_L-Hmg2p-GFP was similar to that of Hmg2p-GFP, and regulation of stability, tested by increasing FPP levels with zaragozic acid or decreasing FPP levels with lovastatin, was unaltered by the added tag. Strains expressing the myc_L-Hmg2p-GFP reporter were used to prepare ER microsomes for study by limited proteolysis.

The proteolysis pattern of myc_L-Hmg2p-GFP indicated that the epitope was, as predicted, in a luminally protected space. Upon exposure of the microsomes to trypsin, the single band of immunoreactivity progressively underwent cleavage into inter-

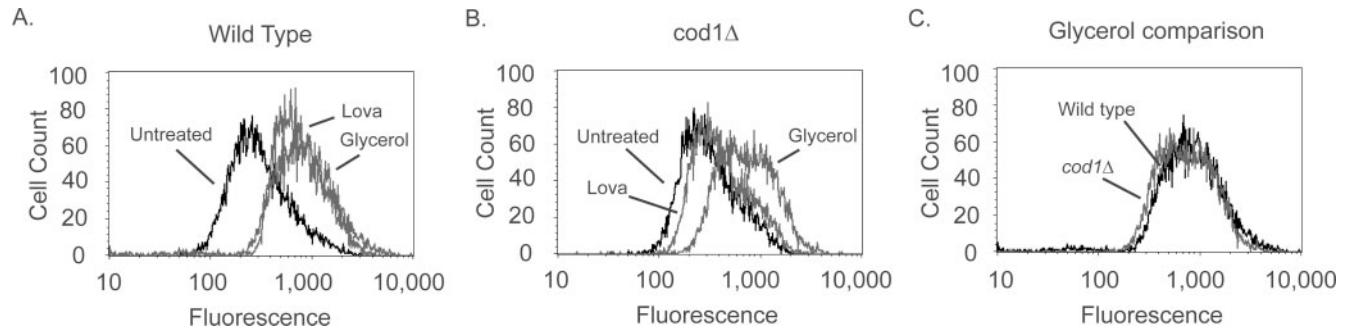

FIG. 2. **Glycerol did not act via the FPP-derived degradation signal.** Wild-type cells (*A*) or *cod1*Δ cells (*B*) were grown for 4 h with 10% glycerol (*Glycerol*) or 25 μg/ml lovastatin (*Lova*) as indicated and analyzed for GFP fluorescence via flow cytometry. *C*, histograms of glycerol-treated wild-type or *cod1*Δ cells overlaid.

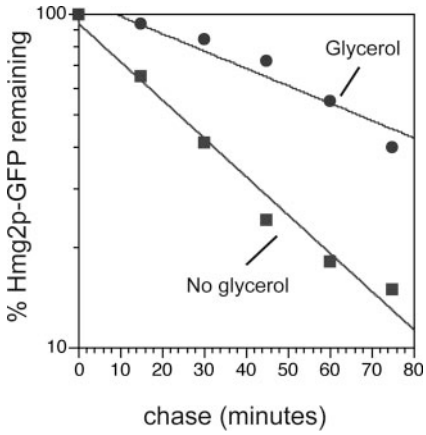

FIG. 3. **The glycerol-induced stabilization of Hmg2p was reversible.** Cells were grown overnight in glycerol, then resuspended in medium with 50 μg/ml cycloheximide, with (*circles*) or without (*squares*) glycerol and allowed to incubate for the indicated times. Hmg2p-GFP levels at each time point were quantified by flow cytometric evaluation of Hmg2p-GFP fluorescence.

mediate bands in a time-dependent manner without loss of overall signal intensity (Fig. 4*B*). In contrast, permeabilization of microsomes with detergent to allow access to the luminal space resulted in loss of immunoreactivity (Fig. 4*C*). The preserved signal strength during proteolysis without detergent and its loss upon proteolysis with detergent are consistent with a luminal location of the first loop bearing the Myc tag. Tests with other proteases gave similar results (Fig. 4*C*). Trypsin provided the most useful digestion pattern for this assay, due to its greater specificity, and so was used for further evaluation of Hmg2p structural features.

