UNIVERSITY OF CALIFORNIA, SAN DIEGO

HMG-CoA Reductase: Protein Regulation by a Quality Control Pathway

A dissertation submitted in partial satisfaction of
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in
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by

Alexander Glennon Shearer

Committee in charge:

Professor Randolph Hampton, Chair
Professor Alexandra Newton
Professor Maho Niwa
Professor Lorraine Pillus
Professor Michael Yaffe

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The dissertation of Alexander Glennon Shearer is approved and is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2004

This work is dedicated to those patient enough to stand by me.

"In short, don't trust scientists! They're immoral, and challenge well-documented ignorance…"

David Rust

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Vita

1999	B.A. Molecular and Cell Biology, University of California, Berkeley
2004	Ph.D. Biology, University of California, San Diego

Publications

Shearer, A.G. and Hampton, R.Y. (2004) Lipid-mediated, reversible misfolding of a sterol-sensing domain (SSD) protein. *Manuscript in submission.*

Flury, I., **Shearer, A.G.,** Rosen, J., and Hampton, R.Y. (2004) Broadly conserved role for INSIG proteins as chaperones for the sterol-sensing domain. *Manuscript in submission.*

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ABSTRACT OF THE DISSERTATION

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by

Alexander Glennon Shearer

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Professor Randolph Hampton, Chair

Protein degradation is a necessity in the living cell, carrying out the key tasks of quality control and regulation. Quality control, requiring recognition of general hallmarks of misfolding, seems quite distinct from regulatory degradation, which targets specific proteins. This work describes the adaptation of quality control to regulation and the mechanism by which it occurs.

HMG-CoA reductase is a rate-limiting enzyme in sterol synthesis. In both yeast and mammals, it is regulated by degradation driven by feedback based on abundance of a pathway intermediate. The yeast isozyme Hmg2p is degraded by a

constitutive, quality-control pathway, implying that Hmg2p undergoes a structural transition to have traits of a misfolded protein in response to that feedback signal.

Studies *in vivo* and *in vitro* showed that Hmg2p regulation was sensitive to factors influencing protein folding. Chemical chaperones, which improve protein folding *in vivo* and *in vitro*, caused a reversible, immediate slowing in Hmg2p degradation consistent with a stabilizing effect on Hmg2p structure. *In vitro*, chemical chaperones caused a dramatic, rapid and reversible change in Hmg2p structure to a less protease-sensitive form. In addition, buildup of *in vivo* degradation signal led to the acquisition of a more sensitive conformation. This suggested that Hmg2p did, indeed undergo significant, reversible structural changes that are relevant to its physiological regulation

Two different *in vitro* structural assays were used to show that the pathway derivative farnesol caused Hmg2p to undergo a change to a less folded structure. The effect was reversible, biologically relevant by numerous criteria, highly specific for farnesol structure, and required an intact Hmg2p sterol sensing domain (SSD). Hmg2p acquired quality control traits on exposure to a pathway derivative, providing a mechanism for its regulated degradation by quality control pathway.

Preliminary work was done on identifying chaperones involved in Hmg2p degradation. Though several were required for stability, only two were necessary for degradation. Finally, the stabilizing structural effects of an Hmg2p chaperone, Nsg1p, were elucidated directly for the first time.

Chapter 1

Adaptation in action: the intersection of quality control and regulation

Protein degradation is vital in the living cell. Many proteins are broken down after defined life spans or under specific regulatory circumstances, facilitating the normal functions of life. Misfolded proteins must also be cleared from the cell by degradation, preventing pathogenic outcomes that can result from buildup of misfolded or unassembled proteins. These two jobs for protein degradation differ markedly in what they require and what they achieve.

Regulation involves precise targeting of normal, functional proteins for degradation as a means of controlling cellular processes, frequently based on discrete polypeptide sequence tags, or "degrons," that mark specific proteins for degradation (Suzuki and Varshavsky, 1999). Examples of regulatory degradation include a number of proteins required for normal cell cycle progression and health, such as CDC34 (Goebl et al., 1988), IkappaB (Chen et al., 1995) and HIF1a (Salceda and Caro, 1997). In all cases, these mechanisms appear to target proteins based solely on their identities, rather than on some general traits that any protein might acquire, thus maintaining the narrow specificity required for proper regulation.

In sharp contrast, quality control degradation is the destruction of proteins that are misfolded or that have failed to correctly assemble into multimeric complexes, thus preventing buildup of these potentially harmful molecules. While any protein has the potential to occasionally misfold, and thus to be a quality control substrate – that is, a protein with an error in folding or assembly that prevents its normal function, may be

toxic to the cell and can be identified for retention or degradation – notable and devastating examples of constitutive quality control substrates include Z-variant alpha-1 antitrypsin (Burrows et al., 2000), which fails to properly exit the ER, causing liver failure and lung damage, CFTR ΔF508 (Ward et al., 1995), where inability to fold properly and reach the cell surface leads to cystic fibrosis and polyglutamine-expanded huntingtin (Bates, 2003), the causative factor in Huntington's disease. Identification of these quality control substrates requires recognition of the poorly understood structural hallmarks that separate abnormal proteins from their correctly folded counterparts independent of sequence or protein identity. Thus, the mechanisms that handle protein quality control degradation somehow identify general traits of misfolding which many proteins can acquire during their initial attempts at folding, but which are typically associated with dysfunctional proteins. It is natural, then, to imagine that quality control degradation would not play a role in normal regulation.

Nature, however, adapts.

Despite their apparent differences, quality control and regulation may still intersect. It is entirely reasonable to imagine that pathways that constitutively degrade misfolded proteins could be adapted to the task of regulation, most likely through regulated entry of a protein into such a constitutive pathway. That is, the regulated protein would, under certain circumstances, take on traits of a misfolded protein and thus be degraded by a relevant quality control mechanism. Research into the regulated

degradation of the endoplasmic-reticulum (ER) enzyme HMG-CoA reductase (HMGR) has unveiled it as a first example of just such a regulatory mechanism.

HMGR Regulation

HMGR catalyzes the conversion of HMG-CoA into mevalonic acid, the rate-limiting step in sterol synthesis. As such, it has been the primary enzymatic target for cholesterol lowering medications for many years (Morris et al., 1997). Lately, its importance has expanded as it has been determined that the HMGR-inhibiting statins impart substantial cardiovascular benefits independent of their ability to lower serum cholesterol levels (Gresser and Gathof, 2004). Reduction of flux through the mevalonate pathway, of which HMGR catalyzes the rate-limiting step, has demonstrable benefits in reducing the deleterious side effects of diabetes (Baghdasarian et al., 2004; Ullom-Minnich, 2004) and in fighting inflammatory and autoimmune conditions, possibly by reducing the farnesylation of proteins involved in activating the appropriate pathways (Marz and Koenig, 2003; Stuve et al., 2003). Thus, on a medical basis alone, the mechanism of HMGR regulation is of substantial interest.

Mammalian HMGR, naturally the most extensively researched of the HMG-CoA reductases, is subject to transcriptional, translational and postranslational regulation (Goldstein and Brown, 1990). In the last case, HMGR undergoes regulated

degradation primarily driven by levels of the downstream pathway product cholesterol. In conjunction with the Insig proteins, which are also required for proper function of the general sterol regulatory pathway, HMGR "senses" sterol levels (Sever et al., 2003a; Sever et al., 2003b; Yang et al., 2002). When cholesterol is abundant, HMGR is subject to ubiquitin-mediated degradation by the proteasome (Ravid et al., 2000). In this way, HMGR undergoes feedback inhibition through loss of the enzyme, helping to modulate activity of the sterol pathway generally. It has also been suggested that pathway derivatives earlier than cholesterol may play a part in regulation by accelerating HMGR degradation. Farnesol and geranylgeraniol, derived from two successive isoprene compounds, have both been posited to do this (Correll et al., 1994; Meigs et al., 1996; Sever et al., 2003a). Recent work suggests that perhaps geranylgeraniol accelerates HMGR degradation as an adjunct to the sterol signal, directly contradicting the earlier studies by observing no role for farnesol. Regardless, it is clear that HMGR is degraded in a regulated fashion as a means of modulating one aspect of cellular metabolism.

Key insights into HMGR regulation have been made by studying the yeast HMGR isozyme Hmg2p, which undergoes similar, feedback-regulated degradation in response to a signal derived from the sterol pathway (Hampton, 2002). Unlike mammalian HMGR, the primary signal in yeast is not sterol based. Extensive genetic and pharmacological analysis of the yeast mevalonate pathway has identified the

pathway intermediate farnesylpyrophosphate (FPP) as the key determinant of Hmg2p degradation rate (Gardner and Hampton, 1999b). When FPP is abundant, Hmg2p is quickly degraded, and when FPP levels are low, Hmg2p is stabilized (Figure 1-1). There is an additional, adjunct signal from an oxysterol that serves to enhance the effectiveness of the FPP-derived signal (Gardner et al., 2001). Thus it appears that the two systems, yeast and mammal, are quite well conserved across millions of years, though possibly with the identity of the primary and adjunct degradation signals reversed. The tractable nature of yeast, however, has allowed insights not readily available in mammalian cells and paved the way for greater understanding of this mode of regulated degradation generally.

Understanding the Mechanism of Hmg2p Degradation

Regulated degradation of Hmg2p occurs through the action of the ubiquitin-proteasome system. The discovery of a requirement for ubiquitination and the proteasome in Hmg2p degradation was the first identification of a role for this system in the degradation of any kind of HMGR (Hampton and Bhakta, 1997; Hampton et al., 1996). The ubiquitin-proteasome system, which bears the burden of much of total cellular protein degradation, begins with covalent tagging of substrate proteins with multiple copies of the 8kDa protein ubiquitin. Thus marked, the substrate is then

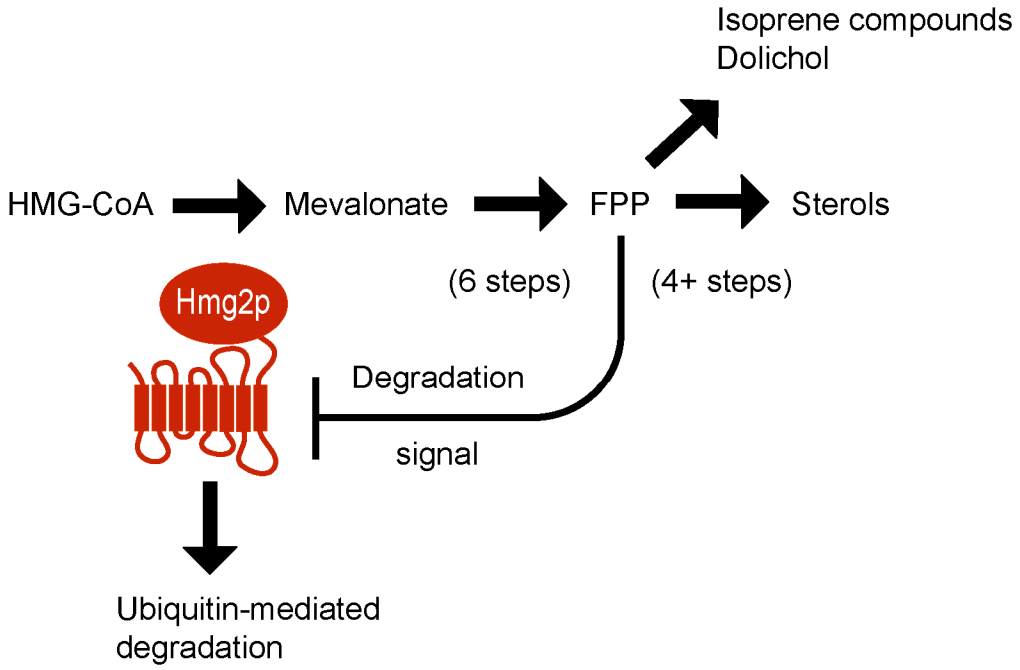

Figure 1-1
HMGR undergoes feedback regulation via degradation.
HMG-CoA Reductase catalyzes the rate-limiting step in the mevalonate pathway, making it a control point for the synthesis of sterols, isoprene compounds and other lipids. In the yeast HMGR isozyme Hmg2p, the degradation signal is based on the level of the pathway intermediate farnesylpyrophosphate (FPP). Under conditions when FPP should be abundant, Hmg2p is rapidly degraded.

recognized and degraded by the 26S proteasome, a multisubunit protease that is abundant in the cell. In fact, the discovery of a role for ubiquitination in Hmg2p degradation refocused attention on a possible role for ubiquitination in HMGR degradation in mammals, leading to more thorough research that reversed an earlier declaration that ubiquitin played no part in mammalian HMGR regulation (McGee et al., 1996).

Subsequently, the HMG-CoA Reductase Degradation (HRD) screen revealed that many of the genes required for Hmg2p degradation are those involved in protein degradation generally and endoplasmic reticulum associated degradation (ERAD) specifically (Hampton et al., 1996). As might be expected from the involvement of ubiquitin, proteasome components make a showing. *HRD2*, for example, encodes Rpn1p, a conserved proteasome component. Venturing away from the proteasome, *HRD4/NPL4* is required for degradation of Hmg2p, but not for its ubiquitination (Bays et al., 2001b). Complexed with Cdc48p and Ufd1p, Hrd4p is required for proteasomal degradation of a number of ERAD substrates, including Hmg2p and its mutants, CPY* and UP*, acting as a retrotranslocation factor to remove substrates from the ER and allow their degradation by the proteasome (Ye et al., 2003). Again, it is perhaps not surprising to find that ER-localized Hmg2p requires the same machinery as other, general ERAD substrates. Neither the proteasome nor Hrd4p represent specificity steps in the pathway of ubiquitin-mediated degradation.

The specificity of ubiquitination is controlled by ubiquitin ligases, and it is here that a surprising discovery about Hmg2p degradation was made. Ubiquitin ligases, or E3s, mediate the recognition of target proteins for ubiquitination, whether through canonical sequence tags and motifs, or general traits, such as those of a quality control substrate. Extensive work following the HRD screen identified Hrd1p as the E3 ubiquitin ligase that mediates Hmg2p ubiquitination (Bays et al., 2001a). Surprisingly, Hrd1p functions as a key quality control ligase in the ER, being required for the degradation of misfolded proteins such as soluble CPY* and membrane-bound Pdr5p*, Sec61-2 and many constitutively degraded Hmg2p mutants, and for maintaining a survivable level of misfolded proteins in that organelle (Figure 1-2) (Bays et al., 2001a; Bordallo et al., 1998; Bordallo and Wolf, 1999; Friedlander et al., 2000; Plemper and Wolf, 1999; Wilhovsky et al., 2000). This begs the question of how a quality control ligase could be responsible for the regulated degradation of a normal protein such as wild type Hmg2p.

Structural Transition Hypothesis

The most direct model of Hmg2p regulation and the hypothesis that drove the research described in this volume is structural transition. That is, in response to a signal based on the abundance of the critical pathway intermediate FPP, Hmg2p would

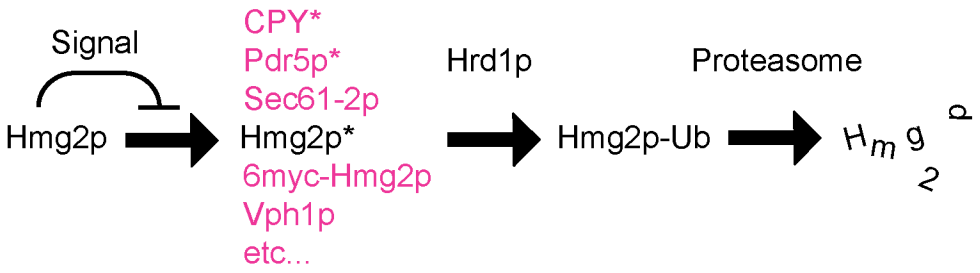

Figure 1-2
Hmg2p is degraded by a quality control pathway.
In response to a metabolic signal, Hmg2p undergoes regulated entry into the constitutive HRD quality control degradation pathway (Hmg2p*), where it is ubiquitinated by the E3 ubiquitin ligase Hrd1p (Hmg2p-Ub), then degraded by the proteasome (broken up Hmg2p). Notably, all other known substrates for this pathway are mutant or misfolded proteins that do not enter it in a regulated fashion.

acquire traits of a quality control substrate (Figure 1-3). This change would necessarily be rapid and occur in mature, folded Hmg2p, since Hmg2p can quickly be shifted to rapid degradation following pharmacological blockade of the sterol pathway, even in the absence of new protein synthesis. Thus, a wild type protein would be shunted down a constitutive quality control pathway by dint of regulated acquisition of quality control traits. But does the standing knowledge about Hmg2p support this idea?

The Specifics of Hmg2p Regulation

Hmg2p has proven resistant to simple, structural-feature based explanations of its regulated degradation. Hmg2p is an ER-resident enzyme with eight transmembrane spans and a substantial, cytosolic catalytic domain that is dispensible for regulation (Hampton and Rine, 1994). Though the transmembrane domain as a whole is necessary and sufficient for that task, extensive analysis of this portion of the protein has had only limited success in finding smaller regions of the domain to which both standards apply. In fact, analysis of over three hundred mutations throughout the transmembrane domain identified many regions of that domain that must have their proper sequence for correct regulated degradation (Figure 1-4) (Gardner et al., 1998; Gardner and Hampton, 1999a). This requirement for sequence integrity across the bulk of the transmembrane domain aligns well with the concept of a structural transition in

Figure 1-3
Much of the Hmg2p transmembrane domain is necessary for regulation.
The transmembrane domain of Hmg2p is necessary and sufficient for regulated degradation, with the soluble catalytic domain (labeled Hmg2p) dispensable for that purpose. Alterations in the transmembrane domain frequently lead to disrupted regulation (portions that do this are labeled in black). Thus, though many parts of the transmembrane domain are necessary for proper regulation, none of them are individually sufficient.

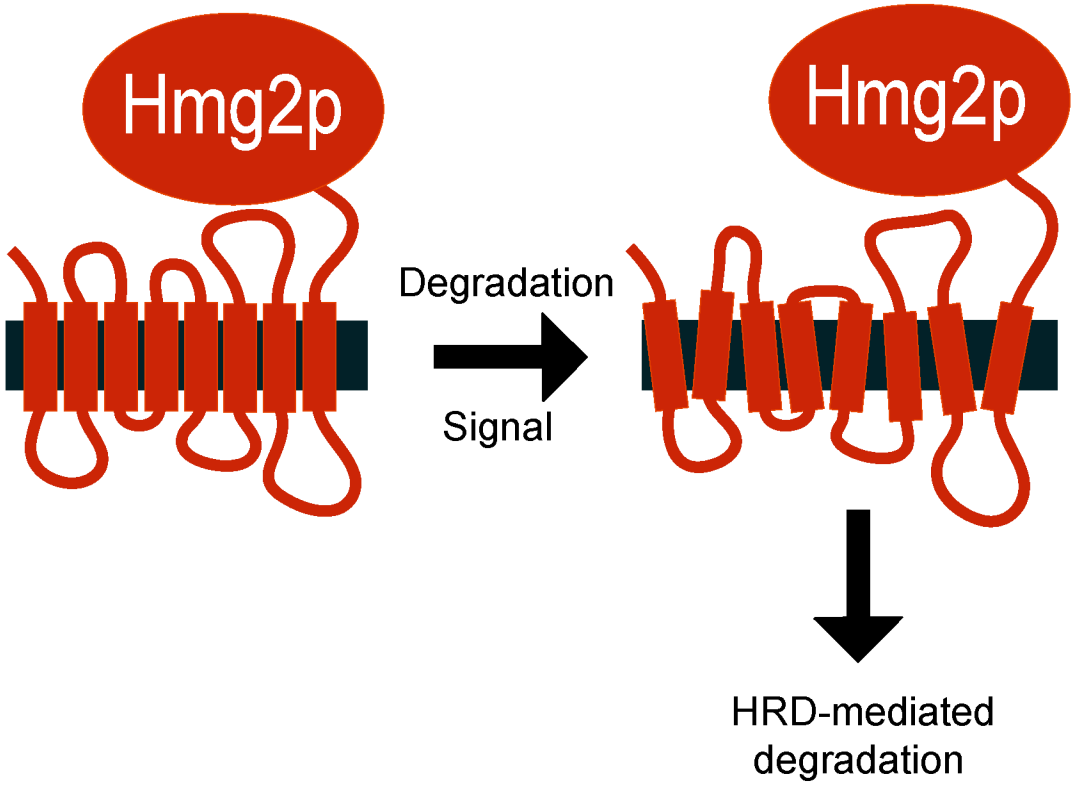

Figure 1-4
The structural transition hypothesis.
The work described in the following chapters is directed toward testing the following hypothesis: Hmg2p can exist in two conformational states. In the absence of signal Hmg2p appears to be a properly folded protein (left), and is not a substrate for the HRD pathway. In the presence of signal Hmg2p undergoes a conformational change to acquire traits of a misfolded protein (right) and is detected and degraded by the constitutive HRD quality control pathway.

response to degradation signal. Whether or not any portion of the protein is involved in direct interaction with the signal, it is clear that variation in any part of the transmembrane domain could potentially disrupt the ability of the entire protein to undergo the structural change required for recognition as a quality control substrate.

Beyond the extensive catalog of *in cis* features required for Hmg2p regulated degradation, only one external component has turned up that is explicitly necessary for Hmg2p regulation. A screen for genes involved in the Control Of reductase Degradation (*COD* genes) turned up exactly one gene, *COD1*, which codes for Cod1p, an ER-resident ATPase and putative ion pump with calcium-related phenotypes (Cronin et al., 2000; Cronin et al., 2002). Though the specific function of Cod1p remains unknown, it has also turned up in screens for genes that are synthetically lethal with a disabled unfolded protein response (UPR) (Vashist et al., 2002). As might be expected from that observation, the UPR is activated when Cod1p is absent. In a *cod1*Δ strain, regulation of Hmg2p in response to changes in pathway flux is almost completely absent. Experiments described in this work shed light on how Cod1p, with its pleiotropic effects on the ER, is so important for Hmg2p regulation.

Molecular Chaperones and Hmg2p

Molecular chaperones are proteins that transiently interact with unfolded or misfolded proteins, both aiding in folding and apparently handing misfolded proteins off to be degraded. If Hmg2p acquires traits of a quality control substrate in the ER, one would also imagine a requirement for one or more molecular chaperones in the process of its degradation. Numerous models have been proposed for which chaperones are required for degradation in the ER depending on whether the substrate is in the lumen, is integral to the membrane, or has its mutations in its cytosolic or lumenal regions (Taxis et al., 2003; Vashist and Ng, 2004; Zhang et al., 2001). However, there has been very little study of natural ERAD substrates and their chaperones. Thus, determining which chaperones are required for Hmg2p degradation is both a matter of understanding its particular circumstance and shedding light on chaperones in ERAD generally.

Outstanding questions

The work described in this volume addresses a number of key issues in understanding how a normal protein can be regulated via a quality control pathway. These include:

1) Whether Hmg2p undergoes a structural transition

2) The identity and mode of action of the degradation signal

3) Whether Hmg2p acquires quality control traits

4) Which chaperones are involved in Hmg2p degradation

The work detailed in the following three chapters definitively answers questions one and three, develops a firm understanding of the answer to question two and provides a starting point from which to develop a thorough comprehension of the answers to question four. In addressing these questions, I hope I have not simply shed light on the specific mechanism of Hmg2p's degradation by a quality control pathway, but also a general biological principle that will turn up repeatedly in years to come.

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Chapter 2

Structural control of ER-associated degradation: effect of chemical chaperones on HMG-CoA reductase

Abstract

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 HMG-CoA reductase (Hmg2p) undergoes ERAD that is physiologically regulated by sterol pathway signals. It has been proposed that Hmg2p degradation occurs following a regulated transition to an ERAD quality control substrate. Consistent with this, Hmg2p is strongly stabilized by chemical chaperones such as glycerol, which improve folding of misfolded proteins *in vivo* and *in vitro*. To understand the features of Hmg2p that permit regulated ERAD, I 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. I also 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 protease-sensitive 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, I used the limited proteolysis assay to examine the effects of changing *in vivo* degradation signals on Hmg2p structure, and 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.

Introduction

Protein degradation has two principle 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 IKB (Chen et al., 1995), HIF1a (Salceda and Caro, 1997), and numerous proteins required for cell cycle progression (Deshaies, 1999; Deshaies et al., 1995; Goebl et al., 1988). 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 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 constutively acting quality control pathway. Previous work on the regulated degradation of HMG-CoA reductase (HMGR), 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 8kDa protein ubiquitin, allowing recognition and proteolysis by the 26S proteasome (Hochstrasser, 1996; Smith et al., 1996). 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 (Ciechanover, 1998; Pickart, 2001; Sakamoto, 2002). A smaller group is known to participate in protein quality control, promoting the ubiquitination of misfolded proteins (Bays et al., 2001; Niwa et al., 2002; Swanson et al., 2001). In *S. 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 lumenal proteins such as CPY* and integral membrane proteins (Bays et al., 2001; Bordallo et al., 1998; Wilhovsky et al., 2000). 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 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 (Friedlander et al., 2000). Along with this clear role in quality control, the HRD complex also functions in regulation of a normal, essential metabolic enzyme, HMG-CoA reductase (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 (Hampton, 2002b). Increases in pathway signals lead to increased degradation and a concomitant reduction in Hmg2p's steady-state level. Conversely, decreased signals lead to decreased degradation and a rise in steady state levels (Gardner and Hampton, 1999b). This degradation leads to rapid, fine control of Hmg2p levels in response to the cell's physiological requirements.

In prior genetic analysis of Hmg2p degradation, the HRD complex was identified as the ubiquitin ligase that mediates Hmg2p regulated degradation (Bays et al., 2001; Gardner et al., 2000). 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 was my goal to understand this novel mechanism of regulation, both to allow clear understanding of HMGR regulation, and to explore the concept of harnessing cellular quality control for regulatory purposes in nature and medicine.

The signal for Hmg2p degradation depends on the abundance of the sterol pathway intermediate farnesyl pyrophosphate (FPP) (Gardner and Hampton, 1999b). 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 minutes to hours. Experiments in which FPP levels are rapidly shifted as a consequence of pathway inhibition 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's entry into the constitutively active HRD quality control pathway? The most direct model is that Hmg2p acquires structural features of a quality control substrate in response to the FPP-derived signal (Gardner et al., 2001; Hampton, 2002a; Hampton, 2002b). This regulated structural transition would occur in mature protein and be reversed rapidly when signal decreases. In other words, Hmg2p's status as a quality control substrate would be switchable, 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, I used studies on the clinically important ΔF508 variant of CFTR as a conceptual guide. CFTR is a multi-spanning 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 ER-associated degradation (ERAD), resulting in insufficient CFTR at the cell surface and the consequent clinical manifestations of cystic fibrosis (Qu and Thomas, 1996; Zheng et al., 1998). 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* (Brown et al., 1996; Welch and Brown, 1996). Similarly, defective trafficking of a mutant version of the HERG potassium channel is corrected *in vivo* following treatment with glycerol (Zhou et al., 1999).