We then tested the effects of glycerol on *in vitro* limited proteolysis of myc_L-Hmg2p-GFP as a direct gauge of chemical chaperone activity. At concentrations that are effective *in vivo*, glycerol significantly decreased the trypsin accessibility of the luminally tagged reporter. Specifically, limited proteolysis was slowed considerably, producing the same pattern of bands but at a markedly slower rate (Fig. 5*A*). This action of glycerol was dose-dependent, with more slowing observed at 20% glycerol than 10%. Extended (3 h) incubation of 20% glycerol-treated microsomes eventually produced the pattern seen after 10 min of trypsinization without glycerol (data not shown). Furthermore, this action of glycerol was rapid: no significant preincubation with glycerol was required to see its effects. These effective concentrations of glycerol had no inhibitory effect on the intrinsic activity of trypsin as measured with the chromogenic substrate TAME. In fact, glycerol slightly stimulated trypsin activity at the highest concentrations tested (Fig. 5*B*). Thus, Hmg2p structure was directly and specifically affected by glycerol.

The *in vitro* effect on Hmg2p structure was also caused by appropriate concentrations of several other chemical chaperones, including sorbitol, mannitol, and TMAO (data not shown). We continued our studies with glycerol in order to make the most meaningful comparisons with the *in vivo* data and the structural aspects of Hmg2p that pertain to its regulated entry into the HRD pathway.

This effect of glycerol on the rate of proteolysis was highly specific for the transmembrane region of myc_L-Hmg2p-GFP. Glycerol had no effect on the digestion rate of GFP liberated from the membrane-bound reporter protein, as evaluated by immunoblotting the proteolysis samples with GFP antibody (data not shown). Furthermore, the proteolytic time course of two other membrane proteins was unaffected by glycerol. 6myc-Hmg2p, a misfolded variant of Hmg2p that is not stabilized by glycerol *in vivo*, was not affected by addition of glycerol to the *in vitro* assay. (Fig. 5*C*). Similarly, immunoblotting of the dnaJ epitope of the endogenous ER membrane protein Sec63p, which occupies a luminal loop (32), also showed trypsin digestion with a pattern and rate unchanged by the presence of glycerol (Fig. 5*D*). These controls indicated that the strong effect of glycerol on *in vitro* proteolysis of Hmg2p was not due to actions on trypsin itself, or effects on general protein accessibility, or general effects on trypsinolysis of membrane proteins.

In living cells, the effect of glycerol on Hmg2p or Hmg2p-GFP degradation occurred rapidly and was rapidly reversible (18). The *in vitro* effect of glycerol was similarly rapid, occurring without preincubation. We next tested the reversibility of this *in vitro* effect. Microsomes with myc_L-Hmg2p-GFP were incubated in 20% glycerol for half an hour, far more time than was sufficient to cause decreased trypsin accessibility as described above. Samples of these glycerol-treated membranes were then centrifuged and resuspended in the presence or absence of 20% glycerol. The dilution of glycerol caused by resuspension in glycerol-free buffer was sufficient to completely restore the limited proteolysis rate to that of a buffer-only control (Fig. 6). Thus, it appeared that the Hmg2p transmembrane region underwent significant, rapid, and reversible alterations in response to chemical chaperones both *in vivo* and *in vitro*.