These and other studies indicate that the effect of chemical chaperones may be used as a criterion for poor folding of a protein. Specifically, the structural transition model predicts that in conditions that promote degradation, Hmg2p would be stabilized by chemical chaperones in the manner of a misfolded protein. Indeed, 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 (Gardner et al., 2001).

In this chapter I have evaluated this action of chemical chaperones on Hmg2p. I show that the *in vivo* action of glycerol is rapid, reversible and highly specific for Hmg2p. To examine the conformational effects of chemical chaperones, I 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, 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 mechanism Hmg2p can be modulated by a cellular housekeeping pathway to serve the cell's regulatory needs.

Results

The sensitivity of Hmg2p to chemical chaperones is an important indicator of the mechanism of its regulated degradation. In this chapter I have characterized this response, both *in vivo* and *in vitro*, using 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 affect Hmg2p structure in a manner unrelated to regulated degradation, a process mediated solely by the N-terminal transmembrane region (Gardner and Hampton, 1999a; Hampton and Rine, 1994)

Glycerol stabilizes Hmg2p undergoing normal, physiologically regulated degradation (Gardner et al., 2001). This effect is most evident in strains with high FPP levels, in which Hmg2p degradation is rapid. Fast degradation is promoted by co-expression of soluble HMGR catalytic domain, providing high HMGR activity and thus a high level of FPP-derived degradation signal. The Hmg2p-GFP is expressed from the constitutive *TDH3* promoter so that any effects on its steady-state level are due to changes in degradation rate (Hampton and Rine, 1994). Hmg2p-GFP stability is thus directly indicated by the steady-state cellular fluorescence of the GFP fusion protein as measured by flow cytometry (Bays et al., 2001; Cronin et al., 2000; Gardner et al., 1998; Gardner and Hampton, 1999a; Gardner and Hampton, 1999b; Gardner et al., 2001; Gardner et al., 2000; Hampton and Bhakta, 1997; Wilhovsky et al., 2000).

When cells expressing rapidly degraded Hmg2p-GFP were 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 (Figure 2-1, A). The extent of the stabilizing effect was similar to that caused by depleting the FPP-derived signal with lovastatin, and the stabilizing effects of glycerol and lovastatin were not additive, as measured by flow cytometry and biochemically (Gardner et al., 2001). Hmg2p-GFP was also stabilized by similar concentrations of other typical chemical chaperones, including sorbitol and mannitol (data not shown). Two other potential chaperones, trimethylamine N-oxide (TMAO) and 4-phenylbutyric acid, blocked cell growth, thus preventing *in vivo* analysis (Grzanowski et al., 2002).

I next examined the specificity of the stabilizing effects of glycerol for Hmg2p. 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 (Gardner et al., 2001). To further evaluate its selectivity I tested glycerol's action on several other ERAD substrates, including CPY*, a non-folding mutant of carboxypeptidase Y (Hiller et al., 1996), Ole1p (Braun et al., 2002), a natural multi-spanning membrane protein that undergoes ERAD that is not dependent on either of two known ER-localized ubiquitin ligases, Hrd1p or Doa10p, and Hrd1p, which undergoes self-ubiquitination and degradation in the absence of Hrd3p (Gardner et al., 2000). In all cases, glycerol had minimal effect

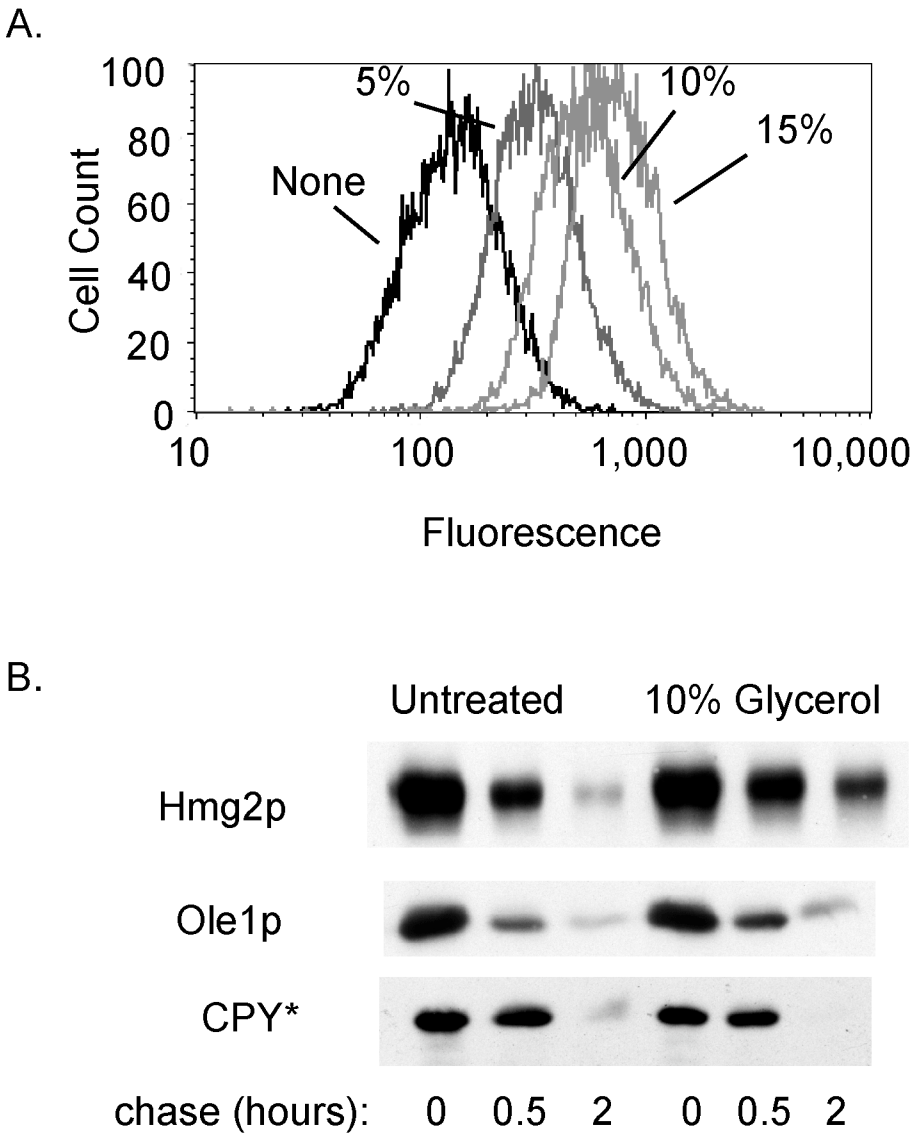

Figure 2-1
Glycerol specifically stabilizes Hmg2p and Hmg2p-GFP *in vivo*.
(A) Early log phase cultures were grown for four hours in the presence of an increasing percentage glycerol as indicated and analyzed for GFP fluorescence via flow cytometry. Each histogram represents 10,000 cells. (B) Cells expressing the indicated ERAD substrates were grown in selective medium, harvested and resuspended in medium with 50μg/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.

on ER degradation of these various test substrates (Figure 2-1, 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 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 (Gardner and Hampton, 1999a). Thus, an alternate model for the specificity of glycerol's effect on Hmg2p is that somehow the FPP-derived signal is made ineffective by chemical chaperones. Flux through the mevalonate pathway is unaffected by glycerol treatment, indicating that there is likely to be normal production of FPP (Gardner et al., 2001). However, any effect of glycerol on the processing of or 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, I employed the *cod1*Δ mutant (Cronin et al., 2000). 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's degradation or steady-state level in this mutant. Incubation of wild type cells in 10% glycerol resulted in stabilization of Hmg2p-GFP to an extent equal to that caused by downregulation of the FPP-derived signal with the pathway inhibitor

lovastatin (Figure 2-2, 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 (Figure 2-2, B). Indeed, Hmg2p-GFP was stabilized to the same steady-state levels in both strains (Figure 2-2, 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.

I next tested the reversibility of glycerol stabilization of Hmg2p. Because the onset of glycerol stabilization was immediate and it acted on mature protein (Gardner et al., 2001), it was reasonable to imagine that the chemical chaperone caused a rapid structural change in the mature Hmg2p protein, rather than any effect on Hmg2p synthesis or *in trans* regulatory mechanisms. According to the structural transition "switching" hypothesis, this effect should 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. 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,

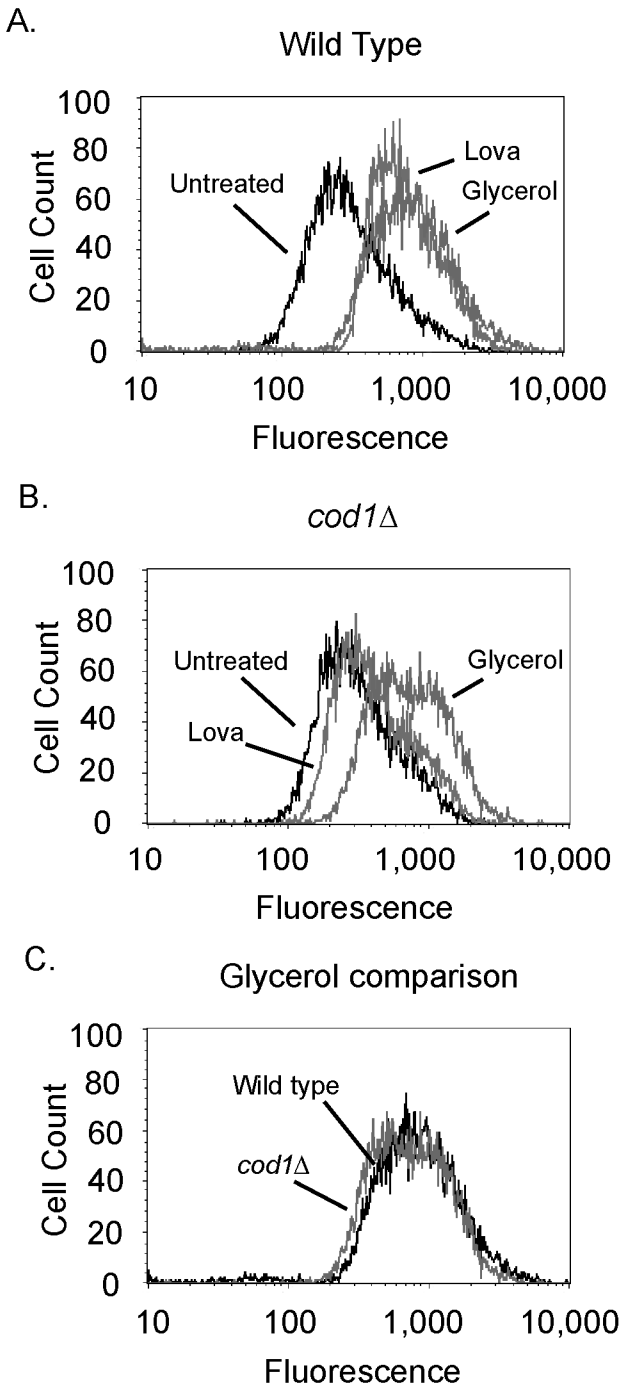

Figure 2-2
Glycerol does not act via the FPP-derived degradation signal.
Wild type cells (A) or *cod1*Δ cells (B) were grown for four hours with 10% glycerol (Glycerol) or 25μg/ml lovastatin (Lova) as indicated and analyzed for GFP fluorescence via flow cytometry. (C) Histograms of glycerol-treated wild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

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 (Figure 2-3). 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. These same studies implied that Hmg2p can undergo structural changes that alter its susceptibility to HRD-dependant degradation. In order to better understand these changes, I developed a limited proteolysis assay to directly study the structure of Hmg2p *in vitro*. The following questions were of particular interest: does glycerol directly affect the structure of Hmg2p, and do these structural effects pertain to the physiological control of Hmg2p ERAD?

Methods exist to produce ER-derived microsomes that are cytosol-side out (Feldheim et al., 1992; Gardner et al., 2001; Gardner et al., 2000). Lumenal 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, I added an epitope to a lumenal region of Hmg2p to allow limited proteolysis-based analysis of its conformational state in microsomes. In making this reporter, it was imperative to not perturb regulated

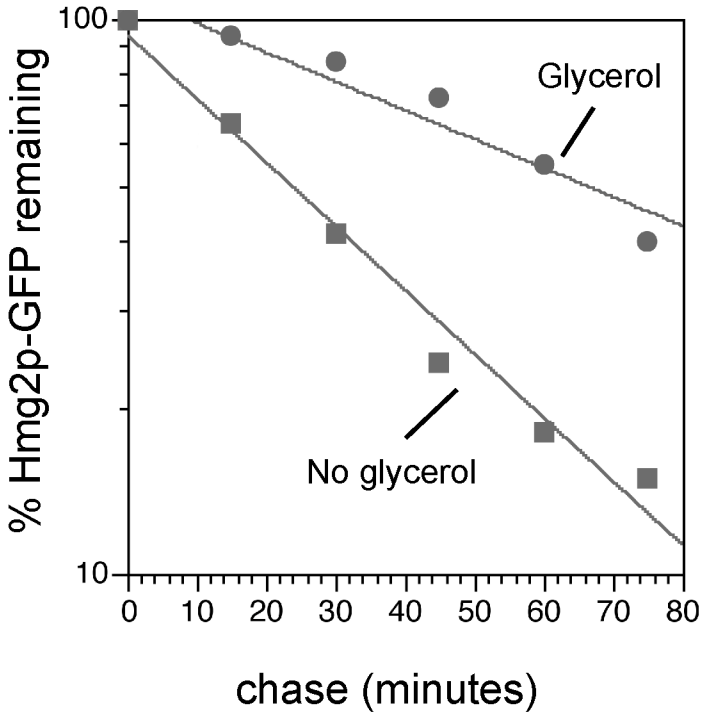

Figure 2-3
The glycerol-induced stabilization of Hmg2p is reversible.
Cells were grown overnight in glycerol, then resuspended in medium with 50μg/ml cycloheximide, with (circles) or without (squares) glycerol and allowed to incubate for the indicated times. Hmg2p-GFP levels at each time point were quantified by flow cytometric evaluation of Hmg2p-GFP fluorescence.

degradation of Hmg2p by the addition of the tag, since the assay was intended to ultimately evaluate Hmg2p regulation.

I used prior analysis of over three hundred Hmg2p mutants to guide epitope placement (Gardner and Hampton, 1999a). These studies revealed that though many lumenal regions of Hmg2p are critical for correct regulation, "cold spots" that can be altered without regulatory consequence exist in certain parts of the protein, most notably in the predicted first lumenal loop. I 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, Figure 2-4). Flow cytometry was then used to verify that the resulting lumenally tagged Hmg2p-GFP was normally regulated (Figure 2-5). 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 lumenally protected space. Upon exposure of the microsomes to trypsin, the single band of immunoreactivity progressively underwent cleavage into intermediate bands in a time-dependent manner without loss of overall signal intensity (Figure 2-6, A). In contrast, permeabilization of microsomes with detergent to allow access to the lumenal space resulted in loss of immunoreactivity (Figure 2-6, B). The preserved signal strength during proteolysis without detergent and its loss upon

Figure 2-4
Design of the limited proteolysis construct, mycL-Hmg2p-GFP.
Based on the available mutational map of Hmg2p (as described in Figure 1-3), the epitope tag (in blue) was swapped in to replace a similarly charged sequence stretch in a regulatory "cold spot" in the first lumenal loop (note again that black areas represent portions of the transmembrane domain that cannot be altered without disrupting regulation). The dispensable cytosolic domain was replaced with green fluorescent protein (GFP) to avoid interactions with drugs and pathway intermediates and to facilitate evaluation of proper regulation.

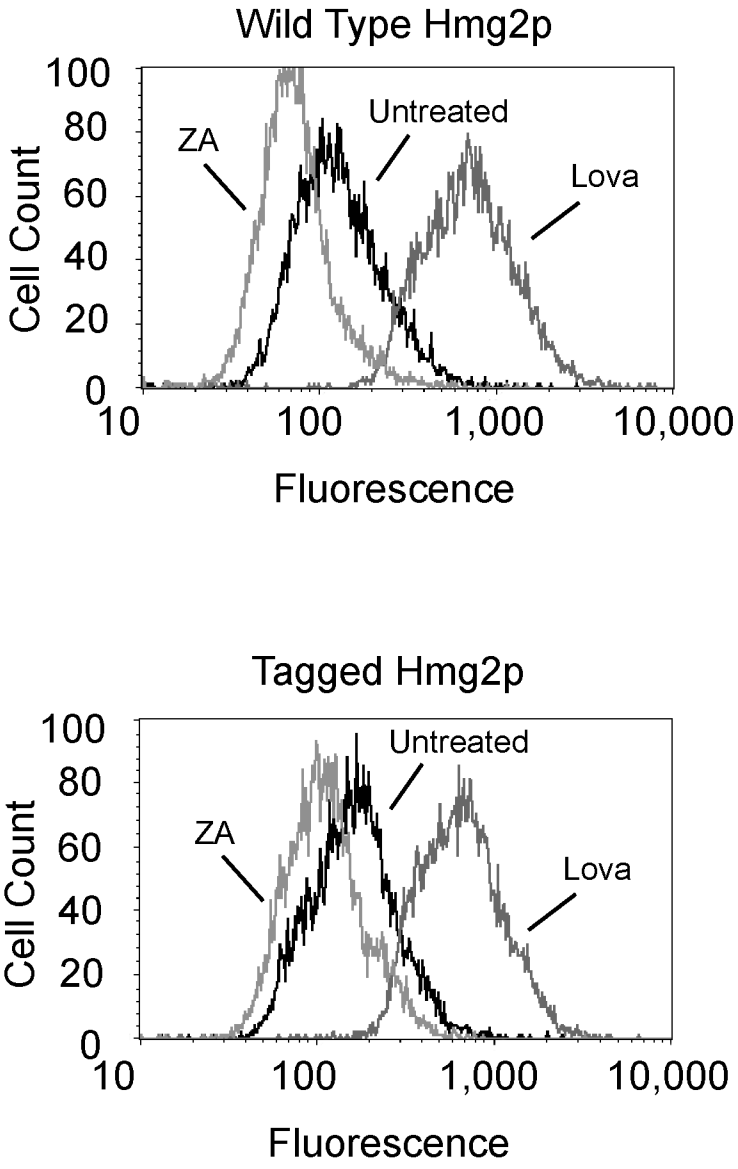

Figure 2-5
The mycL-Hmg2p-GFP epitope-tagged construct is properly regulated
Cells expressing either unmodified Hmg2p-GFP or mycL-Hmg2p-GFP were grown to early log phase and then incubated for four hours with no drug, 25μg/ml lovastatin (Lova) or 10μg/ml zaragozic acid (ZA) as indicated.

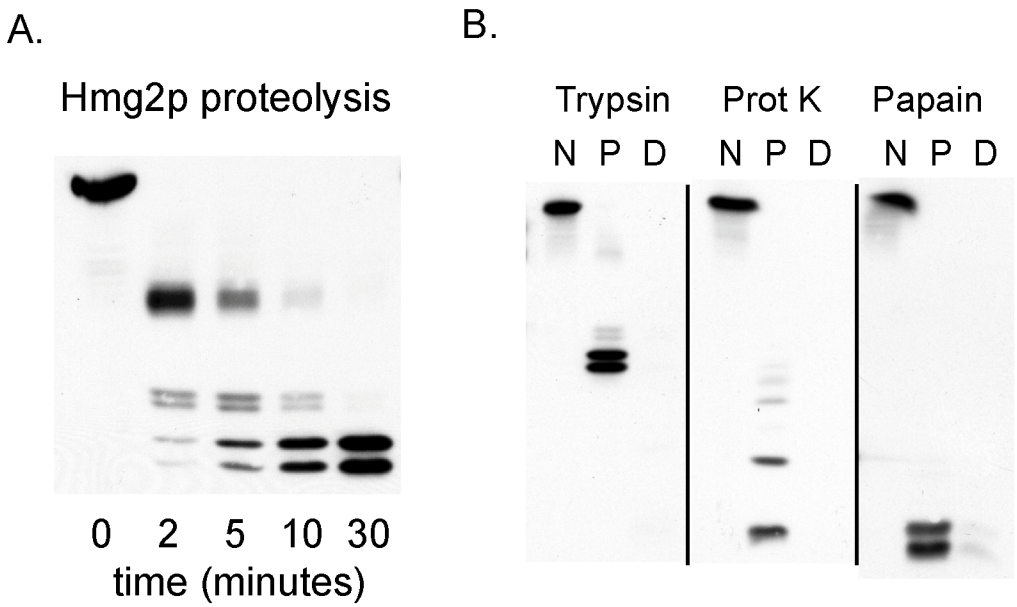

Figure 2-6
The tag is lumenal and yields a characteristic proteolysis pattern.
(A) Limited proteolysis time course of mycL-Hmg2p-GFP. Microsomes bearing mycL-Hmg2p-GFP were prepared and incubated with trypsin (100μg/ml) at 30˚ C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the myc epitope. (B) Effect of detergents on proteolysis. Microsomes containing mycL-Hmg2p-GFP were prepared as described and incubated with trypsin (100μg/ml), proteinase K (10μg/ml) or papain (100μg/ml) for 30 minutes 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.

proteolysis with detergent are consistent with a lumenal location of the first loop bearing the myc tag. Tests with other proteases gave similar results. Trypsin provided the most useful digestion pattern for this assay, due to its greater specificity which resulted in a distinctive cleavage pattern, and so was used for further evaluation of Hmg2p structural features.

Based on the placement of the epitope tag in the first lumenal loop of myc_L-Hmg2p-GFP and the known position of lysines and arginines within putative cytosolic loops, it is possible to make reasonable conclusions about the cutting sites represented in the characteristic trypsin limited proteolysis of this protein. The initial drop in size from ~96 kDa to ~64 kDa represents cleavage within the linker connecting the transmembrane domain and cytosolic GFP (Figure 2-7, site 1). For the pair of doublets, at ~40 kDa and ~36 kDa, each represents a distinct single cut in the third cytosolic loop, at the C- and N-terminal sides respectively (Figure 2-7, sites 2 and 3). The appearance of these cutting products as doublets rather than single bands is most likely due to complementary removal of the cytosolic N-terminus, which is on the other side of the epitope tag (Figure 2-7, site 4). This same "doublet" effect might also be occurring for the ~64 kDa band, but without sufficient resolution at its size and position on the gel to actually discern two bands. Understanding the origin of the characteristic trypsinization pattern of myc_L-Hmg2p-GFP is not only generally useful, it applies directly to understanding the results obtained in a *cod1*Δ background, below.

I first 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

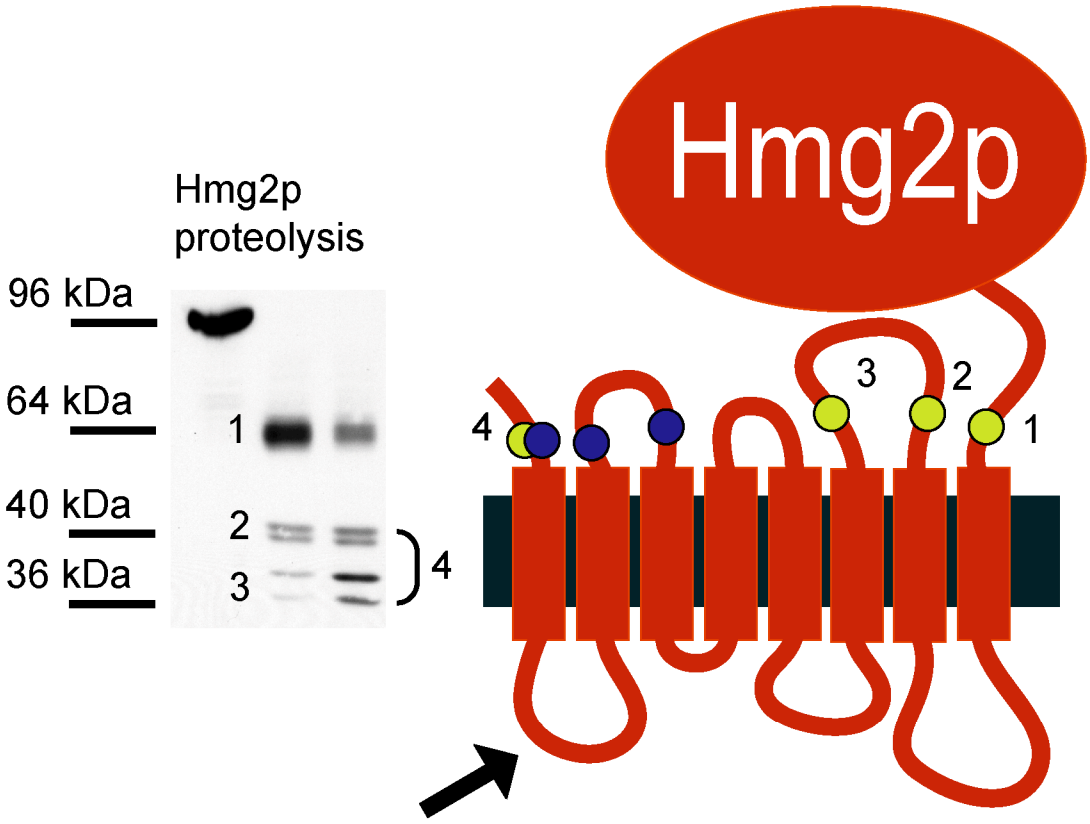

Figure 2-7
Probable trypsin cut sites in mycL-Hmg2p-GFP.
Given the location of the tag in the first lumenal loop (indicated by arrow) and knowledge of the locations of Rs and Ks in Hmg2p sequence, predictions can be made about the cutting sites required to make the observed fragments. Green circles mark probable cut sites in normal Hmg2p, while blue circles mark probable additional cut sites in *cod1*Δ mutant background. Each number marks the site that likely produced the matching band in Hmg2p's characteristic proteolysis profile. Note that sites 2 and 3, although single cuts, are respectively responsible for the doublets at around 40 and 36 kDa. Partial cutting at site 4, N-terminal to the epitope tag, accounts for the existence of these doublets.

are effective *in vivo* (10% and 20% glycerol), glycerol significantly decreased the trypsin accessibility of the lumenally tagged reporter. Specifically, limited proteolysis was slowed considerably, producing the same pattern of bands but at a markedly slower rate (Figure 2-8). This action of glycerol was dose-dependent, with more slowing observed at 20% glycerol than 10%. Extended (3 hour) incubation of 20% glycerol-treated microsomes eventually produced the pattern seen after 10 minutes of trypsinization without glycerol (data not shown). Furthermore, this action of glycerol was rapid: no significant preincubation was required to see its effects. The concentrations of glycerol used had no inhibitory effect on trypsin's intrinsic activity as measured with the chromogenic substrate TAME (Roberts and Burkat, 1966). In fact, glycerol slightly stimulated trypsin activity at the highest concentrations tested (Figure 2-9, C). In addition, as can be seen in every limited proteolysis experiment, the removal of the soluble GFP by trypsin (visible in the loss of the 96 kDa full-sized protein and the appearance of the 64 kDa band) proceeds at the same rate regardless of glycerol concentration, indicating that trypsin was functioning normally in the experiment itself. Thus, Hmg2p's structure was directly and specifically affected by glycerol.