In vivo, Hmg2p expressed in the *cod1*Δ null was stabilized by glycerol, but was not responsive to changes in the FPP-derived signal. We next tested the *in vitro* effect of glycerol on Hmg2p-GFP derived from *cod1*Δ cells. Our prediction was that the glycerol effect on the limited proteolysis assay should still occur, providing a further test of the relevance of the *in vitro* assay to the cellular effects of chemical chaperones. As expected, the limited proteolysis of myc_L-Hmg2p-GFP from *cod1*Δ microsomes showed slowed appearance of several cleavage products upon glycerol treatment (Fig. 7). Importantly, trypsinolysis revealed that the structure of Hmg2p-GFP derived from

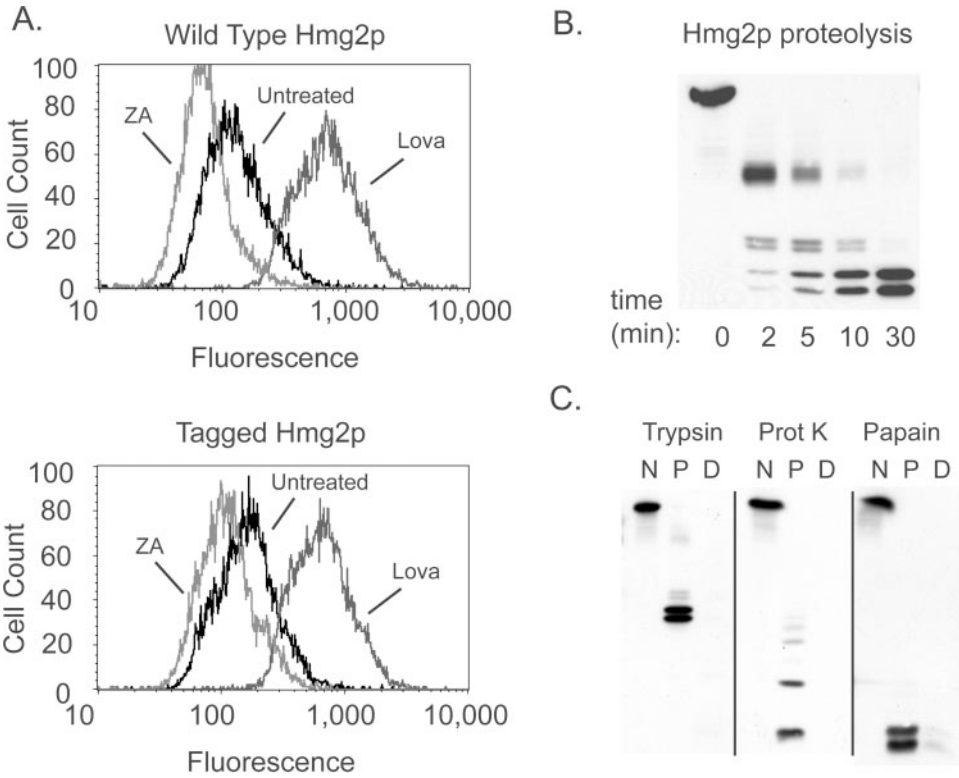

FIG. 4. **The myc_L-Hmg2p-GFP epitope-tagged construct for limited proteolysis assays.** *A*, myc_L-Hmg2p-GFP regulation compared with wild type. Cells expressing either unmodified Hmg2p-GFP or myc_L-Hmg2p-GFP were grown to early log phase and then incubated for 4 h with no drug, 25 μg/ml lovastatin (*Lova*), or 10 μg/ml zaragozic acid (*ZA*) as indicated. *B*, limited proteolysis time course of myc_L-Hmg2p-GFP. Microsomes bearing myc_L-Hmg2p-GFP were prepared and incubated with trypsin (100 μg/ml) at 30 °C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope. *C*, effect of detergents on proteolysis. Microsomes containing myc_L-Hmg2p-GFP were prepared as described and incubated with trypsin (100 μg/ml), proteinase K (10 μg/ml), or papain (100 μg/ml) for 30 min at 30 °C. For each protease type, samples shown are no protease (*N*), protease only (*P*), and protease with 1% Triton-X 100 to permeabilize membranes (*D*). Following incubation, samples were evaluated by SDS-PAGE on a 14% gel and immunoblotting of the Myc epitope.

the *cod1*Δ cells was clearly different (compare with Fig. 4*B*), despite its continued ability to respond to glycerol *in vivo* and *in vitro*. These results indicate that the limited proteolysis assay can discern structural variations in Hmg2p, providing a novel window into the regulation of Hmg2p and other proteins. In addition, this experiment provides an explanation for the lack of Hmg2p regulation in the *cod1*Δ background. Since regulation of Hmg2p is quite sensitive to *in cis* structural changes (26), it is likely that the altered structure of Hmg2p in the *cod1*Δ background underlies its unresponsiveness to the FPP-derived signal.

The *in vitro* results indicated that elevated glycerol caused a "tightening" of the Hmg2p transmembrane domain that altered the rate of limited proteolysis by trypsin *in vitro* and its degradation rate *in vivo*. We wondered if the same structural transition underlies the physiological regulation of Hmg2p by the FPP-derived signal. Accordingly we tested limited proteolysis of Hmg2p-GFP in microsomes derived from otherwise identical cells with high or low FPP-derived signal *in vivo*. This was done as follows: A strain expressing myc_L-Hmg2p-GFP was grown in lovastatin, which lowers FPP levels, or zaragozic acid, which elevates FPP levels. Microsomes were prepared from each experimental sample and subjected to the limited proteolysis assay. In order to maintain comparable amounts of Hmg2p when the levels of degradation signal differed, the experiment was performed in an *hrd1*Δ-null strain in which Hmg2p degradation is completely blocked. In this way, the FPP-derived degradation signal could be altered without causing changes in myc_L-Hmg2p-GFP level.

The results of this "*ex vivo*" experiment are shown in Fig. 8. Elevating FPP in intact, living cells clearly caused a detectable increase in rate in the subsequently measured *in vitro* trypsinolysis. This "*ex vivo*" effect was somewhat smaller, with an average difference of 4.5 ± 1.6, as opposed to 8.5 ± 1.7 with glycerol, as measured by digital densitometry (mean ± S.E., $n = 3$; NIH Image 1.63). Nevertheless, the effect of *in vivo* manipulation of FPP was in the predicted direction: increasing FPP caused increased *in vitro* susceptibility without a change in proteolysis pattern. Thus, it appears that an alteration in structure similar to that seen with chemical chaperones underlies the physiological regulation of Hmg2p *in vivo*.

DISCUSSION

Our previous work on Hmg2p degradation indicated that it undergoes a unique mode of regulation: controlled access to a constitutively active quality control pathway, driven by a physiological degradation signal (15, 19). Thus, in conditions in which Hmg2p is rapidly degraded, it would be expected to acquire features of a quality control substrate. Consistent with this idea, we had demonstrated that Hmg2p is stabilized *in vivo* by the chemical chaperone glycerol (18), which is known to stabilize other quality control substrates (22–24,33). In the work herein we have thoroughly evaluated the effects of chemical chaperones on Hmg2p, with a particular interest in their use in understanding the structural features of Hmg2p regulation. Here we show that these agents cause a direct, reversible effect on Hmg2p structure and we demonstrate that the same kind of transition appears to underlie physiological regulation of Hmg2p.

The stabilizing effect of glycerol was rapid, reversible, and highly specific for Hmg2p. Because FPP regulation of Hmg2p has these features, we tested whether glycerol stabilization