The *in vitro* effect on Hmg2p structure was also caused by similar concentrations of several other chemical chaperones, including sorbitol, mannitol and TMAO (data not shown). Work continued 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.

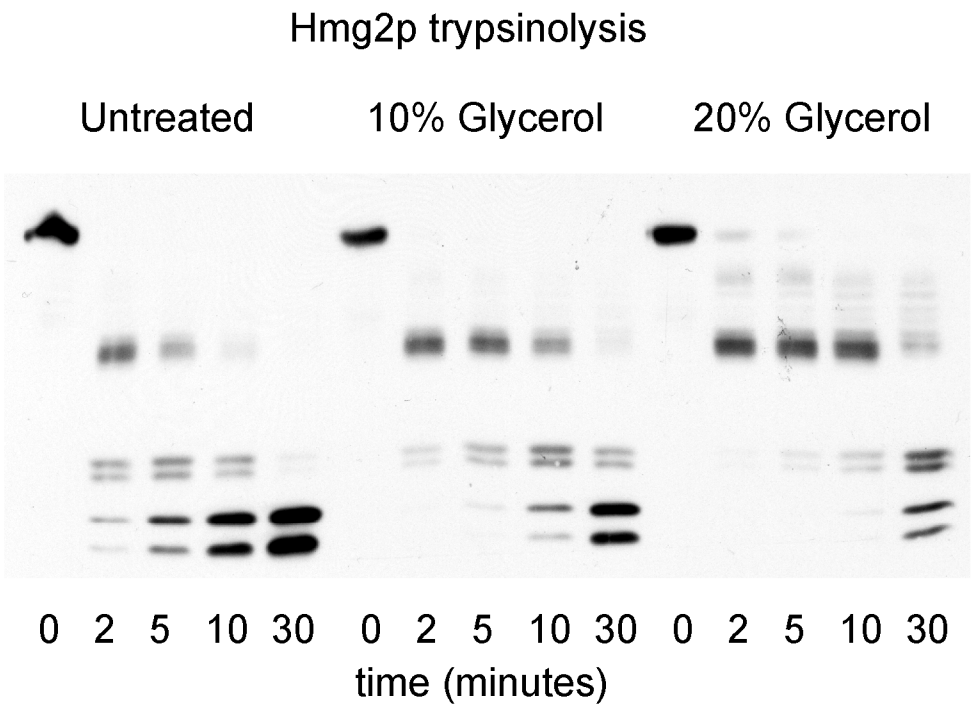

Figure 2-8
Glycerol reduces the proteolytic accessibility of Hmg2p *in vitro*.
Microsomes bearing mycL-Hmg2p-GFP were prepared and resuspended in buffer with no, 10% or 20% glycerol and incubated with trypsin (100μg/ml) at 30° C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the myc epitope.

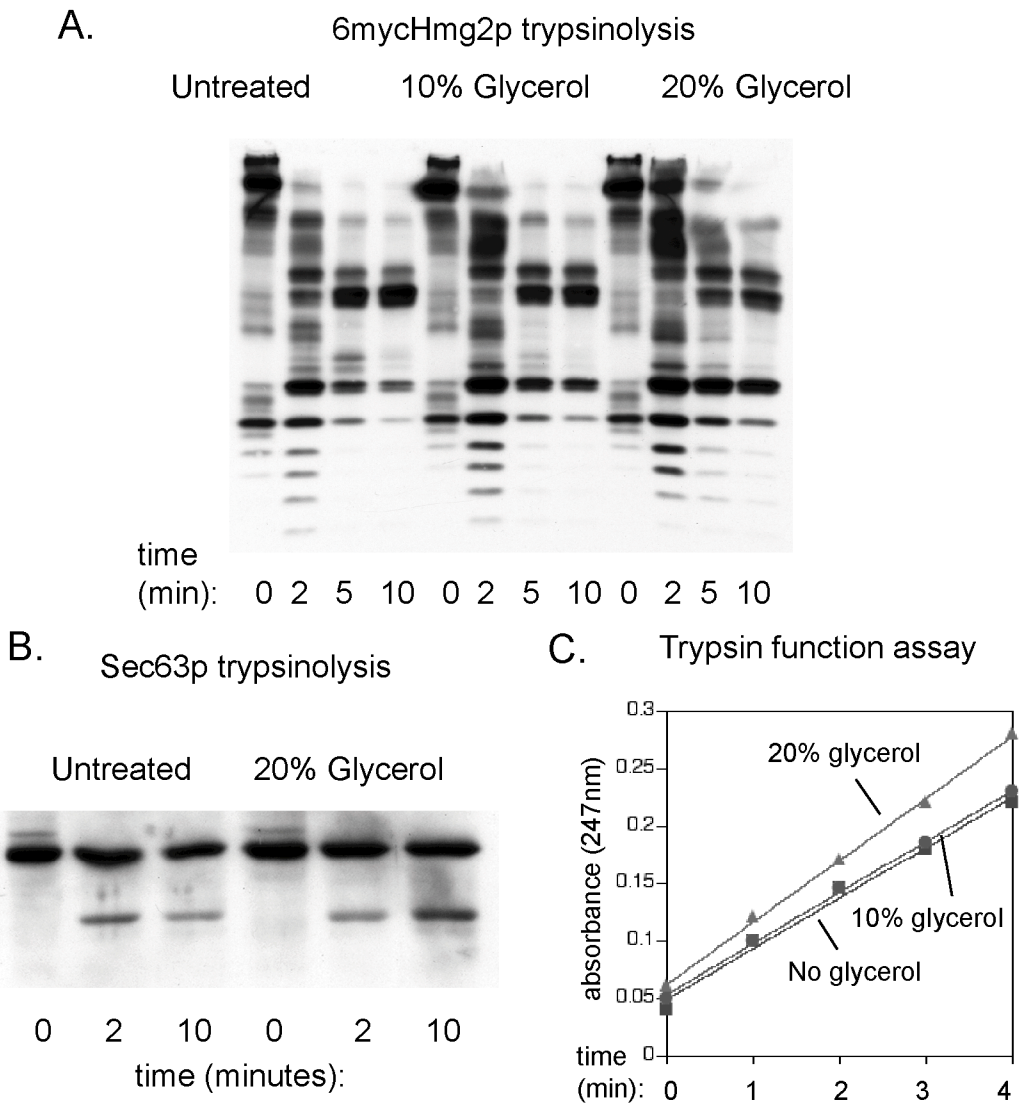

Figure 2-9
Glycerol specifically affects Hmg2p structure.
(A) Glycerol's limited proteolysis of 6myc-Hmg2p. Microsomes bearing 6myc-Hmg2p were prepared and resuspended in buffer with no, 10%, or 20% glycerol and incubated with trypsin (15μg/ml) at 30° C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the myc epitope. (B) Glycerol's effect on proteolysis of the endogenous membrane-spanning ER protein Sec63p. Samples prepared as in Figure 2-8 were evaluated by 14% SDS-PAGE and immunoblotting of the protected lumenal dnaJ epitope of Sec63p. (C) Chromogenic assay of trypsin function. A solution of trypsin (525ng/ml) in reaction buffer with 1mM TAME and with no (squares), 10% (circles) or 20% (triangles) glycerol was incubated and enzymatic product absorbance at 247nm measured at the indicated times.

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 (Figure 2-9, A). Similarly, immunoblotting of the dnaJ epitope of the endogenous ER membrane protein Sec63p, which occupies a lumenal loop (Feldheim et al., 1992), also showed trypsin digestion with a pattern and rate unchanged by the presence of glycerol (Figure 2-9, B). These controls indicate 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 (Gardner et al., 2001). The *in vitro* effect of glycerol was similarly rapid, occurring without preincubation, suggesting it might also be reversible, as *in vivo*. Microsomes with myc_L-Hmg2p-GFP were incubated in 20% glycerol for half an hour, far more time than was necessary 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 (Figure 2-10). 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 mutant was stabilized by glycerol, but was not responsive to changes in the FPP-derived signal. Based on this, I expected that in microsomes derived from *cod1*Δ cells, gycerol's 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 predicted, the limited proteolysis of myc_L-Hmg2p-GFP from *cod1*Δ microsomes showed slowed appearance of several cleavage products upon glycerol treatment (Figure 2-11). Importantly, trypsinolysis revealed that the structure of Hmg2p-GFP derived from the *cod1*Δ cells was clearly different despite its continued ability to respond to glycerol *in vivo* and *in vitro* (see Figure 2-7; the probable additional cut sites are indicated with blue circles). 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 (Gardner and Hampton, 1999a), it is likely that the altered structure of Hmg2p in the *cod1*Δ background underlies its unresponsiveness to the FPP-derived signal.

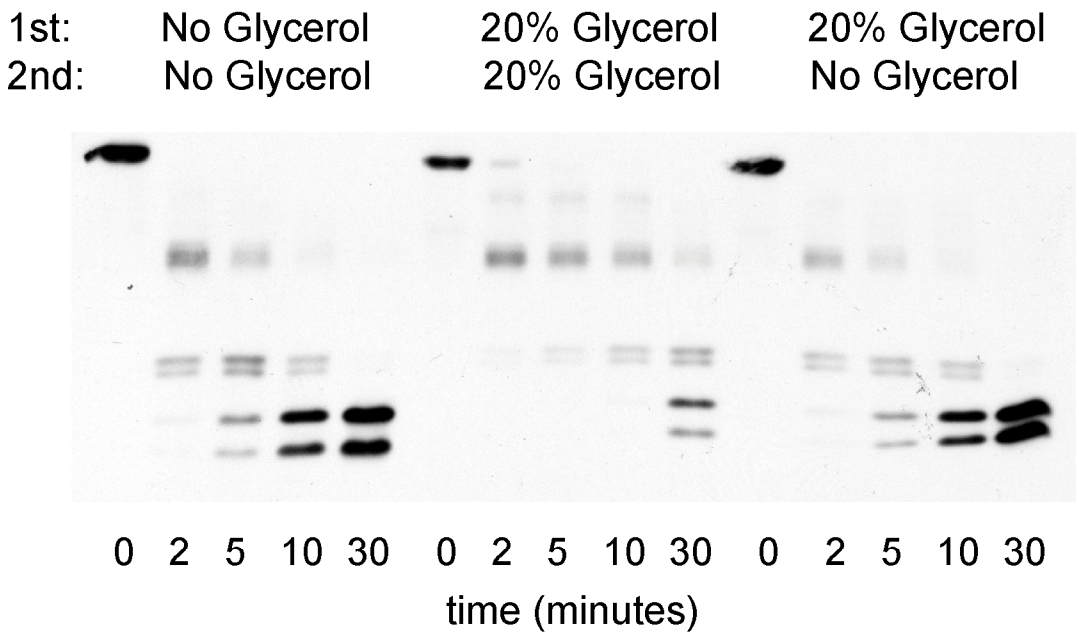

Figure 2-10
Glycerol's *in vitro* effect is reversible.
Microsomes bearing mycL-Hmg2p-GFP were prepared and resuspended in buffer with no or 20% glycerol (1st), incubated for 30 minutes at 30˚ C, then spun down and resuspended in no or 20% glycerol as indicated (2nd), then immediately incubated with trypsin (100μg/ml) at 30˚ C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the myc epitope.

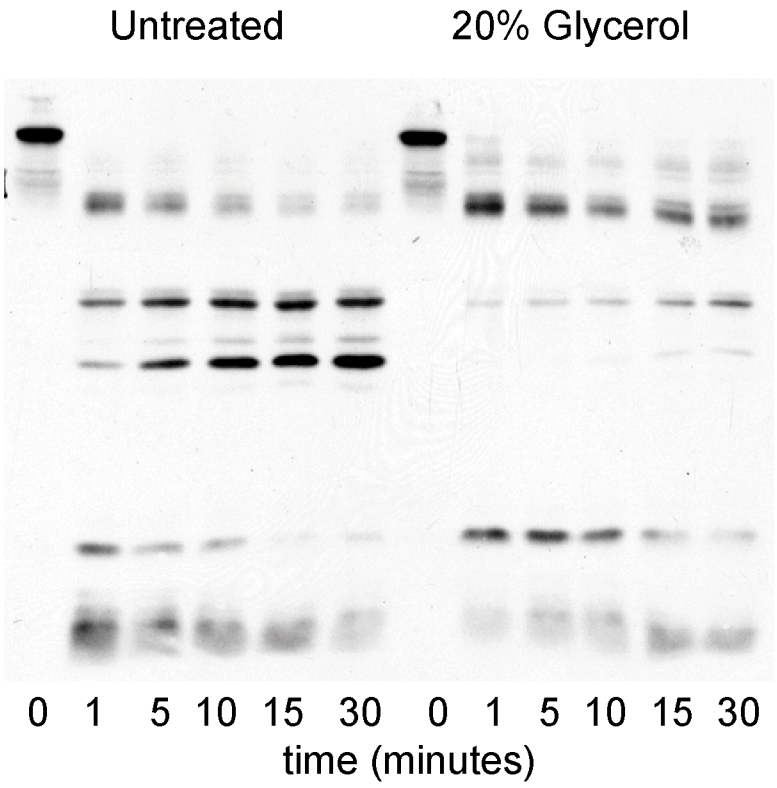

Figure 2-11
Hmg2p is structurally altered yet still sensitive to glycerol in a *cod1*Δ strain.
Microsomes from a *cod1*Δ strain were prepared as described, then resuspended in buffer with no or 20% glycerol and incubated with trypsin (100μg/ml) at 30˚ C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the myc epitope.

The *in vitro* results indicated that glycerol caused a "tightening" of the Hmg2p transmembrane domain that altered the rate of limited proteolysis by trypsin *in vitro* and Hmg2p's degradation rate *in vivo*. To determine if the same structural transition underlies the physiological regulation of Hmg2p by the FPP-derived signal, I carried out 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 a *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 Figure 2-12. Elevating FPP in intact, living cells clearly caused a detectable increase in rate in the subsequently measured *in vitro* trypsinolysis. 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*.

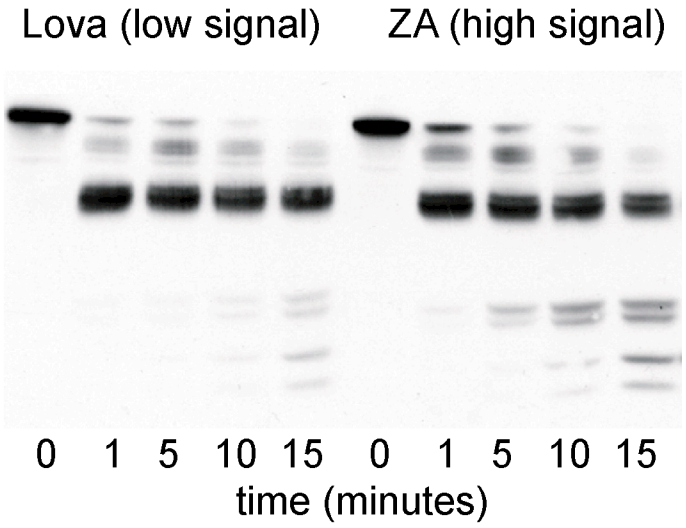

Figure 2-12
Hmg2p accessibility changes in response to *in vivo* degradation signal.
Strains expressing mycL-Hmg2p-GFP in a *hrd1*Δ background were incubated for 16 hours in YPD with 100μg/ml lovastatin (Lova) to reduced FPP-derived degradation signal or 40μg/ml zaragozic acid (ZA) to build up degradation signal. Microsomes were prepared from each culture as described and subjected to limited proteolysis assay as described previously.

Discussion

Prior work on Hmg2p indicated that it undergoes a unique mode of regulation: controlled access to a constitutively active quality control pathway, driven by a physiological degradation signal (Hampton, 2002a; Hampton, 2002b). 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, Hmg2p is stabilized *in vivo* by the chemical chaperone glycerol (Gardner et al., 2001), which is known to stabilize other quality control substrates (Brown et al., 1996; Sato et al., 1996; Welch and Brown, 1996; Zhou et al., 1999). This chapter describes an evaluation of the effects of chemical chaperones on Hmg2p, with a particular interest in their use in understanding the structural features of Hmg2p regulation. These agents caused a direct, reversible effect on Hmg2p structure, and 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 also has these features, I tested whether glycerol stabilization worked through altering the action of the FPP degradation signal. It did not, as glycerol stabilizated Hmg2p in *cod1*Δ cells, where the FPP signal fails to function (Cronin et al., 2000). Importantly, 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 (Gardner and Hampton, 1999a).

These *in vivo* studies indicated that glycerol and other chemical chaperones have a rapid, reversible action on Hmg2p structure. I directly examined this idea by developing a limited proteolysis assay for Hmg2p with a myc epitope tag in the protected first lumenal loop. The new limited proteolysis assay addressed problems inherent in a previously published assay which used untagged Hmg2p and a mixture of polyclonal antibodies that recognized mostly cytosolic epitopes (Gardner et al., 2001). This previous 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 obviates those technical issues and has allowed study of the structural responses described above and the *in vitro* response to signal molecules described in the following chapter.

As posited from the 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* (Brown et al., 1996; Sato et al., 1996), and *in vitro* structural studies of highly purified proteins (Courtenay et al., 2000). 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 (Brown et al., 1996; Sato et al., 1996). The proposed and reasonable model for this effect is glycerol-induced enhancement of the mutant protein's folding. However, the effects of chemical chaperones on CFTR have not been observable *in vitro*[1], 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 (Sato et al., 1996). In contrast, the effect of glycerol on Hmg2p in these studies 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's susceptibility to trypsin. This study appears to be 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 seems to be naturally capable of undergoing structural changes.

[1] Dr. William Skatch, OHSU, personal communication.

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 (Hampton, 2002a; Hampton, 2002b). The observed sensitivity of Hmg2p's regulated degradation to glycerol or other chemical chaperones is consistent with the degraded state having features of a misfolded protein. The particular change 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 sites remain the same, and that the relative accessibility of each site compared to 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. Hmg2p in microsomes from a high-signal environment, where degradation is rapid, was more trypsin-accessible than Hmg2p from a low-signal environment. My 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 (see chapter 3).

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 (Gerhart and Schachman, 1968). Furthermore, there are many proteins that have structures that can vary between more and less folded states. It appears that Hmg2p combines these traits, 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.

Materials and methods

Materials

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 from cells (CRL 1729, American Type Culture Collection) grown in RPMI1640 culture medium (GIBCO BRL) with 10% fetal calf serum and supplements. Anti-HA antibodies were purchased as ascites fluid from Covance. HRP-conjugated goat anti-mouse antibodies were purchased from Jackson Immuno Research. HRP-conjugated goat anti-rabbit antibodies were purchased from Zymed. Chemiluminscence immunodetection reagents were obtained from Perkin Elmer. 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 lumenal loop (TYLSIKPDEL) from a TDH3 promoter (myc_L-Hmg2p-GFP). pRH1581 was constructed using the PCR-mediated overlap extension method as described previously (Gardner et al., 1998),with pRH469 (Hmg2p-GFP behind a TDH3

promoter for constitutive expression) (Gardner et al., 1998), as template (primers are listed in Table 2-3). 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 were grown in minimal media (Difco Yeast Nitrogen Base) with glucose and the appropriate supplements as described previously, unless otherwise noted (Hampton and Rine, 1994). 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 was made by targeted integration of the plasmid pRH1581 at the StuI site of the *ura3-52* locus in RHY519 (Gardner et al., 1998). RHY2803 was derived from RHY2723 by transformation with the *hrd1*Δ*::*KanMX deletion cassette excised from pRH1122 (Gardner and Hampton, 1999b), then selection on YPD with G418.

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 (Cronin et al., 2000). Briefly, cells were grown to early log phase in minimal medium, then incubated with drugs or chemical chaperones 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 2ml 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 50µg/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 (Gardner et al., 1998). Briefly, cells were grown to early log phase then spun down, medium removed bv aspiration and replaced with new medium with cycloheximide (50μg/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 immunoblotting 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 lysis buffer (0.5 ml XL, 2 ml H_2O, 260 nM AEBSF, 100 nM TPCK) per 100 ODs, aliquoted into one 2ml microfuge tube per 10 ODs, mixed with an equal volume 0.5mm acid-washed glass beads and chilled 2 minutes on ice. Cells were lysed by vortexing 6x1 minute, with 1 minute on ice between each vortexing. Debris was pelleted by five second pulses at 16,000xg in 1.5ml microfuge tubes until no pellet was formed (typically three pulses required). From the resulting clarified supernatant, microsomes were pelleted by 30 minutes at 21,000xg at 4˚ C. The supernatant was aspirated and the pellet overlaid twice with fresh XL buffer, with

a 5 minute centrifugation following each wash to ensure pellet cohesion. The resulting microsomal pellet was then resuspended in XL buffer with or without glycerol as described in 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 (20mM Tris, pH 7.5, 500µg/mL BSA, 10mM *b*-mercaptoethanol), followed by a 5 minute spin at 16,000xg to pellet any debris. Supernatant was diluted in additional SED to the desired 20x trypsin stock concentration, then added to the final concentration described. Samples were incubated at 30˚ C, with 30µl aliquots removed at the time points indicated, and the reaction halted by addition of 30µl 2x USB (75mM MOPS, pH 6.8; 4% SDS; 200mM DTT; 0.2mg/ml bromophenol blue; 8M urea) followed by solubilization for ten minutes at 55˚ C.

Samples were resolved on 14% SDS-PAGE, then transferred with 15% methanol to nitrocellulose and immunoblotted for myc epitope as described (Hampton and Rine, 1994).

TAME chromogenic protease activity assay

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

Table 2-1
Yeast strains used in this chapter.

RHY 522	*MATa ade2-101 met2 lys2-801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3-52::URA3::HMG2cd::HMG2-GFP*
RHY2723	*MATa ade2-101 met2 lys2-801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3-52::URA3::HMG2cd::myc$_L$-HMG2-GFP*
RHY2803	*MATa ade2-101 met2 lys2-801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3-52::URA3::HMG2cd::myc$_L$-HMG2-GFP hrd1Δ::KanMX*
RHY2843	*MATa ade2-101 met2 lys2-801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3-52::URA3::HMG2cd::OLE1-3myc*
RHY2853	*MATalpha ade2-101 met2 lys2-801 his3200 ura3-52::URA3::myc$_L$-HMG2-GFP cod1Δ::KanMX*

Table 2-2
Plasmids used in this chapter.

pRH1122	Contains *hrd1*Δ::KanMX deletion cassette
pRH1581	Expresses lumenally myc-tagged *HMG2-GFP* from a TDH3 promoter

Table 2-3
PCR primers used in this chapter.

oRH1412	3’ primer for final PCR of myc tag into *HMG2* (CTCAATCTCCATTTCGTTCCG)
oRH1456	3’ primer for first reaction inserting myc tag into *HMG2* (CAGGTCTTCCTCCGATATCAACTTCTGCTCAGAATACTGATTAGAGTCCAGCTTCC)
oRH1457	5’ primer for second reaction inserting myc tag into *HMG2* (GAGCAGAAGTTGATATCGGAGGAAGACCTGTTTGAAAAATGCACACACTACTATAGGTCT)
oRH1458	3’ primer for second reaction inserting myc tag into *HMG2* (GCATGCAGAGTTTGAAAGAGCAGA)
oRH1468	5’ primer for first reaction inserting myc tag into *HMG2* and for final PCR of myc tag into *HMG2* (CACCAACCATCAGTTCATAGGTCC)

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Chapter 3

Lipid-mediated, reversible misfolding of a sterol-sensing domain (SSD) protein

Abstract

Cellular quality control requires recognition of common features of misfolding, and so is not typically associated with the specific targeting of individual proteins. However, physiologically regulated degradation of yeast HMG-CoA reductase (Hmg2p) occurs by the HRD ER quality control pathway, implying that Hmg2p undergoes a regulated transition to a quality control substrate in response to a sterol pathway molecule. Using *in vitro* structural assays, I show that the pathway derivative farnesol directly causes Hmg2p to undergo a change to a less folded structure. The effect is reversible, biologically relevant by numerous criteria, highly specific for farnesol structure, and requires an intact Hmg2p sterol sensing domain (SSD). This represents a distinct lipid-sensing function for this highly conserved motif that suggests novel approaches to cholesterol management. More generally, the observation of reversible small molecule-mediated misfolding may herald numerous examples of regulated quality control to be discovered in biology or applied in the clinic.

Introduction

Protein degradation fills two distinct roles in the cell: regulation and quality control. In regulation, a normal, functional protein is specifically recognized by degradation machinery to control a particular cellular process. In contrast, quality control requires recognition of structural hallmarks that distinguish the large set of abnormal proteins from their properly folded and assembled counterparts, independent of specific protein sequence and identity. Consequently, quality control and regulation are traditionally seen as unrelated processes. However, it is entirely reasonable that the high specificity of quality control could be harnessed by the cell for protein regulation. The sterol pathway enzyme HMG-COA Reductase (HMGR) is a notable first example of this interface of regulation and quality control (Hampton, 2002a; Hampton, 2002b), and the studies described in this chapter show how a reversible, small molecule-mediated transition to a quality control substrate can be employed in the selective control of protein levels.

HMGR catalyzes a key step in sterol synthesis 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 a specific signal from the sterol pathway (Hampton, 2002b; Ravid et al., 2000; Sever et al., 2003). Increases in the signal lead to increased degradation and a drop in enzyme steady-state level. Conversely, decreased signal leads to decreased degradation and a

rise in steady-state level. This degradation leads to rapid control of Hmg2p level in response to changing demand for sterol pathway products.

In the genetic analysis of yeast Hmg2p regulated degradation, Hrd1p was identified as the E3 ubiquitin ligase that mediates ubiquitination of Hmg2p (Bays et al., 2001; Gardner et al., 2000; Hampton and Bhakta, 1997; Hampton et al., 1996). Surprisingly, Hrd1p is a key quality control E3 of the ER, required for degradation of numerous misfolded proteins, such as soluble CPY* and membrane-bound Pdr5p*, and for maintaining an acceptable level of normally-produced misfolded proteins in the ER (Bays et al., 2001; Bordallo et al., 1998; Friedlander et al., 2000; Wilhovsky et al., 2000). Thus, the degradation of Hmg2p is an intersection between regulation and quality control.