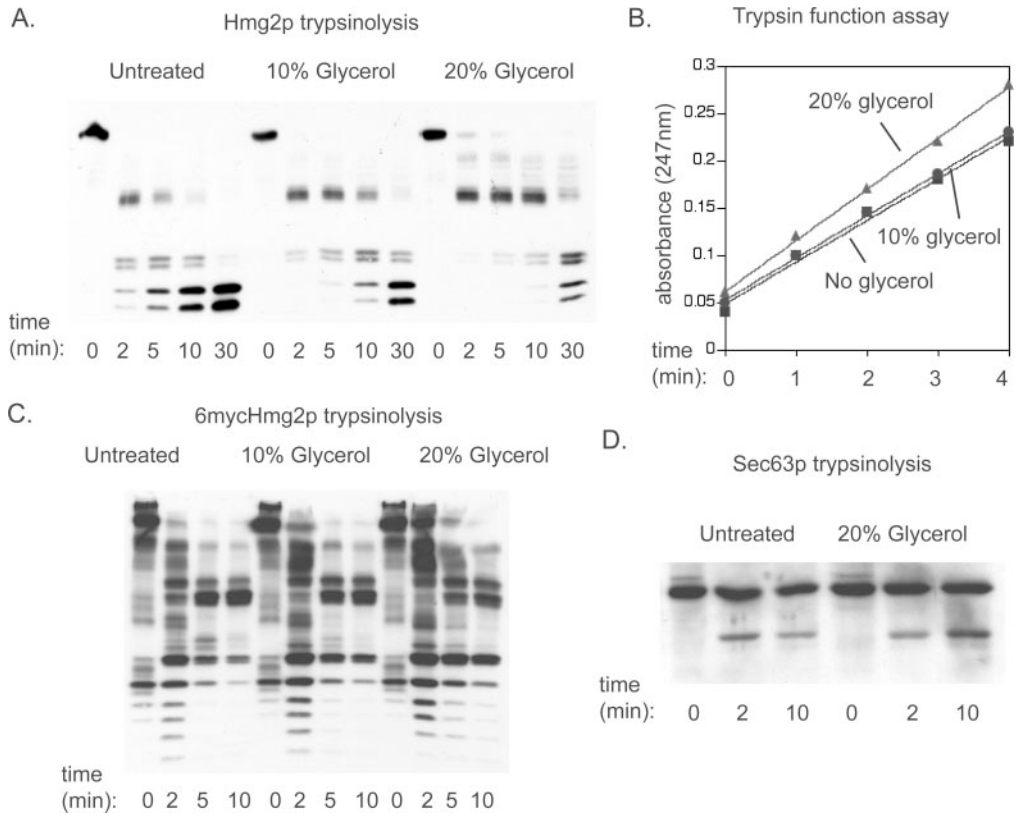

FIG. 5. **Glycerol specifically reduced the proteolytic accessibility of Hmg2p *in vitro*.** *A*, effect of glycerol in the *in vitro* assay. Microsomes bearing myc_L-Hmg2p-GFP were prepared and resuspended in buffer with no, 10% or 20% glycerol and incubated with trypsin (100 μg/ml) at 30 °C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope. *B*, chromogenic assay of trypsin function. A solution of trypsin (525 ng/ml) in reaction buffer with 1 mM TAME and with no (*squares*), 10% (*circles*), or 20% (*triangles*) glycerol was incubated and enzymatic product absorbance at 247 nm measured at the indicated times. *C*, limited proteolysis of glycerol of 6myc-Hmg2p. Microsomes bearing 6myc-Hmg2p were prepared and resuspended in buffer with no, 10%, or 20% glycerol and incubated with trypsin (15 μg/ml) at 30 °C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope. *D*, effect of glycerol on proteolysis of the endogenous membrane-spanning ER protein Sec63p. Samples described in *A* were evaluated by 14% SDS-PAGE and immunoblotting of the protected luminal dnaJ epitope of Sec63p.

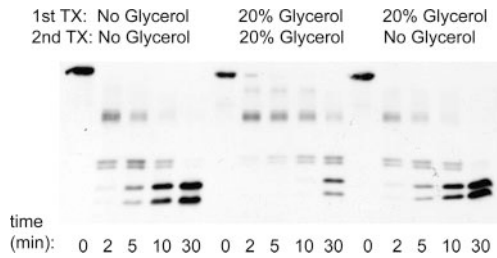

FIG. 6. **The *in vitro* effect of glycerol is reversible.** Microsomes bearing myc_L-Hmg2p-GFP were prepared and resuspended in buffer with no or 20% glycerol, incubated for 30 min at 30 °C, then spun down, and resuspended in no or 20% glycerol as indicated, then immediately incubated with trypsin (100 μg/ml) at 30 °C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope.