Hmg2p catalyzes the synthesis of mevalonic acid, an early molecule in the sterol pathway. The signal for Hmg2p degradation is derived from the later pathway intermediate farnesyl pyrophosphate (FPP). When cellular FPP is abundant, the Hmg2p degradation rate is high; when the FPP level is low, the Hmg2p degradation rate is low. Mature Hmg2p is subject to this stability control and can undergo changes in degradative status rapidly and reversibly following changes in the FPP-derived signal (Gardner and Hampton, 1999b; Hampton and Bhakta, 1997). From *in vivo* studies it was proposed that Hmg2p acquires structural features of a quality control substrate in response to the FPP-derived signal (Gardner et al., 2001b; Hampton, 2002a; Hampton, 2002b). This regulated structural transition would occur in mature protein and be reversed rapidly when signal decreases. By this model, Hmg2p's status

as a quality control substrate is regulated, so that when the FPP-derived signal is abundant Hmg2p acquires traits of a misfolded protein that allow Hrd1p-dependent degradation.

In vivo evidence for this structural transition model was obtained through experiments with chemical chaperones as described in the previous chapter. These small molecules have been used to enhance protein folding *in vivo* and *in vitro* (Brown et al., 1996; Sato et al., 1996; Welch and Brown, 1996). Treatment of yeast with chemical chaperones such as glycerol strongly stabilizes Hmg2p, but has no effect on constitutively degraded ER degradation substrates (Gardner et al., 2001b; Shearer and Hampton, 2004). Hmg2p stabilization by chemical chaperones is rapidly reversible, implying that Hmg2p can undergo reversible changes in structure. Those studies employed a limited proteolysis assay to evaluate the structure of membrane-localized Hmg2p and its response to chemical chaperones (Shearer and Hampton, 2004). As predicted from the *in vivo* stabilization, the presence of chemical chaperones dramatically reduces *in vitro* Hmg2p proteolysis, and this effect on Hmg2p structure is completely and rapidly reversible. Furthermore, altering the *in vivo* FPP-derived degradation signal with drugs has the expected effects on subsequently measured Hmg2p *in vitro* protease sensitivity: Hmg2p protease accessibility is higher in membranes prepared from cells with elevated FPP, and lower in membranes prepared from cells with diminished FPP. Thus, it seems that the *in vivo* degradation signal causes Hmg2p to be better recognized as a Hrd1p substrate.

The Hmg2p N-terminal transmembrane domain is both necessary and sufficient for proper regulated degradation (Gardner and Hampton, 1999a; Hampton and Rine, 1994). The simplest model for Hmg2p regulation is that an FPP-derived signal directly causes the Hmg2p transmembrane domain to adopt structural features of a quality control substrate, thus rendering the protein susceptible to Hrd1p-dependent degradation. This chapter demonstrates the validity of this structural transition model, and shows that the FPP-derived molecule farnesol causes specific, reversible misfolding of Hmg2p in isolated ER microsomes. This *in vitro* conformational change is the same as that observed *in vivo*, biologically relevant by numerous criteria, and requires specific structural features in the signaling lipid. Thus, Hmg2p regulation provides an example of small molecule control of protein structure that allows programmed entry into a constitutive quality control pathway.

Results

In the Chapter 2, I described the development of an *in vitro* limited proteolysis assay to study Hmg2p structure in isolated ER microsomes (Shearer and Hampton, 2004). This assay was used in the studies below, revealing that a biological signal derived from the sterol pathway directly and reversibly alters the folding state of Hmg2p. The assay was developed by constructing myc_L-Hmg2p-GFP, a version of Hmg2p with the catalytic domain replaced with GFP, and a myc tag added to the first lumenal loop as described in the previous chapter (Shearer and Hampton, 2004). The resulting non-catalytic protein, hereafter in this chapter referred to as Hmg2p-GFP, is regulated normally *in vivo*. The use of GFP allows quantitative evaluation of protein levels by fluorescent techniques (Cronin and Hampton, 1999), and restricts the observed effects to the membrane domain, which is necessary and sufficient for FPP-mediated regulation of the entire Hmg2p molecule. Trypsin treatment of this protein in microsomes generates a characteristic time-dependent proteolytic pattern that is sensitive to chemical chaperones and alterations of the *in vivo* degradation signal. Specifically, these treatments cause changes in the rate of *in vitro* trypsinolysis without perturbing the pattern or the final products (Shearer and Hampton, 2004). For example, lowering *in vivo* FPP levels causes slower *in vitro* trypsinolysis of Hmg2p, whereas increasing *in vivo* FPP levels causes hastened *in vitro* trypsinolysis (Shearer and Hampton, 2004). I have now used this assay to investigate the nature and action of the degradation signal.

A central question in Hmg2p regulation is whether the FPP-derived signal works directly on the protein to effect structural change, or whether this occurs through another molecule or mechanism. I addressed this question by testing the effect of this pathway intermediate in the *in vitro* limited proteolysis assay (Figure 3-1, A). FPP induced only a small increase in trypsinolysis rate (1-2 fold), so I moved on to the FPP-derived molecule farnesol.

When FPP is made more abundant in yeast by pharmacological or genetic means, the majority of the FPP is rapidly converted to the 15-carbon neutral lipid farnesol, making it a candidate for the *in vivo* degradation signal (Chambon et al., 1990; Gardner et al., 2001a). Indeed, treatment of ER microsomes with farnesol caused a dose-dependent increase in trypsinolysis rate of the lumenally-tagged reporter that was maximal at 200μM (Figure 3-1, B). Note that this effect involves a change in rate but not proteolytic pattern. Longer incubation of untreated microsomes with trypsin results in the same characteristic quartet of bands seen after a much shorter incubation with farnesol (Shearer and Hampton, 2004). Farnesol's effect on the lumenal reporter was saturable, reaching maximal effect near 200μM, well below concentrations that alter membrane integrity – around 1mM (data not shown). This effect of farnesol was rapid, occurring with no need for preincubation (data not shown).

I next evaluated the structural specificity of this effect. Farnesol is a 15-carbon hydrophobic alcohol that is potentially capable of affecting membrane dynamics independent of its own specific molecular structure. Thus, I examined the effects of

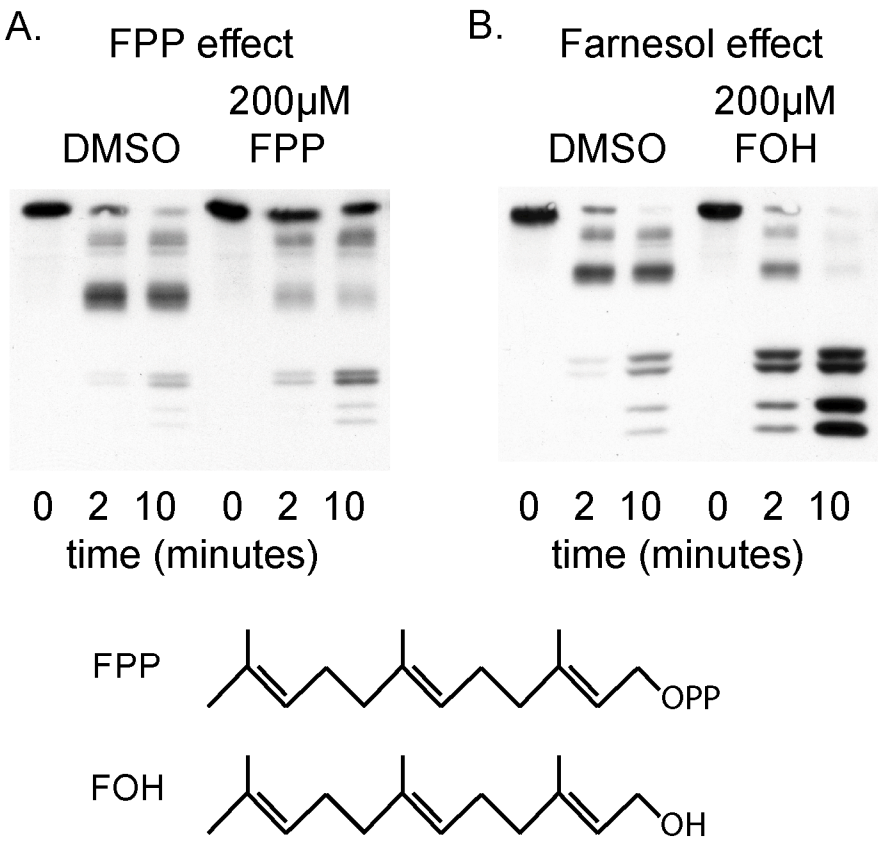

Figure 3-1
Farnesol but not FPP increases Hmg2p-GFP proteolysis rate.
(A) Effect of FPP. Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO or 200μM FPP, then incubated with trypsin (15 μg/ml) at 30° C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the lumenal Myc epitope. (B) Effect of farnesol (FOH). Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO or 200μM FOH, then incubated with trypsin and evaluated as described above.

several closely related lipids using the *in vitro* assay (see Figure 3-2 for structures). In striking contrast to farnesol, the linear 14- and 16-carbon alcohols tetradecanol and hexadecanol had no effect on Hmg2p trypsinolysis (Figure 3-3), indicating that farnesol's effect could not be duplicated by simple lipids with similar properties.

I then tested several more structurally and biologically related compounds. 10-carbon geraniol had no effect on the trypsinolysis rate of Hmg2p, whereas 20-carbon geranylgeraniol caused approximately a two-fold increase (Figure 3-4). Nerolidol, an isomer of farnesol, was as effective as farnesol in causing Hmg2p structural transition (Figure 3-5). Fully-hydrogenated farnesol, 3,7,11-trimethyldodecan-1-ol (TMD), had no effect, demonstrating that farnesol's unique isoprene structure was necessary for promotion of the Hmg2p structural transition (Figure 3-6). Thus, the action of farnesol on Hmg2p-GFP structure was highly specific for a variety of molecular features of this molecule.

Unlike Hmg2p, mammalian HMGR stability is regulated by sterols (Chin et al., 1985; Sever et al., 2003). Accordingly, I also tested the yeast sterols ergosterol and lanosterol in the limited proteolysis assay. As predicted from *in vivo* regulation of Hmg2p stability, neither sterol had any effect on Hmg2p-GFP conformation (Figure 3-6), even when tested at concentrations forty-fold above sterol concentrations that affect mammalian SCAP (Brown et al., 2002).

The *in vivo* regulation of Hmg2p by the FPP-derived signal requires an intact *COD1* gene and correct Hmg2p sequence. As a test of biological relevance, I

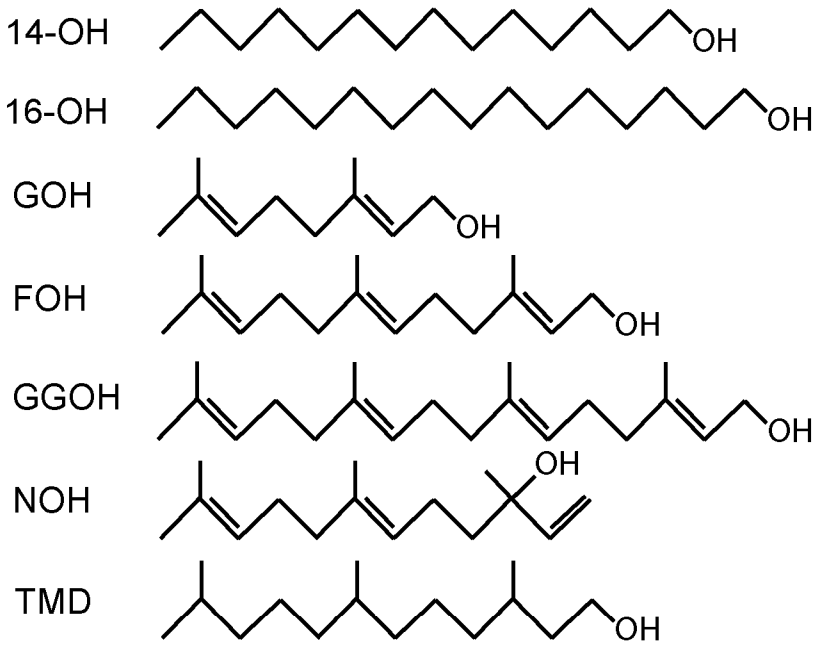

Figure 3-2
Structures of componds tested in the *in vitro* assay.
In addition to FPP, as shown in figure 3-2, the molecules above were all tested in the *in vitro* assay. Included are the acyl alcohols tetradecanol (14-OH) and hexadecanol (16-OH), the isoprene pathway derivatives geraniol (GOH), farnesol (FOH) and geranylgeraniol (GGOH), the farnesol isomer nerolidol (NOH) and fully saturated farnesol (TMD).

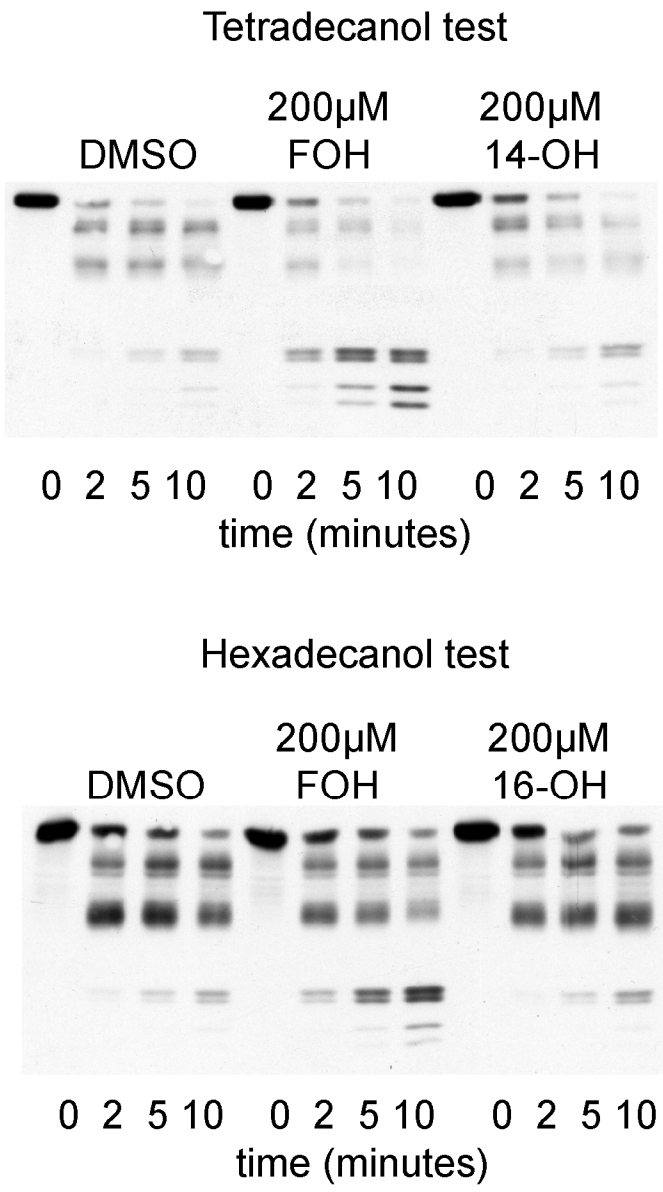

Figure 3-3
Lipid specificity I: similarly sized acyl alcohols have no effect on Hmg2p.
Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO, 200μM FOH, 200μM 14-OH or 200μM 16-OH, then incubated with trypsin (15 μg/ml) at 30˚ C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope.

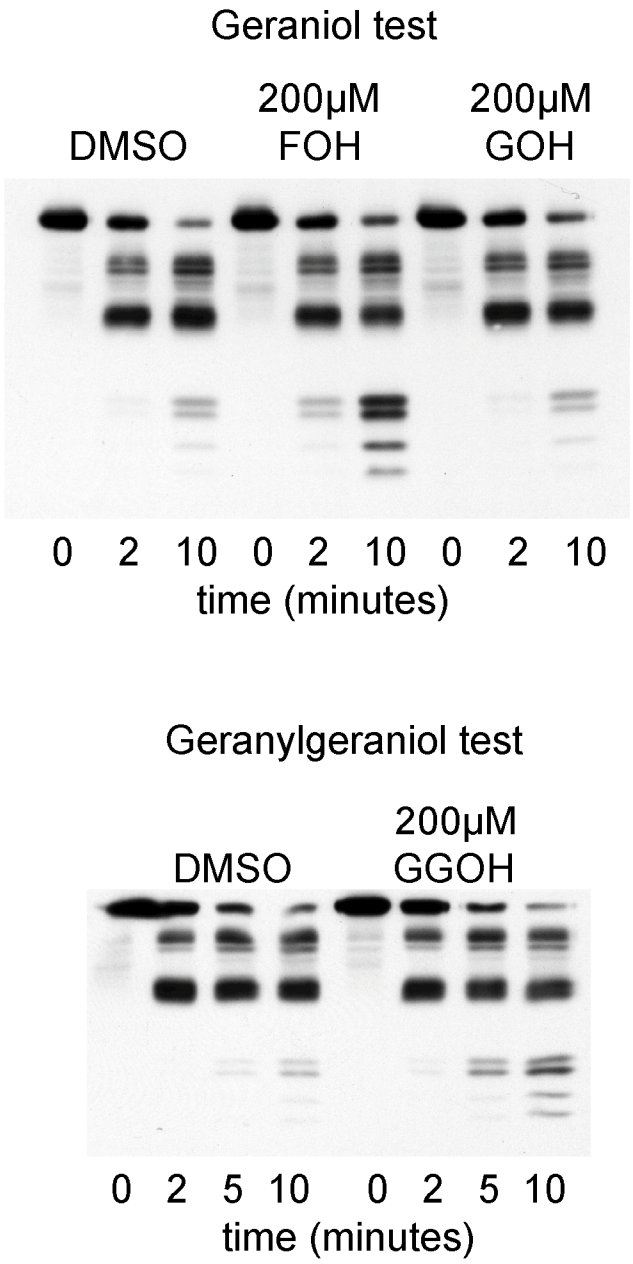

Figure 3-4
Lipid specificity II: other isoprenes have limited or no effect on Hmg2p.
Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO, 200μM FOH, 200μM GOH or 200μM GGOH, then incubated with trypsin (15 μg/ml) at 30˚ C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope.

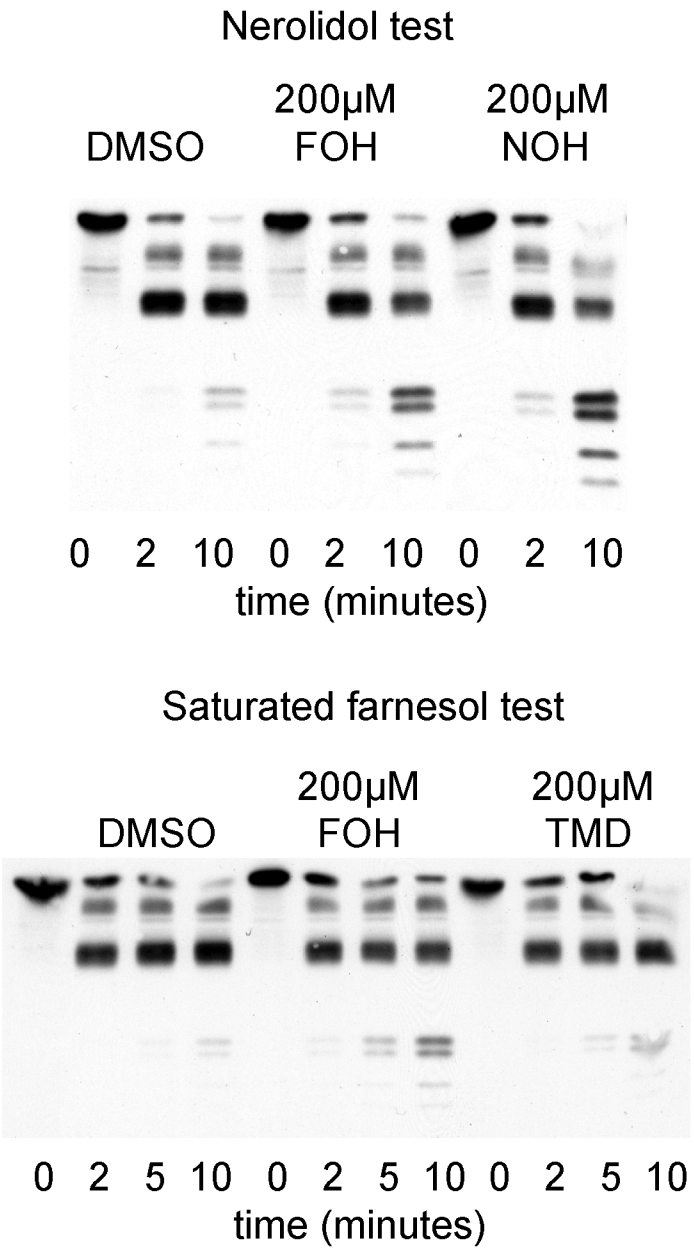

Figure 3-5
Lipid specificity III: an isomer of FOH is effective, saturated FOH is not.
Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO, 200μM FOH, 200μM NOH or 200μM TMD, then incubated with trypsin (15 μg/ml) at 30° C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope.

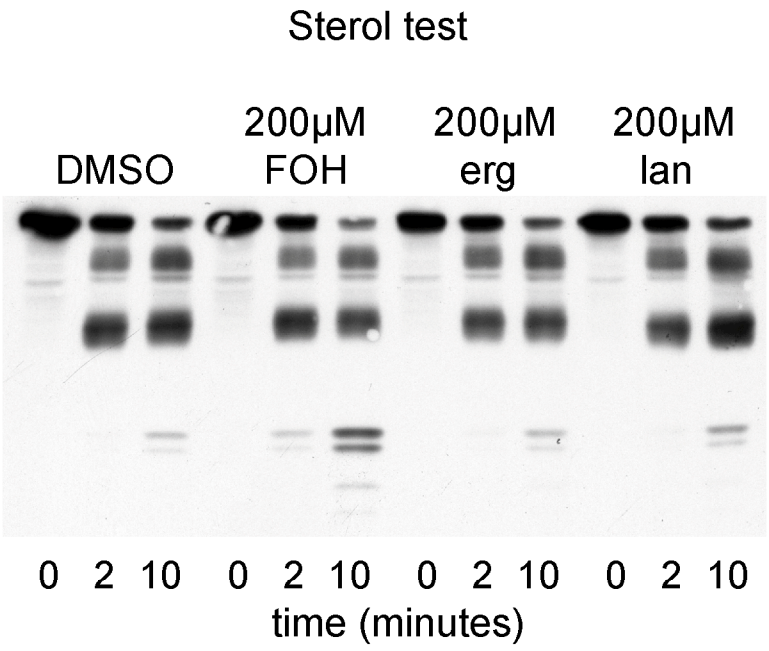

Figure 3-6
Lipid specificity IV: sterols do not induce conformational change in Hmg2p. Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO, 200μM FOH, 200μM ergosterol (erg) or 200μM lanosterol (Lan) then incubated with trypsin (15 μg/ml) at 30° C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope.

evaluated the *in vitro* effect of farnesol on Hmg2p in these two circumstances where regulation is absent.

Cod1p is a P-type ATPase with unknown specificity that was discovered in a screen for genes required for regulation of Hmg2p (Cronin et al., 2000; Cronin et al., 2002). In a *cod1Δ* mutant, Hmg2p undergoes constitutive HRD-dependent degradation that is largely unresponsive to alterations in FPP level. As described in the previous chapter, in the *cod1Δ* genetic background, Hmg2p has structural alterations that probably underlie its unresponsiveness to the degradation signal (Shearer and Hampton, 2004). As predicted from the *in vivo* lack of regulation, farnesol had minimal effect on *in vitro* proteolysis of Hmg2p in microsomes from a *cod1Δ* strain (Figure 3-7). Note also the altered proteolytic pattern of Hmg2p, which is characteristic of the perturbation of Hmg2p structure caused by the *cod1Δ* mutation.

Evaluation of over 350 engineered mutants of Hmg2p revealed that regulated degradation requires numerous sequence elements in the transmembrane domain (Gardner and Hampton, 1999a). To further test the biological relevance of the *in vitro* effect of farnesol I employed an Hmg2p variant that is degraded in a Hrd1-dependent manner, yet is totally unresponsive to the *in vivo* FPP-derived degradation signal. In this variant, residues 348-352 at the end of the sixth transmembrane span are altered from TFYSA to ILQAS. A mutant version of the lumenally-tagged Hmg2p-GFP that incorporated this change, termed NR1-GFP, was expressed in yeast to evaluate the effects of this mutation on the *in vitro* assay. I first confirmed that NR1-GFP was degraded constitutively by the HRD pathway *in vivo*, using flow cytometery as in

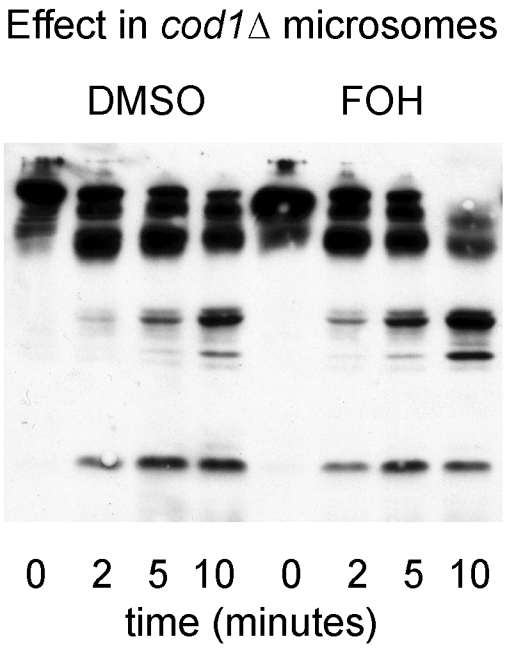

Figure 3-7
Hmg2p derived from a *cod1*Δ background is unresponsive to farnesol.
Microsomes from a *cod1*Δ strain expressing Hmg2p-GFP were resuspended in buffer with DMSO or 200μM FOH, then incubated with trypsin (15 μg/ml) at 30° C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and myc immunoblotting.

many previous studies (Cronin et al., 2000; Gardner and Hampton, 1999a). Addition of cycloheximide to halt protein synthesis caused the expected time-dependent loss of the fluorescent protein (Figure 3-8, "CHX"), which was entirely dependent on the HRD pathway (data not shown). We next confirmed that degradation of NR1-GFP was unregulated. Cells expressing NR1-GFP or Hmg2p-GFP were treated with drugs that alter FPP levels, and hence Hmg2p degradation, and the steady-state cellular fluorescence measured by flow cytometry. Wild type reporter responded in the expected manner to changes in FPP: lovastatin, which reduces FPP levels, caused increased Hmg2p-GFP levels; zaragozic acid, which increases FPP abundance, decreased Hmg2p-GFP levels (Figure 3-8). In contrast, NR1-GFP steady state levels remained unchanged when cells expressing this mutant were subjected to those same treatments. Thus, NR1-GFP underwent normal, HRD-dependent degradation that was completely unresponsive to the *in vivo* regulatory signal.

Microsomes from strains expressing NR1-GFP were tested for the *in vitro* effect of farnesol in the limited proteolysis assay. While a wild type Hmg2p-GFP control showed the expected increase in trypsinolysis, the unregulated NR1-GFP mutant was not responsive to farnesol (Figure 3-9). Importantly, the cleavage pattern of NR1-GFP was identical to that of the wild-type protein, indicating no obvious conformational defect. Nevertheless, NR1-GFP was completely refractory to farnesol *in vitro* just as it was unresponsive to the degradation signal *in vivo*. Several other Hmg2p regulatory mutants were similarly tested, and all showed the same correlation between *in vivo* regulation and *in vitro* response to farnesol (data not shown).

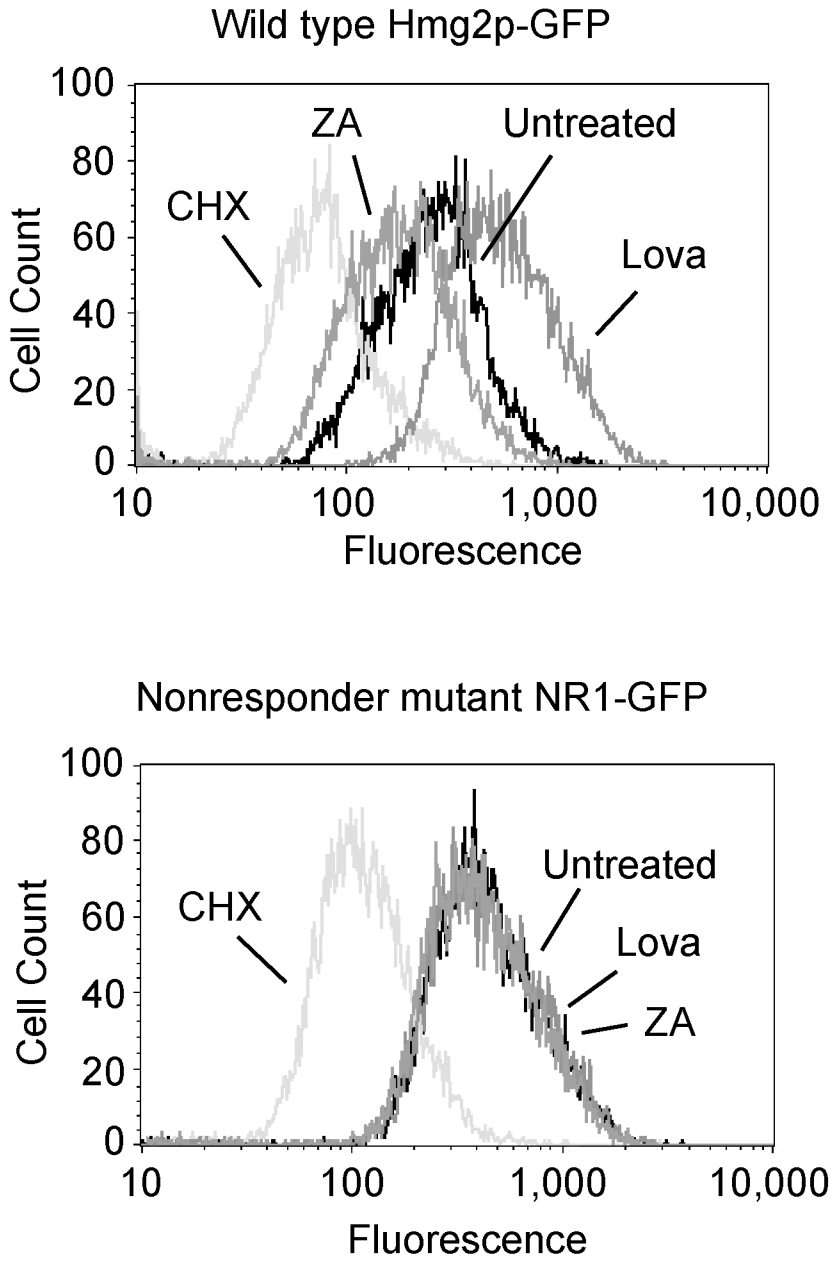

Figure 3-8
Mutant Hmg2p variant NR1-GFP is not regulated *in vivo*.
Cells expressing either Hmg2p-GFP or NR1-GFP were grown to early log phase and then incubated for 4 h with no drug, 25μg/ml lovastatin (Lova), 10μg/ml zaragozic acid (ZA) or 50μg/ml cycloheximide (CHX) as indicated, then assayed via flow cytometry for effects on Hmg2p stability.

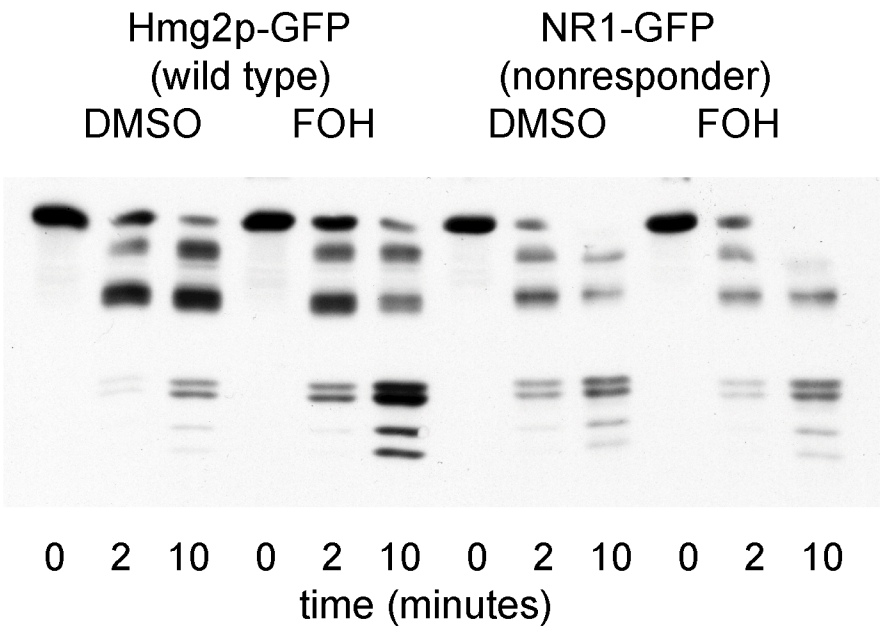

Figure 3-9
Mutant Hmg2p that is not regulated *in vivo* fails to respond to farnesol *in vitro*. Microsomes bearing Hmg2p-GFP or signal nonresponder NR1-GFP were resuspended in buffer with DMSO or 200μM FOH, then incubated with trypsin (15 μg/ml) at 30° C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope.

The *in vivo* degradation rate of Hmg2p changes rapidly in response to alterations in regulatory signal. A sudden increase in FPP level, as when cells are treated with zaragozic acid, causes accelerated Hmg2p degradation (Hampton and Bhakta, 1997). A sudden decrease in FPP, as when cells are treated with lovastatin, causes immediate stabilization of the entire pool of Hmg2p (Gardner and Hampton, 1999b; Hampton and Rine, 1994). Thus, it appears that the effects of the *in vivo* signal in Hmg2p are readily reversible. I next tested whether this was the case for the *in vitro* effect of farnesol as well.

To test for reversibility, farnesol needed to be removed from the microsomes following incubation. I initially tried simple resuspension of farnesol-treated microsomes in fresh buffer, but this did not reverse farnesol's effect. It was possible that the highly hydrophobic farnesol molecule might preferentially partition into the microsomal membrane. With this possibility in mind, to remove farnesol from treated membranes I provided a "lipid sink" in the form of additional microsomal membranes from a strain not expressing Hmg2p-GFP. Microsomes from a strain expressing Hmg2p-GFP were treated with DMSO or farnesol. These microsomes were then split into two samples and mixed with additional microsomes lacking tagged protein (the sink membranes). The additional microsomes were also treated with either DMSO or farnesol, providing four conditions as shown in a diagram of the experiment (Figure 3-10, A). I used a two-fold excess of sink membranes added to the microsomes containing Hmg2p-GFP. Each sample was then resuspended in new buffer and assayed via limited proteolysis. Farnesol-enhanced proteolysis depended entirely on

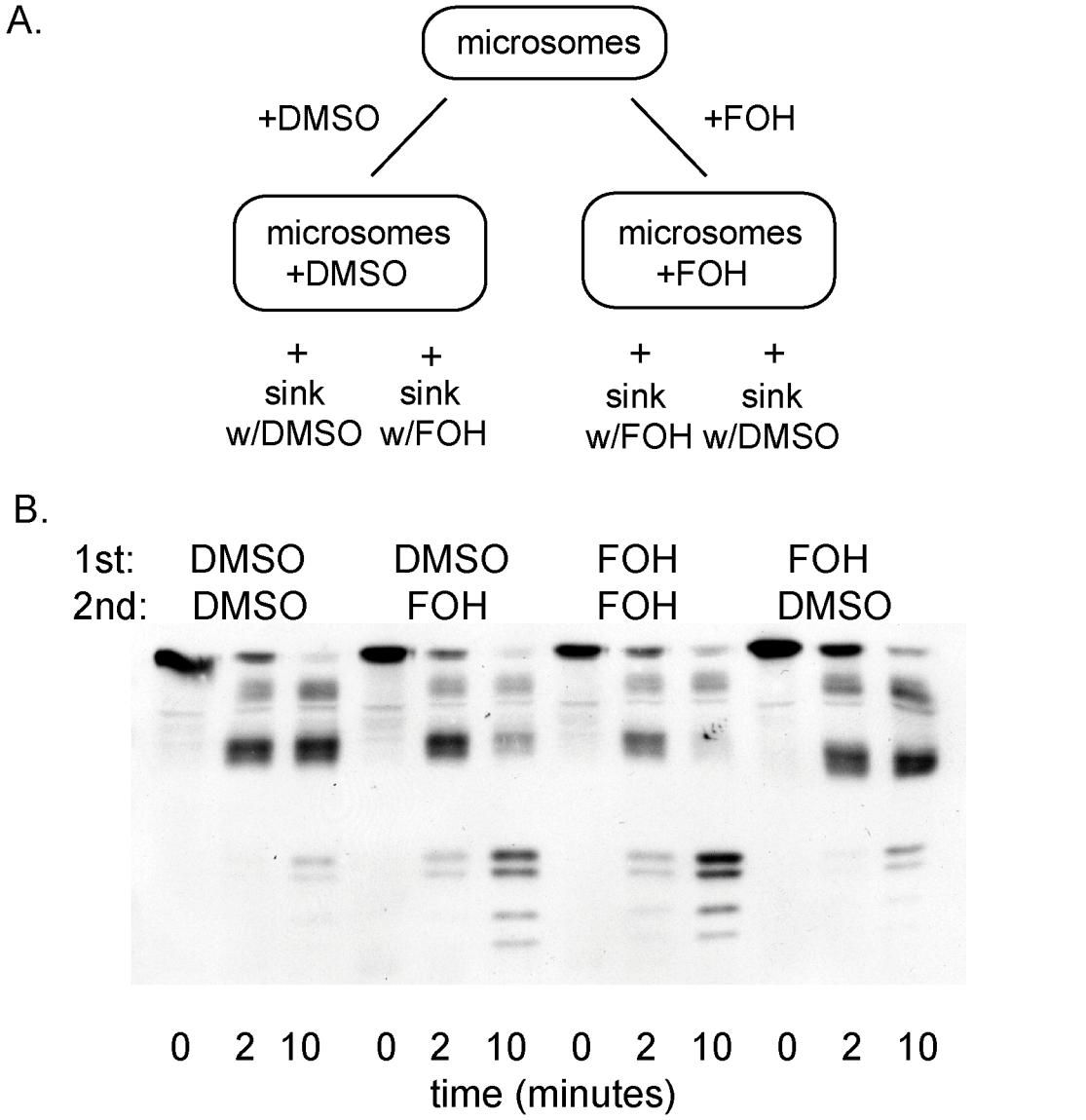

Figure 3-10
The farnesol effect is reversible.
(A) Diagram of the reversibility experiment. Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO or 200μM FOH. Each sample was then split in two and combined with additional sink membranes (lacking Hmg2p-GFP) treated with DMSO or 200μM FOH. (B) Outcome of reversibility treatments. Mixes resulting from the procedure described above were pelleted, resuspended in buffer and then 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. The row labeled "1st" indicates the initial treatment, while the row labeled "2nd" indicates whether sink membranes with or without farnesol were added.

the presence of this molecule at the time of trypsinization. The effects of farnesol treatment on Hmg2p-GFP were abrogated by addition of sink membranes without farnesol (Figure 3-10, B, first and fourth groups), whereas inclusion of farnesol with the sink membranes preserved the *in vitro* effect (Figure 3-10, B, third group). Finally, addition of farnesol only at the time of the addition of sink membranes also caused the *in vitro* effect on Hmg2p-GFP proteolysis (Figure 3-10, B, second group). Thus, pretreament with farnesol could be readily reversed if the properties of the lipid were considered in the procedure.

Taken together, the above observations indicated that farnesol specifically caused Hmg2p to undergo a reversible structural change to a more protease-accessible state. The model of Hrd1p-mediated Hmg2p regulated degradation predicts that farnesol should induce a structural change to a more misfolded state. I addressed this possibility using chemical chaperones and thermal denaturation assays.

Chemical chaperones such as glycerol aid in the proper folding of misfolded proteins. If farnesol were inducing Hmg2p to acquire traits of a misfolded protein *in vitro*, then it follows that chemical chaperones would antagonize this effect, promoting correct folding (Brown et al., 1996; Sato et al., 1996). Accordingly I tested the effect of glycerol on the *in vitro* action of farnesol in Hmg2p. Addition of 20% glycerol to farnesol-treated membranes antagonized the effect of farnesol, restoring the trypsinolysis rate to roughly that of untreated controls. (Figure 3-11). As described in the previous chapter, the presence of 20% glycerol has no effect on the activity of trypsin in this assay (Shearer and Hampton, 2004). Thus, the farnesol-induced

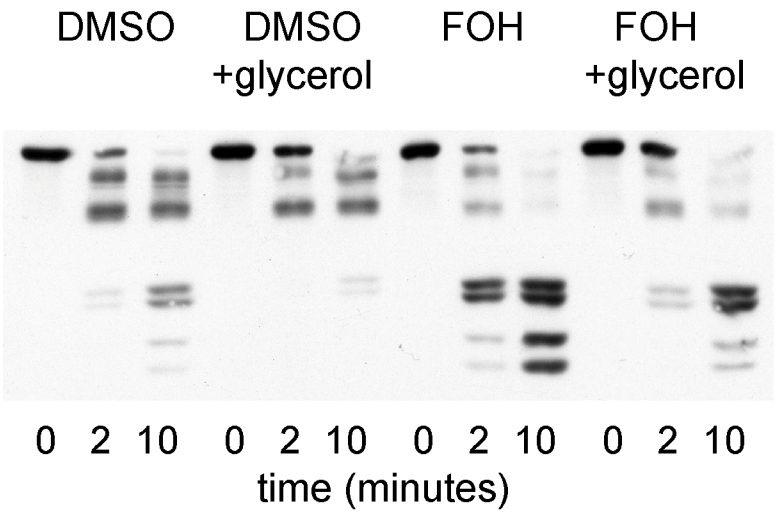

Figure 3-11
The chemical chaperone glycerol antagonizes the farnesol effect.
Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO or 200μM FOH, with or without 20% glycerol, then incubated with trypsin (15 μg/ml) at 30˚ C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the Myc epitope.

alteration in Hmg2p structure was sensitive to chemical chaperones, implying that the protein in this state was less fully folded.

I tested if Hmg2p-GFP in the presence of farnesol was more misfolded by employing a thermal denaturation assay, in which the rate and extent of protein aggregation at elevated temperature under various conditions is determined. Upon heat treatment, poorly folded proteins are more prone to aggregation than those that are fully folded. Thus, if Hmg2p were adopting traits of a misfolded protein in response to farnesol, one would expect to see an increased tendency to aggregate in the presence of farnesol when incubated at high temperatures. Microsomes treated with DMSO or farnesol were incubated at 70° C for the indicated times and subjected to SDS-PAGE gel electrophoresis and immunoblotting for the Myc tag in Hmg2p-GFP. In this assay, the stacking gel is included in the analysis to allow detection of low mobility aggregates that can form after heat denaturation. As shown, incubation of microsomes at 70˚ C caused the time-dependent appearance of such aggregates, indicated first as immunoreactivity at the stack/running gel boundary (Figure 3-12 A, Stack) and later as low mobility immunoreactivity near the top of the stack (Figure 3-12, A, LMI). In the presence of farnesol, Hmg2p-GFP showed a greater proclivity for aggregation, with similar low-mobility aggregates appearing approximately four fold faster than in the untreated samples (Figure 3-12, A). Importantly, farnesol did not cause aggregation of Hmg2p-GFP in the absence of heating, as indicated by the lack of aggregates at time zero, but rather enhanced the rate of heat denaturation. Thus, farnesol treatment caused membrane-localized Hmg2p-GFP to behave more like a

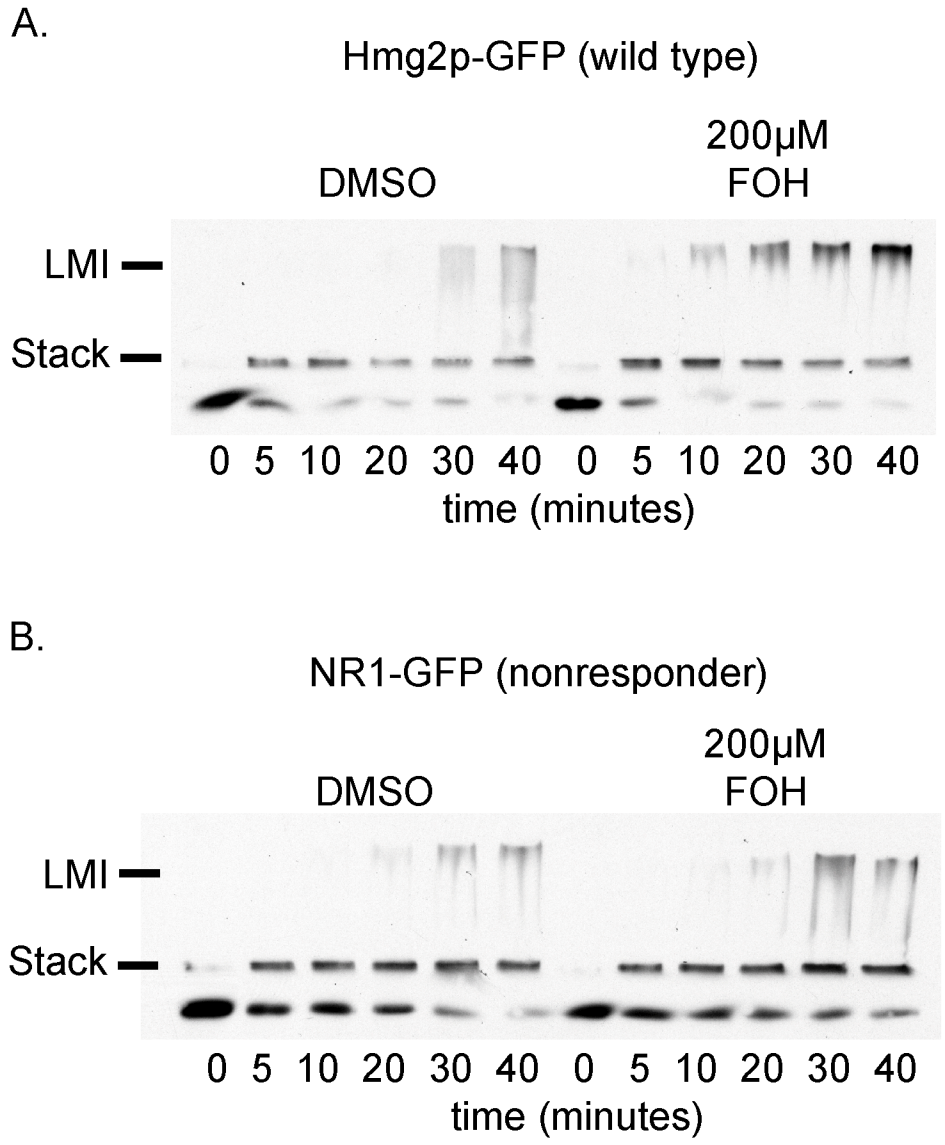

Figure 3-12
Farnesol specifically enhances the thermal denaturation of Hmg2p-GFP.
(A) Effect of farnesol on thermal denaturation of Hmg2p-GFP. Microsomes bearing Hmg2p-GFP were resuspended in buffer with DMSO or 200μM FOH, then incubated at 70° C. Samples were removed at the indicated times and evaluated by SDS-PAGE and immunoblotting of the Myc epitope, including both the 2% stack and the 14% gel. Lines indicate the boundary between the gel and the stack (Stack) and the location of low mobility immunoreactivity (LMI), or Hmg2p-GFP aggregates. (B) Effect of farnesol on thermal denaturation of NR1-GFP. Microsomes bearing NR1-GFP were evaluated for effect of farnesol as described above.

misfolded protein. This unfolding effect of farnesol had the same lipid specificity observed in the *in vitro* proteolysis experiments: Increased aggregation tendency was most strongly caused by farnesol, and to a lesser extent by geranylgeraniol, while geraniol and tetradecanol were without effect (data not shown). Because this independent assay does not require the lumenal epitope tag of Hmg2p-GFP, I also tested native, catalytically active Hmg2p and observed the same effects of farnesol on thermal denaturation (data not shown). Though this is not surprising considering that Hmg2p-GFP regulation matches that of the native protein *in vivo*, it was important to confirm that the effects observed with the reporter generalize to the authentic protein.

I next used the farnesol non-responsive NR1-GFP mutant to evaluate the biological relevance of farnesol-enhanced thermal denaturation. Microsomes bearing NR1-GFP were subjected to the thermal denaturation assay in the presence and absence of farnesol. In contrast to wild-type Hmg2p-GFP, farnesol had no enhancing effect on the thermal denaturation of the unresponsive mutant (Figure 3-12, B). Importantly, NR1-GFP was equally responsive to the non-specific denaturant potassium thiocyanate (KSCN), which caused identical increases in the rate of thermal denaturation in both mutant and wild-type (Figure 3-13, A). Thus, the unresponsiveness of NR1-GFP to farnesol was specific for that molecule, and not due to a general inability of the mutant protein to undergo thermal denaturation.

The effects of farnesol on Hmg2p in the limited proteolysis assay were reversible, when appropriate measures were taken to remove the farnesol from treated microsomes (Figure 3-11). The *in vitro* effects of farnesol in the thermal denaturation

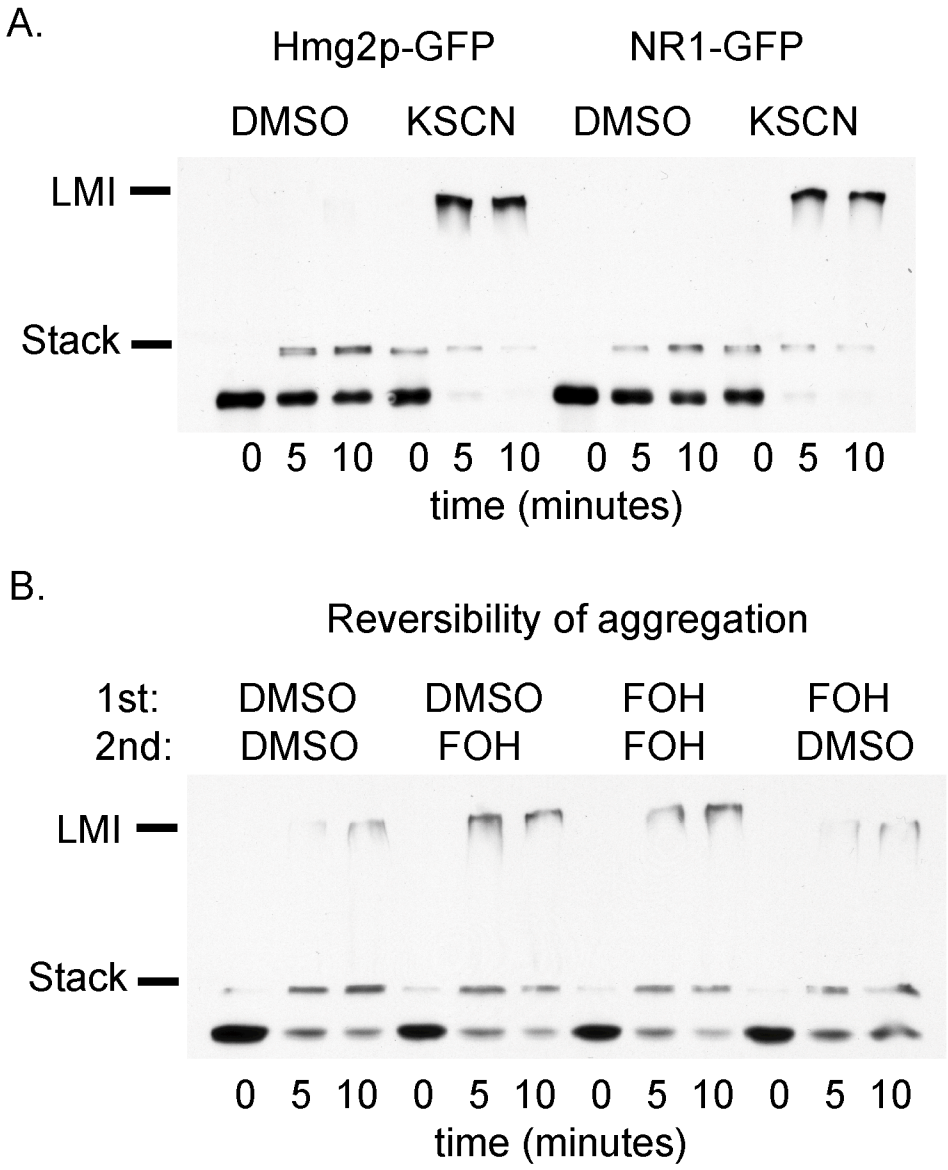

Figure 3-13
Farnesol-promoted thermal denaturation is biologically relevant.
(A) Effect of the chaotrope potassium thiocyanate (KSCN) on thermal denaturation of Hmg2-GFP and NR1-GFP. Microsomes with Hmg2p-GFP or NR1-GFP were resuspended in buffer with or without 125mM KSCN, then incubated at 70˚ C and evaluated as described above. (B) Testing the reversibility of the farnesol effect in the thermal denaturation assay. Microsomes containing Hmg2p-GFP were prepared as described in Figure 3-11, then pelleted, resuspended and incubated at 70˚ C for the indicated times, then evaluated as described above.

assay were similarly reversible. Microsomes were prepared and treated with DMSO or farnesol followed by DMSO or farnesol-treated sink membranes as described above for the limited proteolysis reversibility experiment (as diagrammed in Figure 3-10, A). The resulting samples were then subjected to the thermal denaturation assay. Conditions that reversed the effect of farnesol in the Hmg2p limited proteolysis assay – addition of sink membranes lacking farnesol – similarly reversed its effect in the aggregation assay (FIG 3-13, B). Thus, the farnesol-promoted increase in thermal denaturation was reversible, just as observed with the farnesol-induced conformational change.