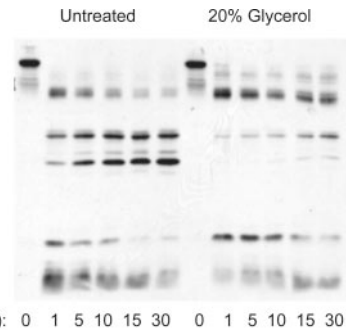

FIG. 7. **Limited proteolysis of myc_L-Hmg2p-GFP derived from a *cod1*Δ strain.** Microsomes from a *cod1*Δ strain were prepared as described, then resuspended in buffer with no or 20% glycerol and incubated with trypsin (100 μg/ml) at 30 °C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope.

worked through altering the action of the FPP degradation signal. It did not, as glycerol stabilized Hmg2p in *cod1*Δ cells, where the FPP signal fails to function (28). Importantly, in wild type cells, the degradation of Hmg2p that had been freed from glycerol stabilization was still normally regulated, indicating that the glycerol pretreatment did not permanently affect Hmg2p structure, since even subtle structural alterations lead to complete abrogation of normal regulation (26).

These *in vivo* studies indicated that glycerol and other chemical chaperones have a rapid, reversible action on Hmg2p structure. We directly examined this idea by developing a limited proteolysis assay for Hmg2p with a Myc epitope tag in the protected first luminal loop. This new limited proteolysis assay removed problems inherent in our previously published assay, which used untagged Hmg2p and a mixture of polyclonal antibodies that recognized mostly cytosolic epitopes (18). That assay was very sensitive to levels of remaining protein and digestion conditions, due in large part to the proteolysis-dependent loss of a majority of the signal (over 90%). The new proteolytic assay described herein obviates those technical issues and has allowed us to study the structural responses described above, and the *in vitro*

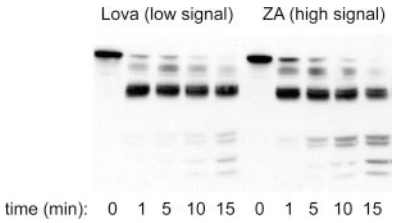

FIG. 8. **Limited proteolysis assay of myc_L-Hmg2p-GFP derived from cells with high or low degradation signal.** Strains expressing myc_L-Hmg2p-GFP in a *hrd1*Δ background were incubated for 16 h in YPD with 100 μg/ml lovastatin (*Lova*) to reduced FPP-derived degradation signal or 40 μg/ml zaragozic acid (*ZA*) to build up degradation signal. Microsomes were prepared from each culture as described and subjected to limited proteolysis assay as described previously.

response to signal molecules.[2]

As predicted from our live cell studies, the Hmg2p transmembrane region underwent a substantial change in trypsin susceptibility when treated with glycerol or other chemical chaperones. A number of controls showed that this action of glycerol was not a general effect on the trypsin or its ability to digest soluble or membrane bound proteins. Rather, the Hmg2p transmembrane region is highly responsive to the action of chemical chaperones. As was the case *in vivo*, the *in vitro* action of glycerol on Hmg2p was rapid and reversible. Taken together, these studies indicate that Hmg2p can undergo fairly drastic, yet reversible, structural changes. The consistency of the effects between the *in vivo* and *in vitro* experiments lends credence to the idea that Hmg2p undergoes a structural transition that allows increased susceptibility to the HRD quality control pathway.