Taken together, these results indicated that the enhanced thermal sensitivity of Hmg2p caused by farnesol had the same biologically relevant properties as its action in the limited proteolysis assay: reversibility, lipid specificity and a requirement for correct Hmg2p sequence.

Discussion

Hmg2p is the first example of a normal protein that undergoes small molecule-regulated quality control degradation. As discussed in prior chapters, it has been suggested that Hmg2p undergoes a structural transition in response to an FPP-derived molecular degradation signal, acquiring traits of a misfolded protein. In this state it would be recognizable by the HRD quality control pathway, and thus ubiquitinated and degraded.

Consistent with this idea, I have shown that Hmg2p undergoes a structural transition when the *in vivo* FPP-derived signal for degradation is abundant, as described in the previous chapter (Shearer and Hampton, 2004). Using two independent *in vitro* assays I have demonstrated that this is a direct effect on the Hmg2p transmembrane domain. The *in vitro* structural transition was quite specific for the FPP-derived molecule farnesol, met all criteria tested for *in vivo* relevance, was reversible, and did indeed involve a transition between a more and less folded form of Hmg2p.

I initially evaluated the possibility that the degradation signal directly affected Hmg2p structure with the *in vitro* limited proteolysis assay. The neutral FPP derivative farnesol had a dramatic effect on the limited proteolysis of Hmg2p. Multiple studies have shown that farnesol is produced in abundance when FPP levels are elevated by drugs or genetic manipulation (Chambon et al., 1990; Gardner et al., 2001a), making it a reasonable candidate for the FPP-derived signal that regulates Hmg2p stability *in vivo*. As observed with the *in vivo* signal, farnesol acted rapidly and reversibly in this

in vitro assay. The concentrations of farnesol that affect Hmg2p structure (50-200 μM) are in the same range as those required for another *in vivo* function of farnesol – quorum sensing in the yeast *Candida albicans* (30-300 μM) (Hornby et al., 2001; Ramage et al., 2002). It is quite possible, however, that the concentrations of farnesol required for *in vivo* regulation are less then those needed for the *in vitro* effect. While the *in vitro* assay demands changes in the whole pool of Hmg2p, *in vivo* regulation of Hmg2p levels may occur by continuous regulated degradation of a small fraction of the Hmg2p pool at a given time.

The biological relevance of the farnesol-induced structural transition was confirmed by analysis of pertinent *cis* and *trans* regulatory mutants. In a *cod1Δ* mutant, Hmg2p is mostly unresponsive to the *in vivo* degradation signal (Cronin et al., 2000), due to alterations in its structure that likely abolish regulation (Shearer and Hampton, 2004). In the same manner, limited proteolysis of Hmg2p in microsomes derived from a *cod1Δ* strain was largely unaffected by farnesol. An independent test of relevance was provided by the unregulated *in cis* NR1-GFP mutant of Hmg2p. This variant of Hmg2p was completely unregulated *in vivo*, and was completely unresponsive to farnesol in the limited proteolysis assay. These results with unregulated mutants demonstrated that the *in vitro* effect of farnesol adhered closely to the *in vivo* properties of Hmg2p regulation. Furthermore, these experiments showed that the actions of farnesol were not due to general effects on membranes or membrane proteins, since its effects were highly dependent on the correct structure and sequence of Hmg2p.

The structural transition model of Hmg2p regulation predicts that farnesol promotes acquisition of quality control traits in Hmg2p, a prediction borne out by these *in vitro* studies. The chemical chaperone glycerol antagonized farnesol's effect *in vitro*, which implied that farnesol was promoting a less folded state. Farnesol also promoted enhanced thermal aggregation of Hmg2p-GFP, indicating that the protein was more misfolded in the presence of farnesol. Enhancement of Hmg2p-GFP thermal denaturation was farnesol specific, did not occur with the NR1-GFP unresponsive mutant, and was fully reversible. Thus, farnesol had precisely the same features in both *in vitro* assays of its effect on Hmg2p.

Farnesol has previously been implicated in the degradation of mammalian HMGR, where it has been shown to increase degradation rate when added to intact or permeablized cells (Correll et al., 1994; Meigs et al., 1996). However, in some circumstances, farnesol appears to cause non-specific aggregation of membrane proteins in permeabilized cells (Meigs et al., 1996). These concerns do not pertain to the studies described in this chapter. In both the limited proteolysis and thermal denaturation assays, the effects of farnesol were reversible. Furthermore, farnesol did not itself cause Hmg2p aggregation, but only enhanced denaturation of Hmg2p at high temperature as a specific readout of tendency to misfold. Finally, farnesol had no effect on the unresponsive NR1-GFP mutant or on Hmg2p in *cod1*Δ membranes, ruling out a generalized effect of farnesol on ER membrane proteins in these studies.

Taken together, the work presented above showed that Hmg2p underwent a rapid, reversible, farnesol-dependent transition to a more misfolded state, one that

presumably is better recognized by the Hrd1p quality control pathway. It will be important to next determine if this altered state is indeed recognized by the Hrd1p ubiquitination machinery, which is a topic for future studies.

The effect of farnesol on Hmg2p structure was quite specific for this lipid. A variety of other lipids, including linear alcohols of similar size, other isoprene compounds, fully hydrogenated farnesol, and the natural sterols ergosterol and lanosterol, were largely ineffective at altering Hmg2p structure. Thus, the effect of signal lipids on the Hmg2p transmembrane domain had strict structural requirements, presenting the possibility that the signal is recognized by the protein in a specific manner.

The transmembrane domain of Hmg2p mediates regulated degradation (Gardner and Hampton, 1999a), and this portion of the molecule, as found in Hmg2p-GFP, is sufficient for the *in vitro* effects of farnesol. This portion of the Hmg2p protein has a conserved sequence motif known as the sterol sensing domain (SSD), which is found in a number of proteins involved in lipid regulation, including mammalian HMGR, the sterol regulator SCAP, the developmental proteins Patched and Dispatched, and the lipid storage-related protein NPC1 (Carstea et al., 1997; Hua et al., 1996; Kuwabara and Labouesse, 2002). Based on the functions of these proteins, the SSD was hypothesized to sense cholesterol or related sterols. However, later work with Drosophila SCAP indicated that the SSD in that protein specifically responds to a lipid signal unrelated to sterols (Dobrosotskaya et al., 2002), implying that the SSD has a broader role in recognition of diverse lipid signals. Adding to this

idea, the studies discussed in this chapter indicate that the Hmg2p SSD is involved in farnesol sensing. This concept is supported by the existence of numerous mutations in the conserved SSD that alter the *in vivo* response of Hmg2p to the degradation signal (Gardner and Hampton, 1999a). In particular, the dramatic, completely unresponsive NR1-GFP mutation is a conservative five amino acid replacement (from TFYSA to ILQAS) that alters a phenylalanine found in nearly all SSDs, and one that is required for sterol regulation of HMGR in mammals (Xu and Simoni, 2003). When comparison is restricted to only HMGR from various organisms, several additional identities appear in this region (consensus TFxxAxxS), perhaps indicating a particular role in HMGR regulation for this conserved SSD segment. It has been shown that SCAP undergoes an SSD dependent conformational change in the presence of sterols (Brown et al., 2002). The SCAP change is qualitatively different from that seen in Hmg2p, causing an altered proteolytic pattern rather than a global change in accessibility. Furthermore sterols do not cause SCAP degradation. Regardless, structural response to lipid signals may prove to be a general trait of SSD proteins. Thus, the SSD is most likely an ancestral lipid sensor that has evolved to discriminate a variety of lipid signals. In this sense, SSDs have a broad conservation of purpose, rather than conservation of specific effector lipid. The slight variation of SSD to mean "signal sensing domain," or perhaps "state sensing domain" might thus be appropriate for this ancient membrane motif.

How related are regulation of Hmg2p and mammalian HMGR? Both are subject to regulated degradation via the ubiquitin-proteasome machinery, driven by

specific pathway lipids, and requiring an intact SSD. Hmg2p degradation depends primarily on the FPP-derived signal, with little or no dependence on sterols (however see (Gardner et al., 2001a) for an explanation of the minor role played by an oxysterol). Mammalian HMGR degradation is regulated primarily by sterols, but it too shows a separate requirement for an early isoprene, which has been proposed to be farnesol (Correll et al., 1994; Meigs et al., 1996) or more recently, geranylgeraniol (Sever et al., 2003). The Insig proteins play a key role in HMGR regulated degradation (Sever et al., 2003), and the distant yeast homologues *NSG1* and *NSG2*, play a distinct, but related role in Hmg2p degradation (Flury I, Shearer AG, Rosen J, Hampton RY, manuscript in preparation). Given these similarities, it is conceivable that quality control machinery could be involved in the regulated destruction of HMGR as well. If mammalian HMGR is recognized by a quality control pathway, this would be another example of what is likely to be a common regulatory strategy: small-molecule-promoted entry into constitutive quality control degradation pathways.

Regulated entry into a constitutive quality control pathway represents a novel yet reasonable combination of two common elements of cellular physiology. Many proteins undergo reversible structural change in response to a small molecules, often allosteric alterations that regulate their activities (Gerhart and Schachman, 1968; Monod et al., 1963; Pardee and Reddy, 2003). Quality control is similarly ubiquitous, being found across all kingdoms of life (Dougan et al., 2002; Hampton, 2002a; Volker and Lupas, 2002). Regulated degradation of Hmg2p could be considered in a sense an allosteric transition to a misfolded state. Given the large number of examples of small

molecule regulation of protein structure and the ubiquity of quality control pathways, many examples of small-molecule regulated quality control will likely be discovered.

Beyond this probable natural role, small molecule regulation of protein quality control also holds promise for drug development. From the data described above, it is quite conceivable that compounds could be developed to modulate the degradation rate of a single target protein by specifically causing the appropriate structural changes. The most immediate application would be in designing molecules that stimulate the degradation of HMGR, providing another, novel mechanism to lower cholesterol and achieve the many other benefits currently associated only with inhibitors of HMGR enzymatic activity, the statins (Gresser and Gathof, 2004; Marz and Koenig, 2003; Stuve et al., 2003). The ubiquity of quality control pathways, and the specificity with which they operate implies that this strategy of small-molecule protein targeting could have many uses in the laboratory and the clinic. Pharmaceutical adaptation of this approach could include targeting of proteins that normally are not degraded by such pathways, or the enhancement of quality control degradation for cases, such as numerous neurodegenerative diseases, in which natural quality control of key proteins is not sufficiently robust. The required knowledge of how quality control can be harnessed will come with the identification of more proteins that, like Hmg2p, selectively enter these pathways in a regulated manner.

Materials and methods

Materials

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. Lovastatin and zaragozic acid were generously provided by Dr. Samuel Wright (Merck, Rahway, NJ). 9E10 cell culture supernatant was produced from cells (CRL 1729, American Type Culture Collection) grown in RPMI1640 culture medium (GIBCO BRL) with 10% fetal calf serum and supplements. HRP-conjugated goat anti-mouse antibodies were purchased from Jackson Immuno Research. 3,7,11-trimethyldodecan-1-ol was synthesized in the lab of Seiichi Matsuda (Rice University). Chemiluminscence immunodetection reagents were obtained from Perkin Elmer. All other chemical reagents were obtained from Sigma or Fisher.

Plasmid construction and DNA manipulation

Plasmid pRH1692 (integrating) expressed the NR1-GFP unregulated variant of lumenally-myc-tagged Hmg2-GFP with amino acids 348-352 switched from TFYSA to ILQAS, and was constructed by replacing the SphI-BglII segment of pRH1581, the plasmid expressing Myc_L-Hmg2p-GFP (Shearer and Hampton, 2004) with the SphI-BglII fragment from pRH902 (Gardner and Hampton, 1999a).

Yeast culture and strains

Yeast strains were grown in minimal media (Difco Yeast Nitrogen Base) with glucose and the appropriate supplements as described previously, or in YPD, if so noted (Hampton and Rine, 1994). Experiments were performed at 30° C unless otherwise noted. Yeast was transformed with plasmid DNA by the LiOAc method.

RHY2842 was made by targeted integration of the plasmid pRH1692 at the StuI site of the *ura3-52* locus in RHY519 (Gardner et al., 1998), then selection for Ura+ prototrophy.

RHY2866 was derived from RHY2842 by transformation with the *hrd1*Δ*::KanMX* deletion cassette excised from pRH1122 (Gardner and Hampton, 1999a), then selection on YPD with G418.

Flow cytometry of yeast cells

Flow cytometry was carried out as described previously (Cronin et al., 2000). Briefly, cells were grown to early log phase (OD_{600} 0.1) in minimal medium, then incubated with drugs 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.

Microsome preparation

Microsomes were prepared as described previously (Shearer and Hampton, 2004). Briefly, cells were grown to mid log phase in YPD, pelleted, and subjected to hypoosmotic lysis. The resulting lysates were cleared of debris, then centrifuged to pellet microsomes, which were then resuspended in physiological buffer at 6µl buffer per OD of starting cells.

Limited proteolysis assay on microsomes

The assay was performed on microsomes as described (Shearer and Hampton, 2004). Briefly, resuspended microsomes were treated with indicated lipids (dissolved in DMSO) or with a DMSO-only vehicle control, then an aliquot was removed as a no trypsin control. Trypsin was then added and the remaining microsomes incubated at 30˚ C. Aliquots were removed at the indicated times and the reaction halted by addition of SDS-urea sample buffer.

Samples were resolved on 14% SDS-PAGE, then transferred with 15% methanol to nitrocellulose and immunoblotted for myc epitope, all as described (Hampton and Rine, 1994).

Thermal denaturation assay

Microsomes were prepared as described above, then treated with lipids as indicated. Following treatment, 30µl sample aliquots were transferred to ultra-thin wall PCR tubes, one per desired time point, and placed on ice for twenty minutes. Tubes were then placed in a Perkin Elmer GeneAmp PCR System 2400 thermocycler holding at 70˚ C and incubated for the indicated times. At the end of the incubation, samples were placed on ice for two minutes, then prepared for electrophoresis by addition of 30µl 2x USB (75mM MOPS, pH 6.8; 4% SDS; 200mM DTT; 0.2mg/ml bromophenol blue; 8M urea) followed by solubilization for ten minutes at 55˚ C.

Samples were resolved on 14% SDS-PAGE with a 2% stacking gel, then transferred from both gel and stack with 15% methanol to nitrocellulose and immunoblotted for myc epitope as described (Hampton and Rine, 1994).

Table 3-1
Yeast strains used in this chapter.

RHY2723	*MATa ade2-101 met2 lys2-801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3-52::URA3::HMG2cd::myc$_L$-HMG2-GFP*
RHY2803	*MATa ade2-101 met2 lys2-801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3-52::URA3::HMG2cd::myc$_L$-HMG2-GFP hrd1Δ::KanMX*
RHY2842	*MATa ade2-101 met2 lys2-801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3-52::URA3::HMG2cd::NR1-GFP*
RHY2853	*MATalpha ade2-101 met2 lys2-801 his3200 ura3-52::URA3::myc$_L$-HMG2-GFP cod1Δ::KanMX*
RHY2866	*MATa ade2-101 met2 lys2-801 his3Δ200 hmg1::LYS2 hmg2::HIS3 ura3-52::URA3::HMG2cd::NR1-GFP hrd1Δ::KanMX*

Table 3-2
Plasmids used in this chapter.

pRH1122	Contains *hrd1*Δ::KanMX deletion cassette
pRH1581	Expresses lumenally myc-tagged *HMG2-GFP* from a TDH3 promoter
pRH1692	Expresses lumenally myc-tagged *HMG2-GFP* bearing the TFYSA to ILQAS patch mutant from a TDH3 promoter

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The text of Chapter 3 is in part a reprint of the material as it has been submitted for publication. I was the primary author of this publication and carried out all the research described in this chapter.

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Chapter 4

Key factors in ERAD of a natural substrate: the role of molecular chaperones in HMG-CoA reductase degradation

Introduction

The complex process of endoplasmic-reticulum-associated degradation (ERAD) requires many additional factors beyond the core components of the ubiquitin-proteasome pathway. Molecular chaperones, which aid in the folding of nascent proteins and the refolding of misfolded proteins, have been tapped as key players in this process (Nishikawa et al., 2001). While various molecular chaperones appear to be required for successful degradation of particular ERAD substrates, entirely consistent rules to identify the chaperones required based on substrate type have not yet been elucidated. Models have been proposed, but very few natural substrates have been evaluated.

Research to date suggests that the requirements for soluble and integral membrane proteins are at least partially different, but even this may not be entirely clear. The mammalian protein CFTR, which has twelve transmembrane spans and is subject to ERAD, has been expressed in yeast as a model integral membrane substrate. In this context, CFTR is stabilized by loss of Hsp70 function whereas diminished Kar2p has no effect (Zhang et al., 2002; Zhang et al., 2001). The Hsp70s are ATP-dependent chaperones that are required for degradation of a number of ERAD substrates, including both soluble and integral membrane proteins (Taxis et al., 2003; Zhang et al., 2001). Kar2p/BiP is another ATP-dependent protein that acts as a

chaperone, aiding in the folding of ER proteins and import of proteins into the ER, as well as buffering against buildup of misfolded proteins and activating the unfolded protein response (Okamura et al., 2000; Sanders et al., 1992; Simons et al., 1995; Umebayashi et al., 1999). By comparison, ERAD of the soluble, mutant ER protein CPY* shows no need for Hsp70 but does require Kar2p and the combined activities of the DnaJ proteins Jem1p and Scj1p. However, this apparently clean functional divide is blurred by the model integral membrane substrate CT*, which fuses CPY* to a single transmembrane span yet still does not require Hsp70 (Taxis et al., 2003). It has also been suggested that certain chaperone requirements depend on the presence or absence of a tightly folded cytosolic domain. This proposal is based on work with CTG*, which is CT* with a GFP on its cytosolic face. CTG* degradation requires Kar2p, Hsp70 and to some degree Jid1p, Hlj1p, Cwc23p and Hsp104p (Taxis et al., 2003). Unfortunately, all this information fails to paint a clear picture of chaperone requirements based on substrate.

Notably, none of the membrane proteins described above represent natural substrates for yeast ERAD. Hmg2p, on the other hand, is a natural yeast protein that undergoes ERAD via a quality control pathway. As described in chapter three, Hmg2p adopts traits of a quality control substrate in the presence of the degradation signal, farnesol. Given this, it is reasonable to imagine that molecular chaperones would play a role in Hmg2p's degradation. In addition, Hmg2p represents a natural, if switchable,

quality control substrate, and thus could shed light on actual chaperone requirements for the degradation of true integral membrane proteins.

I set out to evaluate which chaperones, if any, were required for degradation of Hmg2p. Though the results described below are preliminary, they highlight differences between chaperone requirements for the natural substrate Hmg2p and two model integral membrane substrates, as well as providing valuable information about the mode of action of the Nsg proteins.

Results

The general strategy for identifying chaperones involved in Hmg2p degradation relied on observing changes in the steady state level and degradation rate of the fluorescent reporter Hmg2p-GFP. In this normally regulated version of Hmg2p, the catalytic domain, which is not required for regulation, has been replaced with green fluorescent protein (GFP) (Gardner and Hampton, 1999; Hampton and Rine, 1994). The resulting protein allows quantitation of steady-state levels and degradation via flow cytometry (Cronin and Hampton, 1999). Specifically, in all cases below where two strains are compared, the mean per-cell GFP fluorescence of equal numbers of otherwise isogenic mutant and wild type cells were determined and compared, yielding a quantitative difference in Hmg2p-GFP abundance between the strains in question. In this way, phenotypes relating to Hmg2p degradation can be assessed in a facile manner for a substantial number of genes.

I began my evaluation of chaperone involvement in Hmg2p degradation by examining the requirement for cytosolic Hsp70s. There are four key cytosolic Hsp70s in yeast, encoded by the genes *SSA1-4*. Perhaps because Hsp70 activity is required for viability, the four genes appear to be functionally redundant as evaluated by cell survival (Werner-Washburne et al., 1987). To test the requirement for *SSAs* in Hmg2p degradation, I employed strains with *SSA2-4* deleted, and *SSA1* present in either its

wild type form or as a temperature-sensitive allele, called *ssa1-45* (Becker et al., 1996). Growth of both strains at the permissive temperature (30˚ C) resulted in differing steady-state levels of Hmg2p-GFP (Figure 4-1, A), with somewhat more present in the *ssa1-45* strain (1.4 fold greater). However, growth at the nonpermissive temperature (37˚ C), while yielding little effect on Hmg2p-GFP in the wild type background, led to reduced Hmg2p-GFP steady-state levels in the temperature sensitive strain, leaving both strains with more similar levels of Hmg2p-GFP (Figure 4-1, B. These same strains were grown at the permissive temperature into early log phase and then cycloheximide was added to halt protein synthesis, allowing observation of degradation effects only. When cells were subsequently shifted to the nonpermissive temperature, no alteration in degradation rate was observed in either the wild type or sensitive strain (Figure 4-2). When degradation was allowed to occur at the permissive temperature, however, it did proceed more slowly in the temperature-sensitive strain. Thus, Hsp70 may play some small, positive role in Hmg2p-GFP degradation, as revealed by the deficiency of the temperature-sensitive allele at normal temperatures.

The transcription factor *HSF-1* exerts general control over heat shock proteins, most notably the inducible forms of Hsp90 (Morano et al., 1999). Strains expressing Hmg2p-GFP and either wild type or mutant *HSF-1* were used to determine the requirement for these heat shock proteins in Hmg2p degradation. The specific mutant

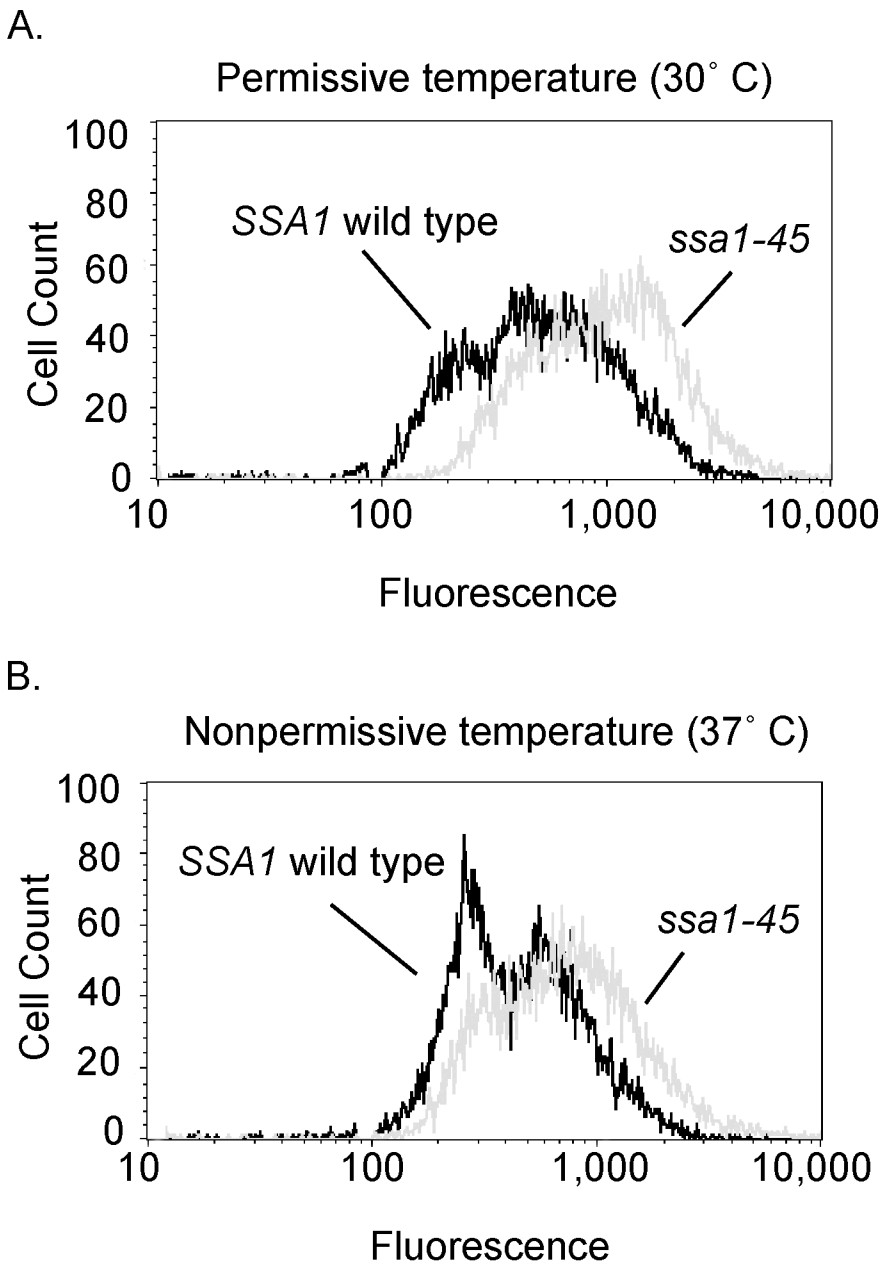

Figure 4-1
Hmg2p-GFP is marginally stabilized in an *ssa1* ts strain at either temperature. (A) Strains expressing Hmg2p-GFP with either wild type *SSA1* or the temperature-sensitive *ssa1-45* allele were grown to early log phase at the permissive temperature of 30° C and analyzed for GFP fluorescence via flow cytometry. Each histogram represents 10,000 cells. (B) The same two strains were grown overnight at 30° C, then incubated for four hours at the nonpermissive temperature of 37° C and then analyzed as in A.