There have been many studies on chemical chaperone effects on proteins, including studies of protein function *in vivo* (22, 33), and *in vitro* structural studies of highly purified proteins (34). These agents have been reported in numerous cases to "cure" or correct mutant phenotypes by promoting the function of mutant proteins, with the most salient example being the glycerol-mediated treatment of mammalian cells to spare the misfolded CFTR-ΔF508 mutant from ERAD (22, 33). The proposed and reasonable model for this effect is glycerol-induced enhancement of the mutant protein folding. However, the effects of chemical chaperones on CFTR have not been observable *in vitro*,[3] and the effect on CFTR function in living cells takes twenty-four hours to occur, with only a fraction of the total ΔF508 protein actually folding properly and avoiding ERAD (33). In contrast, the effect of glycerol on Hmg2p was dramatic, rapid, and direct. The entire pool of Hmg2p was immediately affected in a strong but reversible manner, and our *in vitro* studies showed the direct structural action of chemical chaperones, resulting in a strong reduction in Hmg2p susceptibility to trypsin. As far as we can tell, this is the first demonstration of the action of chemical chaperones via limited proteolysis, an assay often used to evaluate protein structure and conformation. Based on these observations, Hmg2p appears to be naturally capable of undergoing structural changes. In fact, although the studies on CFTR-ΔF508 provided a conceptual template, the Hmg2p protein response to chemical chaperones is far more striking, reversible, and easily detected *in vitro*.

The ability of Hmg2p to undergo changes in conformation that alter its degradation rate is a feature predicted from the role of the HRD quality control pathway in Hmg2p regulation (15, 19). The observed sensitivity of Hmg2p-regulated degradation to glycerol or other chemical chaperones is consistent with the degraded state having features of a misfolded protein. The particular change we observed, a slowing of the rate of limited proteolysis without a change in the pattern of band appearance or mobility, suggests that Hmg2p acquires a "tighter" version of its structure but that the exposed cleavage sights remain the same, and that the relative accessibility of each site compared with the others is also unchanged because the cleavages occur in the same order over time.

This glycerol-induced change in structure could reasonably result in less susceptibility of the resulting form of Hmg2p to the HRD pathway, and a similar structural transition appeared to underlie FPP-regulated degradation. We compared the *in vitro* limited proteolysis of myc_L-Hmg2p-GFP in microsomes derived from cells with high or low levels of the FPP-derived signal. Hmg2p in microsomes from a high signal environment, where degradation is rapid, was more trypsin accessible than Hmg2p from a low-signal environment. Our subsequent work with direct *in vitro* analysis of effects of signals on Hmg2p structure lends strong support to the idea that the natural regulatory response is the same as that observed with chemical chaperones.[4]

There are many proteins that undergo changes in structure mediated by small molecule signals, with the best-studied being enzymes and transcription factors that are regulated allosterically (35). Furthermore, there are many proteins that have structures that can vary between more and less folded states. It appears that Hmg2p combines these properties, and thus can undergo regulated changes in structure from a more to a less folded state in a manner that allows rapid, fine control of its cellular abundance in response to metabolic needs. These studies on Hmg2p demonstrate the extent and quality of this structural range, and most likely reflect a single example of a novel regulatory axis that is widely represented in biology.

Acknowledgments—We thank members of the Hampton laboratory, past and present, for generous intellectual, material, and emotional input. We also thank Robert Rickert for the near-continuous use of his FACScaliber flow cytometer. R. Y. H. wishes further to acknowledge Frank Canino for advice, support, and the best bass lines on the Eastern Seaboard.

REFERENCES

1. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) *Genes Dev.* **9,** 1586–1597
2. Salceda, S., and Caro, J. (1997) *J. Biol. Chem.* **272,** 22642–22647
3. Goebl, M. G., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., and Byers, B. (1988) *Science* **241,** 1331–1335
4. Deshaies, R. J., Chau, V., and Kirschner, M. (1995) *EMBO J.* **14,** 303–312
5. Deshaies, R. J. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 435–467
6. Sakamoto, K. M. (2002) *Mol. Genet. Metab.* **77,** 44–56
7. Pickart, C. M. (2001) *Mol. Cell* **8,** 499–504
8. Ciechanover, A. (1998) *EMBO J.* **17,** 7151–7160
9. Swanson, R., Locher, M., and Hochstrasser, M. (2001) *Genes Dev.* **15,** 2660–2674
10. Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A., and Hampton, R. Y. (2001) *Nat. Cell Biol.* **3,** 24–29
11. Niwa, J., Ishigaki, S., Hishikawa, N., Yamamoto, M., Doyu, M., Murata, S., Tanaka, K., Taniguchi, N., and Sobue, G. (2002) *J. Biol. Chem.* **277,** 36793–36798
12. Wilhovsky, S., Gardner, R., and Hampton, R. (2000) *Mol. Biol. Cell* **11,** 1697–1708
13. Bordallo, J., Plemper, R. K., Finger, A., and Wolf, D. H. (1998) *Mol. Biol. Cell* **9,** 209–222
14. Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., and Sommer, T. (2000) *Nat. Cell Biol.* **2,** 379–384
15. Hampton, R. Y. (2002) *Annu. Rev. Cell Dev. Biol.* **18,** 345–378
16. Gardner, R. G., Swarbrick, G. M., Bays, N. W., Cronin, S. R., Wilhovsky, S., Seelig, L., Kim, C., and Hampton, R. Y. (2000) *J. Cell Biol.* **151,** 69–82
17. Gardner, R. G., and Hampton, R. Y. (1999) *J. Biol. Chem.* **274,** 31671–31678
18. Gardner, R. G., Shearer, A. G., and Hampton, R. Y. (2001) *Mol. Cell. Biol.* **21,** 4276–4291
19. Hampton, R. Y. (2002) *Curr. Opin. Cell Biol.* **14,** 476–482
20. Zheng, F., Kartner, N., and Lukacs, G. L. (1998) *Nat. Struct. Biol.* **5,** 180–183

[2] A. Shearer and R. Hampton, manuscript in preparation.
[3] Dr. William Skatch, OHSU, personal communication.
[4] A. Shearer and R. Hampton, manuscript in preparation.

21. Qu, B. H., and Thomas, P. J. (1996) *J. Biol. Chem.* **271,** 7261–7264
22. Brown, C. R., Hong-Brown, L. Q., Biwersi, J., Verkman, A. S., and Welch, W. J. (1996) *Cell Stress Chaperones* **1,** 117–125
23. Welch, W. J., and Brown, C. R. (1996) *Cell Stress Chaperones* **1,** 109–115
24. Zhou, Z., Gong, Q., and January, C. T. (1999) *J. Biol. Chem.* **274,** 31123–31126
25. Gardner, R., Cronin, S., Leader, B., Rine, J., and Hampton, R. (1998) *Mol. Biol. Cell* **9,** 2611–2626
26. Gardner, R. G., and Hampton, R. Y. (1999) *EMBO J.* **18,** 5994–6004
27. Hampton, R. Y., and Rine, J. (1994) *J. Cell Biol.* **125,** 299–312
28. Cronin, S. R., Khoury, A., Ferry, D. K., and Hampton, R. Y. (2000) *J. Cell Biol.* **148,** 915–924
29. Hampton, R. Y., and Bhakta, H. (1997) *Proc. Natl. Acad. Sci. (U. S. A.)* **94,** 12944–12948
30. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) *Science* **273,** 1725–1728
31. Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002) *EMBO J.* **21,** 615–621
32. Feldheim, D., Rothblatt, J., and Schekman, R. (1992) *Mol. Cell. Biol.* **12,** 3288–3296
33. Sato, S., Ward, C. L., Krouse, M. E., Wine, J. J., and Kopito, R. R. (1996) *J. Biol. Chem.* **271,** 635–638
34. Courtenay, E. S., Capp, M. W., Anderson, C. F., and Record, M. T., Jr. (2000) *Biochemistry* **39,** 4455–4471
35. Gerhart, J. C., and Schachman, H. K. (1968) *Biochemistry* **7,** 538–552