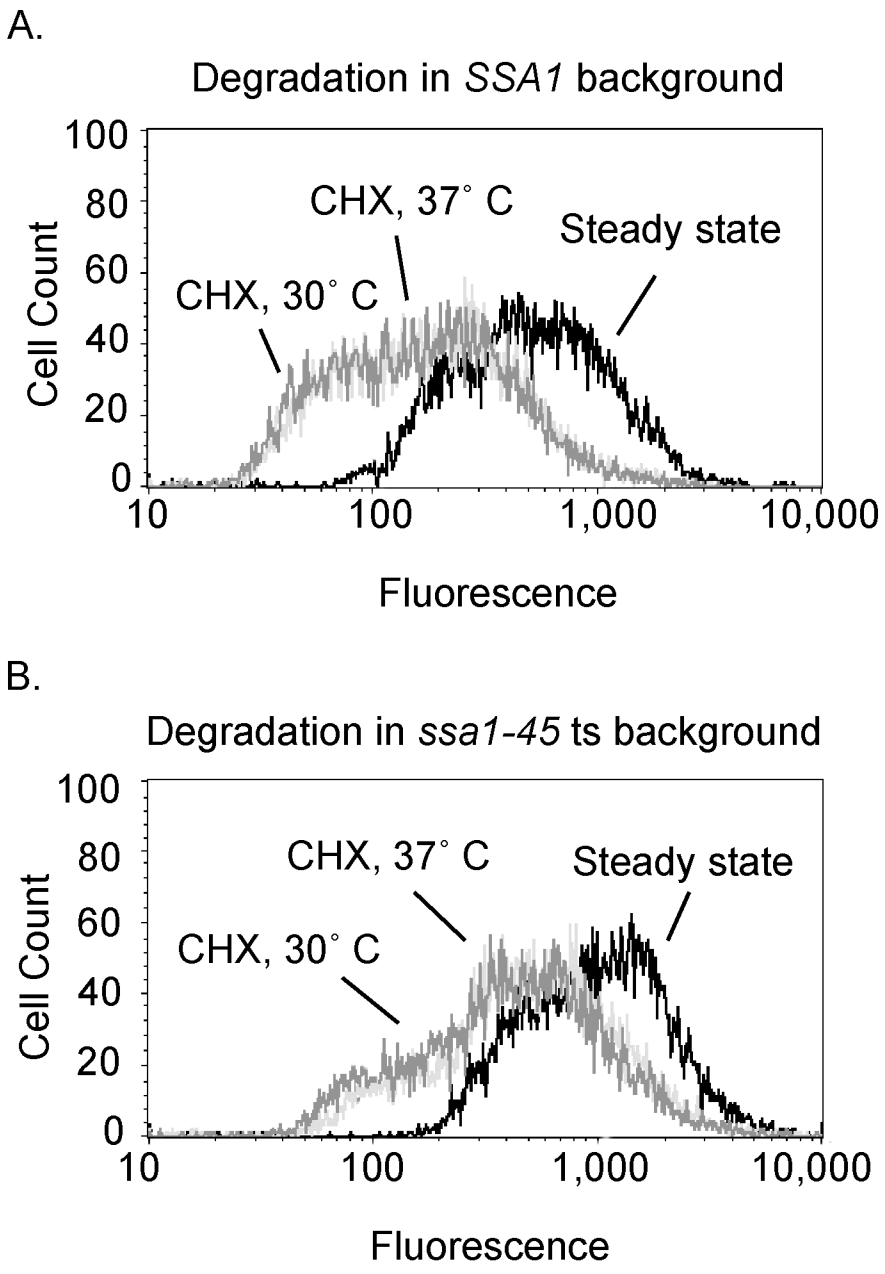

Figure 4-2
Hmg2p-GFP degradation is somewhat slower in the *ssa1-45* ts background.
(A) Cells expressing Hmg2p-GFP with wild type *SSA1* were grown to early log phase at the permissive temperature of 30° C, then some cultures were treated with 50μg/ml cycloheximide (CHX) to halt protein synthesis and incubated for two hours at either 30° C or 37° C. All cultures were then analyzed for GFP fluorescence via flow cytometry. Each histogram represents 10,000 cells. (B) The procedure described in A was applied to cells expressing Hmg2p-GFP with the *ssa1-45* ts allele.

allele of *HSF-1*, termed *hsf-1[1-583]*, lacks a transactivation domain and leads to inviability at 37˚ C when it is the only version of *HSF-1* present in the cell. Some of the effects of *hsf-1[1-583]* may be due to an inability to induce *HSP82*, an Hsp90 gene that is induced as part of the heat shock response in yeast (Morano et al., 1999). Hmg2p-GFP was expressed in strains with wild type *HSF-1* or the mutant *hsf-1[1-583]* allele. Both strains were initially treated with the benzoquinoid ansamycins macbecin and geldanamycin, which disrupt the chaperone and related functions of Hsp90s (Panaretou et al., 1998). No effect of either drug was observed on Hmg2p-GFP steady-state levels or degradation in the wild type or the temperature-sensitive mutant (data not shown). However, during growth at the nonpermissive temperature (Figure 4-3), the steady-state level of Hmg2p-GFP in the temperature sensitive strain was slightly lower (1.4 fold). Both strains were subsequently grown at 30° C, then either left at 30˚ C or shifted to the nonpermissive temperature of 37° C for four hours in the presence of cycloheximide (Figure 4-4). In both strains, degradation occurred somewhat faster at 37˚ C (1.3 fold), and regardless of temperature, degradation was more rapid in the temperature sensitive strain (1.5 fold). The small but significant difference in steady-state levels at 37˚ C and degradation at all temperatures indicated that the chaperones for which *HSF-1* is a transcription factor were involved in Hmg2p-GFP stability, with their absence leading to accelerated degradation. The

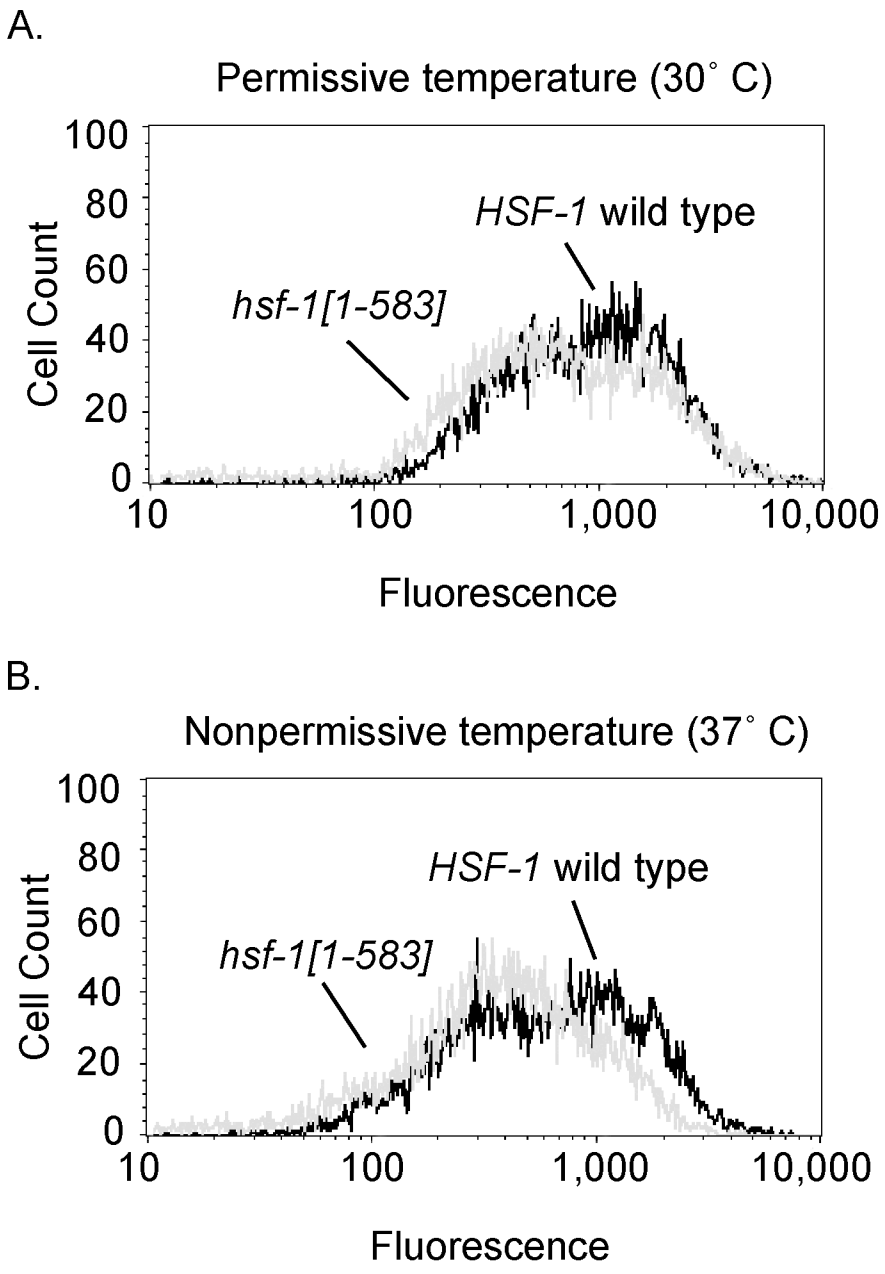

Figure 4-3
Hmg2p-GFP stability is somewhat affected by loss of *HSF-1*.
(A) Strains expressing Hmg2p-GFP with either wild type *HSF-1* or the nonfunctional *hsf-1[1-583]* allele were grown to early log phase at the permissive temperature of 30˚ C and analyzed for GFP fluorescence via flow cytometry. Each histogram represents 10,000 cells. (B) The same two strains were grown overnight at 30˚ C, then incubated for four hours at the nonpermissive temperature of 37˚ C and then analyzed as in A.

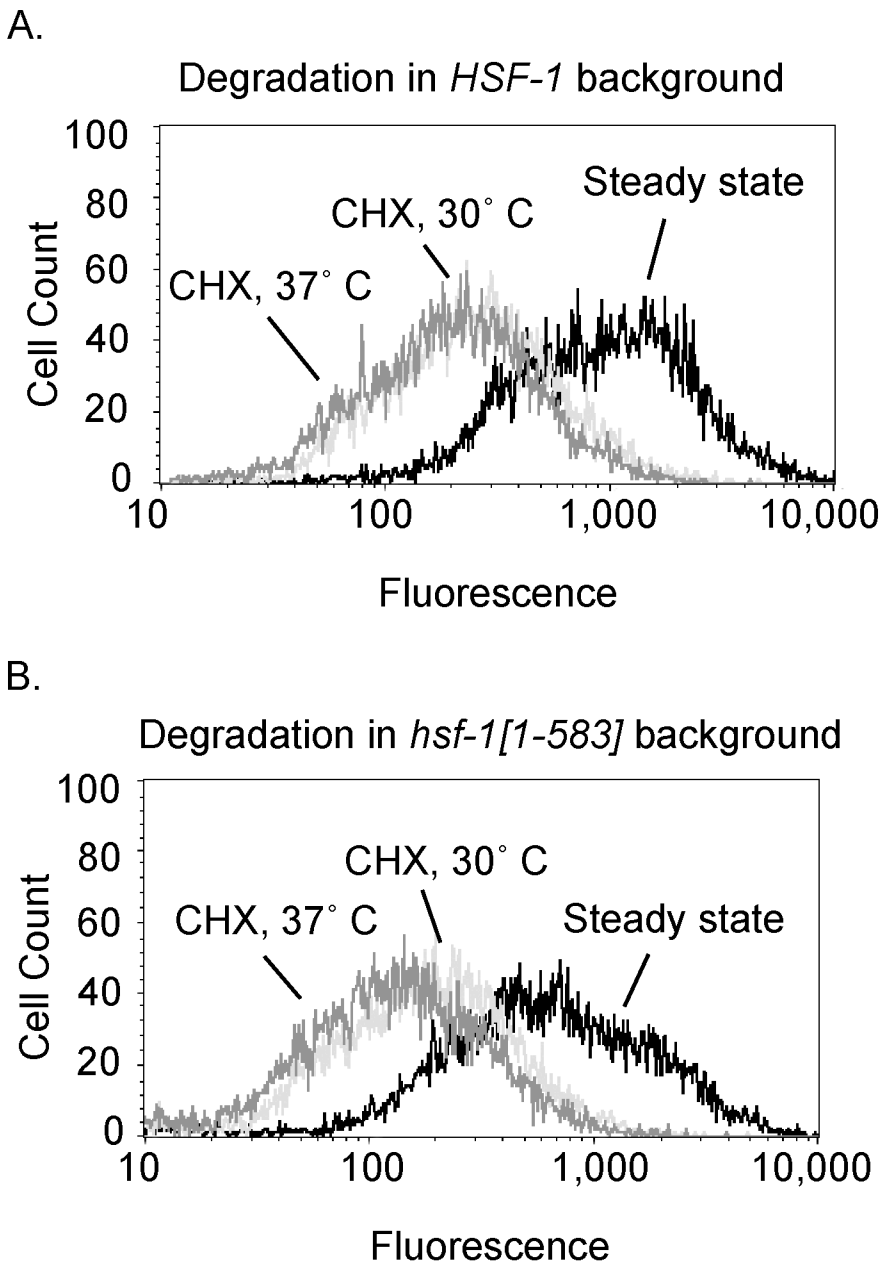

Figure 4-4
Hmg2p-GFP degradation is faster in the *hsf-1[1-583]* background.
(A) Cells expressing Hmg2p-GFP with wild type *HSF-1* were grown to early log phase at the permissive temperature of 30° C, then some cultures were treated with 50μg/ml cycloheximide (CHX) to halt protein synthesis and incubated for two hours at either 30° C or 37° C. All cultures were then analyzed for GFP fluorescence via flow cytometry. Each histogram represents 10,000 cells. (B) The procedure described in A was applied to cells expressing Hmg2p-GFP with the *hsf-1[1-583]* allele.

increased degradation rate in the *hsf-1[1-583]* strain regardless of temperature indicates that this involvement was not just limited to the heat-inducible targets of *HSF-1*.

As mentioned above, other genes were recently identified as being involved in the ERAD of a model transmembrane substrate (Taxis et al., 2003). Among these were several bearing DnaJ domains, and Hsp104. To evaluate the role of these and other genes, I compiled a list of putative chaperones based on homology to DnaJ, evidence of involvement in degradation of the model substrate discussed above and homology to previously characterized chaperone proteins. Strains from the Saccharomyces Genome Deletion Consortium heterozygous diploid deletion collection lacking a copy of the indicated genes were dissected to recover haploids (Winzeler et al., 1999). Of the 31 strains so dissected, 25 were able to germinate and live as viable haploids and 20 of these grew sufficiently robustly to allow testing (see Tables 4-1, 4-2 and 4-3). These strains were then transformed with a plasmid expressing Hmg2p-GFP, and compared with Hmg2p-GFP in two wild type strains from the same background.

Flow cytometry was again employed to determine whether the deleted genes had any involvement in Hmg2p-GFP stability. The twenty mutant strains and two wild type strains from the same background were grown to early log phase then their Hmg2p-GFP levels determined. Four of the strains tested, which featured deletions of *JJJ1*, *JJJ2*, *XDJ1* or *SSE2*, had Hmg2p-GFP steady-state levels somewhat below wild type (1.6 fold, 1.3 fold, 1.5 fold and 1.3 fold, respectively, shown in Figures 4-5 and 4-

6). The strains bearing deletions in *SSZ1* or *ZUO1* had substantial reductions in Hmg2p-GFP steady state levels (2.4 fold and 6.2 fold, respectively, shown in Figure 4-7). Only one deletion, the loss of *HLJ1*, resulted in an increased level of Hmg2p-GFP above wild type (2.1 fold, Figure 4-8, A).

As noted above, not all of the heterozygous diploid collection strains were successfully dissected and grown as haploids. In some of these cases, a PCR-mediated deletion approach was used in the lab strain background. *CWC23*, *SSB1*, *SSE1* and *YDJ1* were all deleted by this method, with no apparent effect. To test the requirement for Hsp104, a plasmid expressing Hmg2p-GFP was transformed into a pair of strains with *HSP104* either present or deleted (Figure 4-8, B). The absence of Hsp104 had a dramatic effect on Hmg2p-GFP steady-state levels, resulting in a substantial decrease (5.3 fold). To attempt to determine whether this effect involved Hmg2p-GFP synthesis or enhanced degradation of the mature protein, both the wild type and *hsp104*Δ strains were treated with lovastatin for four hours, to reduce degradation signal and stabilize Hmg2p-GFP. In both cases, Hmg2p-GFP steady-state levels rose by the same amount (data not shown), suggesting that while Hsp104 was required for Hmg2p-GFP synthesis and initial folding, it did not affect its ability to respond to regulation.

Table 4-1.
DnaJ homolog deletions.
Strains to evaluate the effects of absence of the genes indicated below were prepared from the deletion collection or by PCR in a lab background, as indicated. Under "Fold change," NE stands for No Effect (fold did not change), while a number with a multiplier indicates a fold increase (if positive) or decrease (if negative) in Hmg2p-GFP abundance in the deleted strain relative to a wild type control.

Gene	Tetrad-derived	PCR-derived	Fold change
APJ1	Viable	-	NE
CAJ1	Viable	-	NE
CWC23	Not viable	Viable	NE
DJP1	Viable	-	NE
ERJ5	Viable	-	NE
HLJ1	Viable	-	+2.1x
HUA1	Viable	-	NE
JAC1	Not viable	-	Not tested
JEM1	Viable	-	NE
JID1	Viable	-	NE
JJJ1	Viable	-	-1.6x
JJJ2	Viable	-	-1.3x
JJJ3	Viable	-	Not tested
MDJ1	Barely viable	-	Not tested
SCJ1	Viable	-	NE
XDJ1	Viable	-	-1.5x
YDJ1	Not viable	Viable	NE
ZUO1	Viable		-6.2x

Table 4-2.
BiP-related gene deletions.
See Table 4-1 for additional explanation.

Gene	Tetrad-derived	PCR-derived	Fold change
ECM10	Viable	-	NE
LHS1	Viable	-	Not tested
SSB1	Not viable	Viable	NE
SSE1	Not viable	Viable	NE
SSE2	Viable	-	-1.3x
SSQ1	Not viable	-	Not tested
SSZ1	Viable	-	-2.4x

Table 4-3.
Other deletions.
See Table 4-1 for additional explanation.

Gene	Tetrad-derived	PCR-derived	Fold change
EUG1	Viable	-	NE
HSF1	NA	NA	-1.4x
HSC82	Viable	-	NE
HSP78	Viable	-	NE
HSP82	Viable	-	NE
HSP104	NA	NA	-5.3x
MGS1	Viable	-	Not tested
SSA1	NA	NA	+1.4x

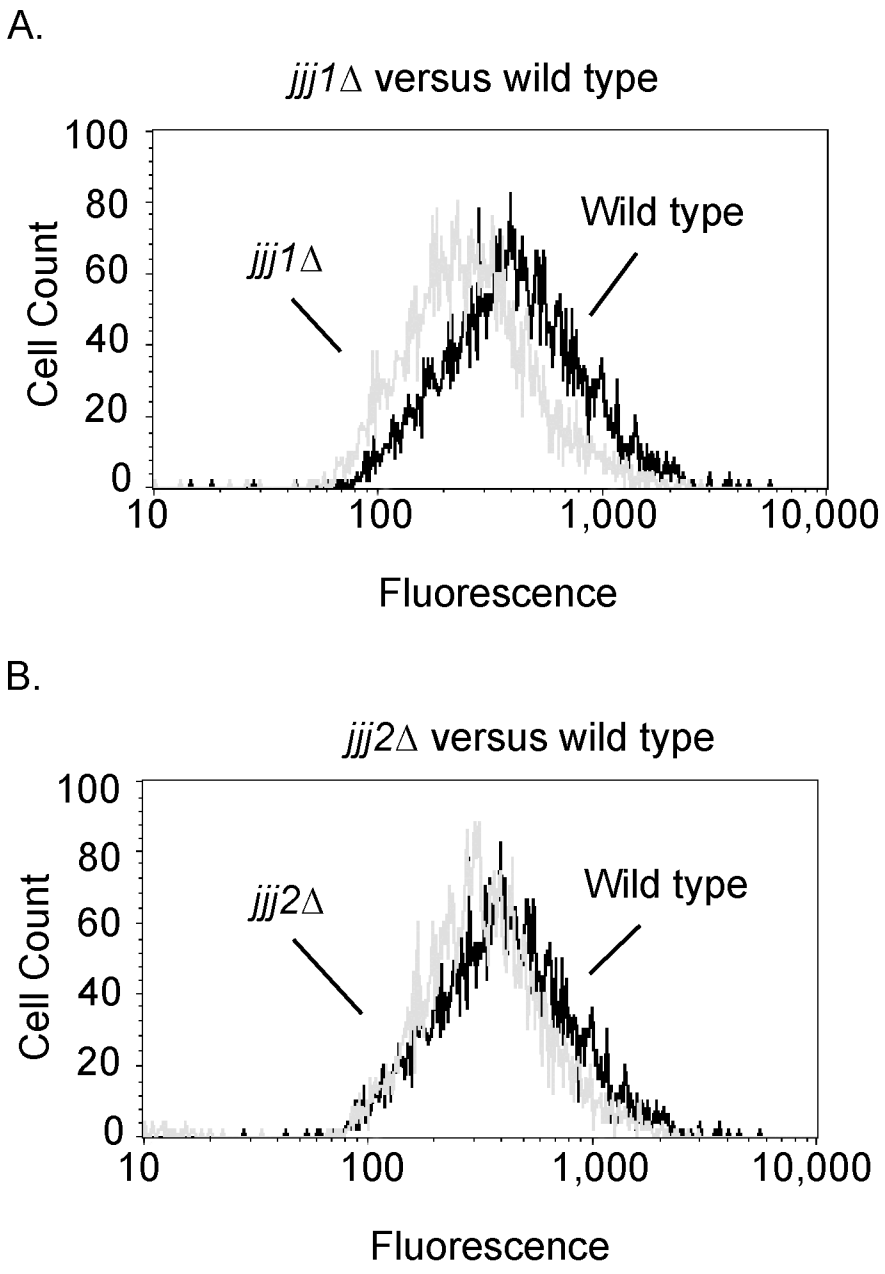

Figure 4-5
Deletions I: *jjj1*Δ and *jjj2*Δ have small effects on Hmg2p-GFP stability.
(A) Cells expressing Hmg2p-GFP in a wild type or *jjj1*Δ background were grown to early log phase and then analyzed for GFP fluorescence via flow cytometry. Each histogram represents 5,000 cells. (B) Hmg2p-GFP was tested in a wild type or *jjj2*Δ background as described in A.

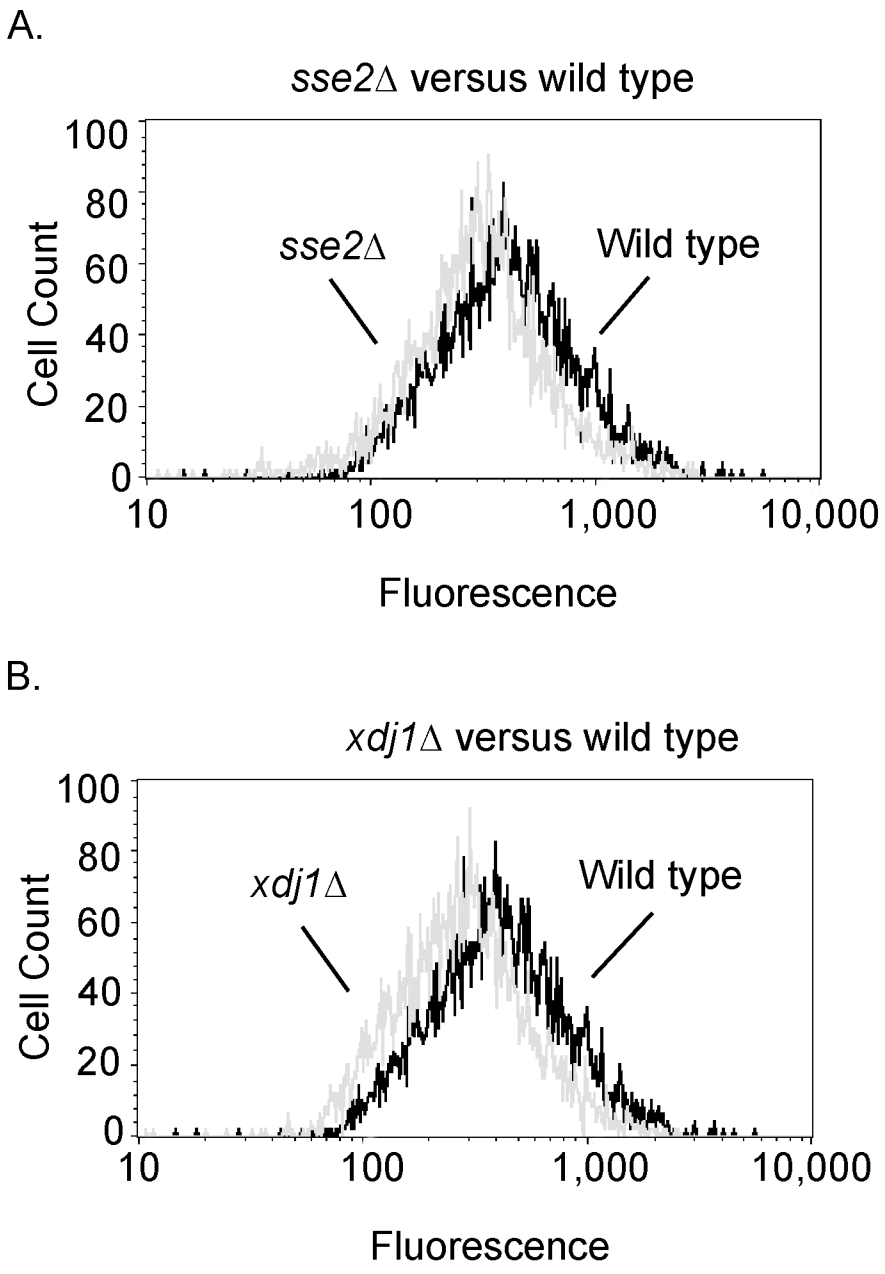

Figure 4-6
Deletions II: *sse2*Δ and *xdj1*Δ have small effects on Hmg2p-GFP stability.
(A) Cells expressing Hmg2p-GFP in a wild type or *sse2*Δ background were grown to early log phase and then analyzed for GFP fluorescence via flow cytometry. Each histogram represents 5,000 cells. (B) Hmg2p-GFP was tested in a wild type or *xdj1*Δ background as described in A.

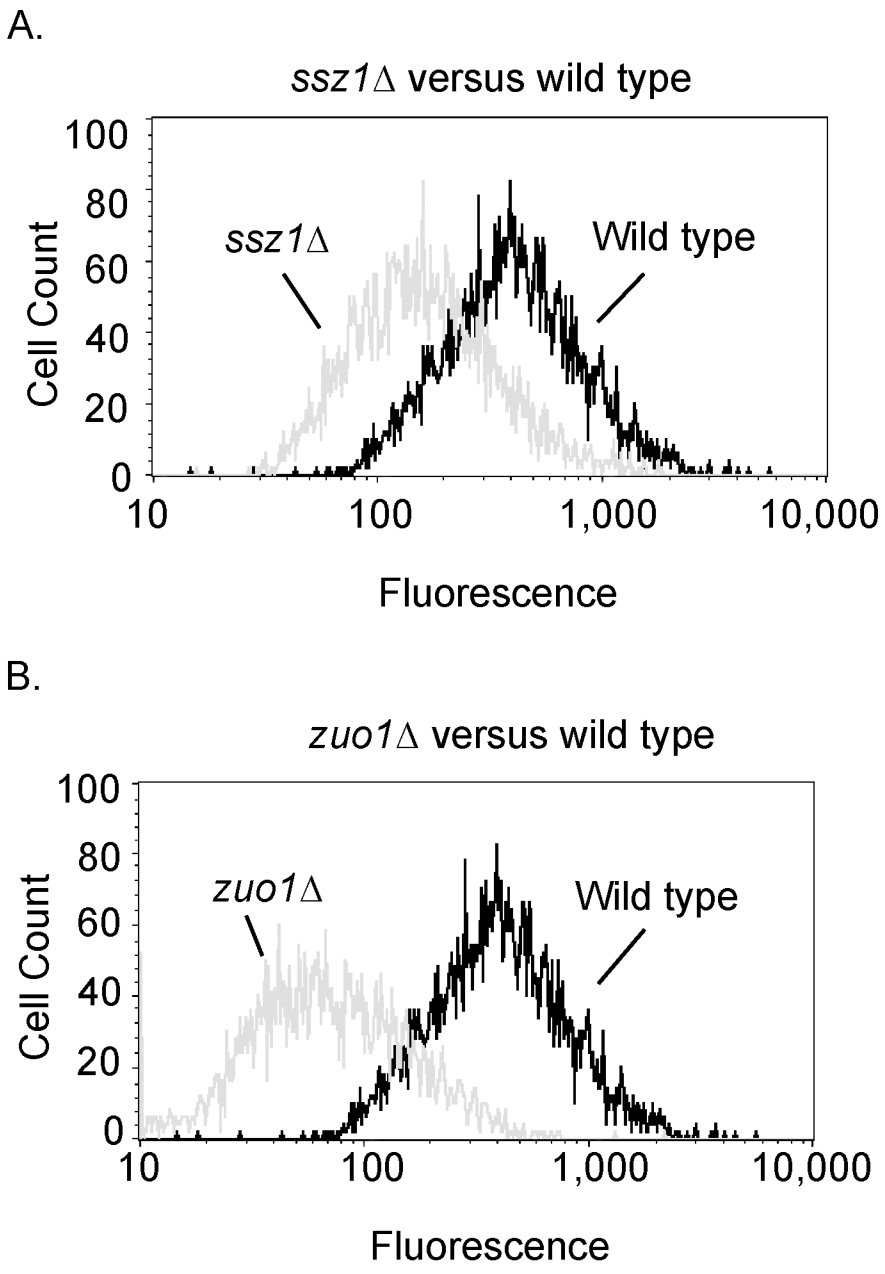

Figure 4-7
Deletions III: *ssz1*Δ and *zuo1*Δ have substantial effects on Hmg2p-GFP stability. (A) Cells expressing Hmg2p-GFP in a wild type or *ssz1*Δ background were grown to early log phase and then analyzed for GFP fluorescence via flow cytometry. Each histogram represents 5,000 cells. (B) Hmg2p-GFP was tested in a wild type or *zuo1*Δ background as described in A.

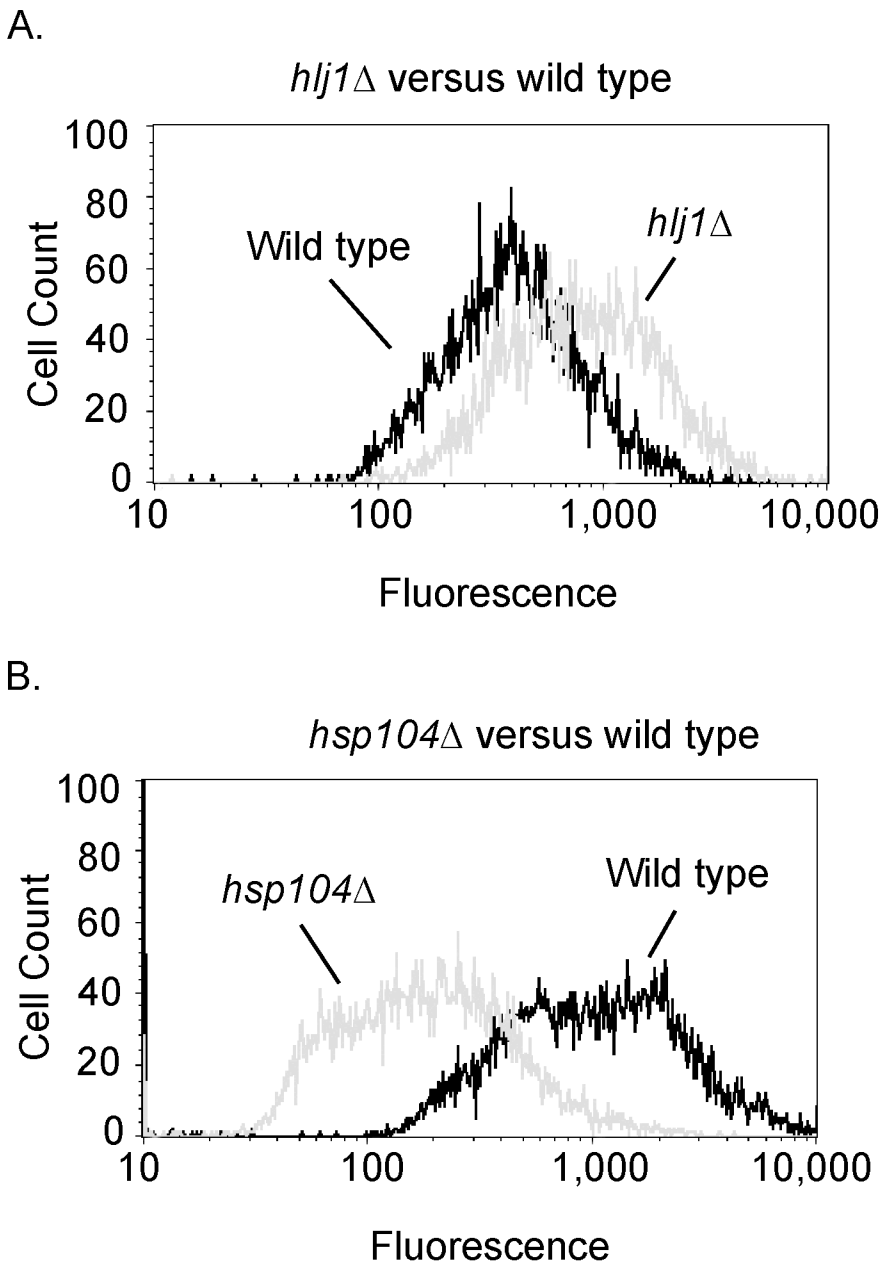

Figure 4-8
Deletions IV: *hlj1*Δ and *hsp104*Δ have dramatic effects on Hmg2p-GFP stability. (A) Cells expressing Hmg2p-GFP in a wild type or *hlj1*Δ background were grown to early log phase and then analyzed for GFP fluorescence via flow cytometry. Each histogram represents 5,000 cells. (B) Hmg2p-GFP was tested in a wild type or *hsp104*Δ background as described in A.

Though the strains already tested and awaiting tests (see Tables 4-1, 4-2 and 4-3) comprise a substantial number of known or putative chaperones involved in general ERAD, there is evidence for additional proteins that might have a chaperone-like activity particularly geared toward Hmg2p specifically. The *NSG* genes were originally discovered during an overexpression screen by Stephen R. Cronin, and additional research has defined their role in Hmg2p regulation (Flury, I, Shearer AG, Rosen J, Hampton RY, manuscript in preparation). These genes are required for stabilization of Hmg2p when it is expressed at genomic levels. When the *NSGs* are overexpressed in the presence of similarly overexpressed Hmg2p, the protein is stabilized. These facts strongly suggested a chaperone role for the Nsg proteins. To directly test for Nsg1p chaperone activity, I employed a limited proteolysis assay, as described in detail in chapter two. Strains were developed which expressed a normally regulated variant of Hmg2p-GFP bearing a myc epitope tag in its first lumenal loop and with wild type *NSG1* expression or with additional *NSG1* expressed from a *TDH3* promoter. Microsomes were prepared from both strains and subjected to limited proteolysis with trypsin to detect changes in conformation or general proteolytic rate (Figure 4-9). In microsomes from the *NSG1* overexpressing strain, Hmg2p-GFP showed a markedly decreased proteolytic rate, similar to what is seen when a chemical chaperone such as glycerol is present (Shearer and Hampton, 2004).

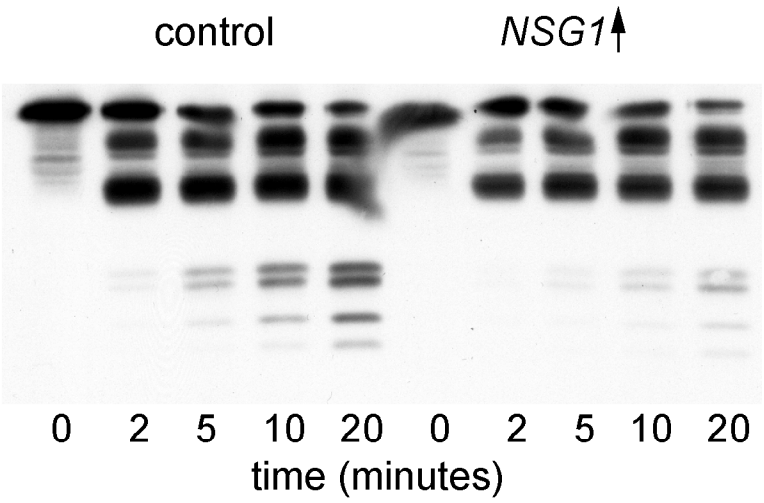

Figure 4-9
Overexpressed *NSG1* reduces proteolytic accessibility of Hmg2p-GFP.
Microsomes were prepared from strains expressing Hmg2p-GFP with or without overexpressed *NSG1* then incubated with trypsin (15 μg/ml) at 30° C. Samples were removed at the indicated times and evaluated by 14% SDS-PAGE and immunoblotting of the lumenal Myc epitope.

Discussion

The role of chaperones in ERAD, especially as it pertains to integral membrane proteins, is still unclear. General conclusions have been made based on work with such proteins as mammalian CFTR and the constructed model substrate CTG* (Taxis et al., 2003; Zhang et al., 2001). In the latter case, degradation appears to depend on Kar2p and Ssa1p, and to a lesser extent on Hsp104p, Cwc23p, Hlj1p and Jid1p. The work described in this chapter offers a first look at which chaperones are involved in this process in a natural ERAD substrate in its normal, cellular context.

Based on this preliminary work, a number of chaperones are involved in some way with Hmg2p-GFP steady-state levels, but few appear to have a major influence on its degradation. The Hsp70 *SSA1* appears to have some role in Hmg2p-GFP based on steady-state results at 30˚ C, but inactivation of the chaperone at 37˚ C had no additional effect. It is possible that Hmg2p, which is itself sensitive to increased temperatures, loses any requirement for Hsp70s at 37˚ C. There is a major requirement for Hsp104p in maintaining normal steady-state levels of Hmg2p, but it is unclear at the moment whether this represents a role in synthesis or if Hmg2p is in constant need of Hsp104p's chaperone activity to prevent spontaneous misfolding. It is clear, however, that what Hmg2p does exist in an *hsp104*Δ strain is properly regulated. Deletion of another four putative chaperones, *JJJ1*, *JJJ2*, *XDJ1* and *SSE2*, resulted in

slight reduction in Hmg2p-GFP steady-state levels in the same vein, if not to the same magnitude as the *hsp104*Δ strain, possibly indicating a similar requirement in folding of nascent Hmg2p or in maintenance of the mature protein, though additional degradation testing is required to confirm this, as chaperones may also be required for maintenance of mature protein. Though deletions of *SSZ1* and *ZUO1* led to dramatically decreased Hmg2p-GFP levels, they are known to work together in a complex with the ribosome, assisting folding of newly synthesized proteins, and thus are also likely to play their part during synthesis and folding rather than degradation (Gautschi et al., 2001).

It is still entirely possible that some of the reduced steady-state levels are due to enhanced degradation, as was the case in the *hsf-1[1-583]* strain. Note that it remains unclear which of the genes controlled by this transcription factor play a part in stabilizing Hmg2p-GFP. The two yeast Hsp90s, one of which is a target of *HSF-1*, showed no effect upon individual deletion. It is quite possible that *HSF-1* controls a body of chaperones that provide a safety net for proteins, catching errant misfolders, and the loss of any single target gene will not have an appreciable effect. Perhaps double mutants in the Hsp90s might result in some destabilization of Hmg2p-GFP. Still, even with a definite role outside of nascent protein folding, the targets of *HSF-1* are not required for degradation, since Hmg2p-GFP disappears more rapidly in their the transcription factor's absence. Rather, they, and possibly others among the proteins discussed above, may be present to allow refolding of transiently misfolded

proteins, reducing the loss of otherwise functional protein. This fits well with the role of heat shock proteins in responding to cellular stresses.

Only one of the genes tested was definitively required for ERAD of Hmg2p, as demonstrated by the marked stability of Hmg2p-GFP in its absence. As shown above, deletion of *HLJ1* led to higher Hmg2p-GFP steady state levels. Again, this has not been specifically confirmed as a degradation effect, but it is difficult to imagine how deletion of a chaperone could increase a protein's abundance unless its normal role were retention or binding of substrates for degradation.

How then, does Hmg2p-GFP compare with the other integral membrane substrates in terms of chaperone requirements? Like both CFTR and CTG*, it was stabilized by loss of Hsp70, though nowhere near as dramatically as those model substrates. It differs from CTG* in a lack of requirement for Jid1p, Cwc23p and Hsp104p (note that while loss of Hsp104p reduced Hmg2p-GFP abundance, its absence stabilizes the CTG* model substrate). The main point of similarity between Hmg2p-GFP and CTG* is a requirement for Hlj1p. This is especially interesting in that Hmg2p-GFP is exactly what CTG* was intended to represent as a class – a transmembrane ERAD substrate with a tightly folded cytosolic domain. In fact, they share the very same cytosolic domain – GFP. There are obvious differences between the two substrates, of course. Most blatantly, Hmg2p-GFP has eight transmembrane spans and CTG* has only one. CTG* is also a point mutant, whereas Hmg2p-GFP

can selectively acquire vaguely defined traits of misfolding. Still, it is unclear why CTG* degradation depends on many more known chaperones than Hmg2p-GFP degradation, though it merits mention that the studies on the CTG* model substrate were carried out using pulse-chase analysis rather than whole-pool degradation. There is precedent from prior work with the ERAD substrate Hmg2p for significant discrepancy between stabilization effects as measured by pulse-chase and whole-pool degradation methods, though the reasons for this difference remain unclear (Hampton and Rine, 1994). Additional study of the chaperone dependence of the degradation of Hmg2p-GFP mutants that are constitutive ERAD substrates would be illuminating on this topic, as would a revised approach to the artificial model substrate.

The *NSG* genes offer some insight into the mechanism of chaperone action on Hmg2p. Identified as part of a high copy screen for factors that led to increased Hmg2p in the cell, the *NSGs* are required at their normal, genomic levels for proper stabilization of this protein (Flury, I, Shearer, AG, Rosen, J, Hampton, RY, manuscript in preparation). These genes, coding for ER-resident, multi-spanning transmembrane proteins, are homologues of the mammalian Insig proteins, which are required for the general cholesterol regulation pathway and the cholesterol-dependent degradation of HMGR (Sever et al., 2003a; Sever et al., 2003b; Yang et al., 2002). Whereas in mammals the Insigs interact with the regulatory protein SCAP and with HGMR, the yeast Nsgs have so far only been implicated in the specific stabilization of

Hmg2p. It is intriguing to note that the deletion singly of either *NSG1* or *NSG2* has no detectable outcome on the abundance of overexpressed Hmg2p, suggesting that perhaps other factors could also be present which are critical to wild type levels of the protein, yet which have been missed in screens and tests that use only overexpressed Hmg2p (Flury, I, Shearer, AG, Rosen, J, Hampton, RY, manuscript in preparation). Indeed, this concern applies to any ERAD substrate, including CTG* and other model substrates, which are similarly evaluated when expressed at well above normal levels. Countering this idea, at least in the case of Hmg2p, is the fact that the *NSGs* were the only genes found to be high copy stabilizers of Hmg2p. The limited proteolysis results of *NSG* overexpression were striking, featuring a reduction in proteolysis quite similar to that achieved with chemical chaperones such as glycerol. This was the first evidence that *NSG*s achieved stabilization of Hmg2p through a chaperone activity. It would be interesting and educational to carry out a similar assay on other strains with altered chaperone function, to see whether or not they also have direct outcomes on Hmg2p structure.

These preliminary results suggest that additional information is necessary before a coherent picture of chaperone requirements in ERAD can be assembled. The axes already proposed – soluble versus integral, lumenal versus cytosolic misfolding, with or without a tightly folded cytosolic domain – fall short of representing all the possible variations in degradation substrates (Taxis et al., 2003; Vashist and Ng, 2004;

Zhang et al., 2001). Comparing required chaperones across multiple substrates provides a means of understanding the situation in the ER. One possible method would make use of the extensive library of Hmg2p mutants, many of which yield constitutively degraded versions of the protein. These mutants could be tested for their chaperone requirements, and subsequently for the effect of those same chaperones or their absence in the limited proteolysis assay. In this way, a natural substrate would be used to tease out a more specific understanding of the role of molecular chaperones in ERAD.

Materials and Methods

Materials

Restriction enzymes was obtained from New England Biolabs. 9E10 cell culture supernatant was produced from cells (CRL 1729, American Type Culture Collection) grown in RPMI1640 culture medium (GIBCO BRL) with 10% fetal calf serum and supplements. HRP-conjugated goat anti-mouse antibodies were purchased from Jackson Immuno Research. Chemiluminscence immunodetection reagents were obtained from Perkin Elmer. All other chemical reagents were obtained from Sigma or Fisher.

Yeast culture and strains

Yeast strains were grown in minimal media (Difco Yeast Nitrogen Base) with glucose and the appropriate supplements as described previously, or in YPD, if so noted (Hampton and Rine, 1994). Experiments were performed at 30˚ C unless otherwise noted. Yeast was transformed with plasmid DNA by the LiOAc method.

Hsp70 tester strains RHY2815 and RHY2816 were respectively derived from *SSA1* and *ssa1-45* strains provided by the lab of Jeffrey L. Brodsky at the University

of Pittsburgh. The Hmg2p-GFP testing strains were created by targeted integration of pRH469 at the StuI site of the *ura3-52* locus in both of the strains.

HSF-1 tester strains RHY2840 and RHY2841 were respectively derived from *HSF-1[1-583]* and *HSF-1* strains provided by the lab of Kevin A. Morano at the University of Texas Houston Medical School. The Hmg2p-GFP testing strains were created by targeted integration of pRH469 (Gardner et al., 1998) at the StuI site of the *ura3-52* locus in both of the strains.

NSG1 overexpression and control strains were derived from the lumenal-tag tester strain RHY2723 (Shearer and Hampton, 2004) by targeted insertion of either the *TDH3*-driven *NSG1*plasmid pRH1791 or the *ADE2* control plasmid pRH1579 at the HpaI site of the *ade2* locus. These strains were evaluated by flow cytometry for the expected stabilization of Hmg2p-GFP by overexpressed *NSG1*, then the limited proteolysis tester strains RHY3485 and RHY2486 were made by deletion of *HRD1* with the *hrd1*Δ*::KanMX* deletion cassette from pRH1122 (Gardner and Hampton, 1999).

Haploid deletion strains were derived from the Saccharomyces Genome Deletion Consortium heterozygous diploid collection by sporulation and dissection of tetrads, then replica plating and selection on appropriate selective media. The Hmg2p-GFP testing strains were then created by targeted integration of the plasmid pRH613

at the PflmI site of the *ADE2* locus into each of the appropriate derived haploid strains RHY3609-3633.

HSF-1 tester strains RHY2840 and RHY2841 were respectively derived from *HSF-1[1-583]* and *HSF-1* strains provided by the lab of Kevin A. Morano at the University of Texas Houston Medical School. The Hmg2p-GFP testing strains were created by targeted integration of pRH469 at the StuI site of the *ura3-52* locus in both of the strains.

Hsp104 tester strains RHY3607 and RHY3608 were respectively derived from *HSP104* and *hsp104*Δ strains provided by the lab of Susan Lindquist at the Whitehead Institute for Biomedical Research. The Hmg2p-GFP testing strains were created by targeted integration of pRH469 at the StuI site of the *ura3-1* locus in both of the strains.

The PCR-deletion strains were made by PCR amplification of the target locus from the appropriate heterozygous diploid strain. The resulting DNA was then transformed into RHY 522 and deleted isolates selected on YPD with G418. Primers for each locus are listed on Table 4-7.

Flow cytometry of yeast cells

Flow cytometry was carried out as described previously (Cronin et al., 2000). Briefly, cells were grown to early log phase (OD_{600} 0.1) in minimal medium, then incubated with drugs for the times indicated and analyzed for individual cell fluorescence with a BD Biosciences FACSCalibur flow cytometer. All histograms in this chapter represent results from 5,000 or 10,000 cells.

Microsome preparation

Microsomes were prepared as described previously (Shearer and Hampton, 2004). Briefly, cells were grown to mid log phase in YPD, pelleted, and subjected to hypoosmotic lysis. The resulting lysates were cleared of debris, then centrifuged to pellet microsomes, which were then resuspended in physiological buffer at 6µl buffer per OD of starting cells.

Limited proteolysis assay on microsomes

The assay was performed on microsomes as described (Shearer and Hampton, 2004). Briefly, a no-trypsin control aliquot was removed from the microsomes, then

trypsin was added and the remaining microsomes incubated at 30° C. Aliquots were removed at the indicated times and the reaction halted by addition of SDS-urea sample buffer.

Samples were resolved on 14% SDS-PAGE, then transferred with 15% methanol to nitrocellulose and immunoblotted for myc epitope, all as described.

Table 4-4.
Yeast strains used in this chapter, part one.

RHY522	*MATa ade2-101 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 lys2-801 met2-1 ura3-52::URA3:HMG2-GFP*
RHY2815	*MATalpha his3-11,15 leu2-3,112 ssa2-1 ssa3-1 ssa4-2 trp1-Δ1 ura3-52::URA3::HMG2-GFP*
RHY2816	*MATalpha his3-11,15 leu2-3,112 ssa1-45 ssa2-1 ssa3-1 ssa4-2 trp1-Δ1 ura3-52::URA3::HMG2-GFP*
RHY2840	*MATalpha ade2-1 can1-100 his3-11,15 hsf1Δ::LEU2 trp1 ura3-52::URA3::HMG2-GFP (with pRS314-HSF[1-583])*
RHY2841	*MATalpha ade2-1 can1-100 his3-11,15 hsf1Δ::LEU2 trp1 ura3-52::URA3::HMG2-GFP (with pRS314-HSF)*
RHY3359	*MATa ade2::ADE2 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 lys2-801 met 2 ura3-52::URA3::HMG2cd::myc$_L$-HMG2-GFP*
RHY3360	*MATa ade2::ADE2::NSG1 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 lys2-801 met 2 ura3-52::URA3::HMG2cd::myc$_L$-HMG2-GFP*
RHY3485	*MATa ade2::ADE2 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX lys2-801 met2 ura3-52::URA3::HMG2cd::myc$_L$-HMG2-GFP*
RHY3486	*MATa ade2::ADE2::NSG1 his3Δ200 hmg1Δ::LYS2 hmg2Δ::HIS3 hrd1Δ::KanMX lys2-801 met2 ura3-52::URA3::HMG2cd::myc$_L$-HMG2-GFP*
RHY3607	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1::URA3:HMG2-GFP*
RHY3608	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1::URA3:HMG2-GFP hsp104Δ::LEU2*

Table 4-5.
Yeast strains used in this chapter, part two.
For the strains on this table, the status of the *MET15* locus and the mating type have not been determined.

RHY3609	*apj1Δ::KanMX his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
RHY3610	*caj1Δ::KanMX his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
RHY3611	*djp1Δ::KanMX his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
RHY3612	*ecm10Δ::KanMX his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
RHY3613	*erj5Δ::KanMX his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
RHY3614	*eug1Δ::KanMX his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
RHY3615	*his3Δ1 hlj1Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3616	*his3Δ1 hsc82Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3617	*his3Δ1 hsp78Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3618	*his3Δ1 hsp82Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3619	*his3Δ1 hua1Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3620	*his3Δ1 jem1Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3621	*his3Δ1 jid1Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3622	*his3Δ1 jjj1Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3623	*his3Δ1 jjj2Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3624	*his3Δ1 jjj3Δ::KanMX leu2Δ0 lys2Δ0 ura3Δ0*
RHY3625	*his3Δ1 leu2Δ0 lys2Δ0 mgs1Δ::KanMX ura3Δ0*
RHY3626	*his3Δ1 leu2Δ0 lys2Δ0 scj1Δ::KanMX ura3Δ0*
RHY3627	*his3Δ1 leu2Δ0 lhs1Δ::KanMX lys2Δ0 ura3Δ0*
RHY3628	*his3Δ1 leu2Δ0 lys2Δ0 sse2Δ::KanMX ura3Δ0*
RHY3629	*his3Δ1 leu2Δ0 lys2Δ0 ssz1Δ::KanMX ura3Δ0*
RHY3630	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 xdj1Δ::KanMX*
RHY3631	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 zuo1Δ::KanMX*
RHY3632	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*
RHY3633	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*

Table 4-6.
Plasmids used in this chapter.

pRH613	Expresses *HMG2-GFP* from a *TDH3* promoter
pRH1122	Contains *hrd1*Δ::KanMX deletion cassette
pRH1581	Expresses lumenally myc-tagged *HMG2-GFP* from a *TDH3* promoter
pRH1579	*ADE2*-marked control
pRH1692	Expresses lumenally myc-tagged *HMG2-GFP* bearing the TFYSA to ILQAS patch NR1-GFP mutant from a *TDH3* promoter
pRH1791	Expresses *NSG1* from a *TDH3* promoter

Table 4-7.
PCR primers used in this chapter.

oRH1815	5’ for *CWC23* knockout (ATCAAAATCCTCTCCCCACGA)
oRH1816	3’ for *CWC23* knockout (AACCAGGAGTAACTCTCTTTGCAG)
oRH1819	5’ for *JAC1* knockout (TACAACAATTCCTCAACGGG)
oRH1820	3’ for *JAC1* knockout (ACAAGGAATTTGGAACGTGC)
oRH1823	5’ for *SSB1* knockout (ACACACGGGACTTGATCGAA)
oRH1824	3’ for *SSB1* knockout (CCGAAGGTTTGCCAGTTAAA)
oRH1827	5’ for *SSE1* knockout (ATGTTAGCGTGTGCAAACTACC)
oRH1829	3’ for *SSE1* knockout (AAGGCTGGGAAGATATTCCTGT)
oRH1831	5’ for *YDJ1* knockout (AGATGTGAAGTCGCTGGTTTG)
oRH1832	3’ for *YDJ1* knockout (CAGTCAAAGCCAAAAAGGGA)

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Chapter 5

Toward the future

In this text, I have described a mechanism by which a natural, wild type protein undergoes a structural change in the presence of a small molecule, becomes more misfolded as a consequence of that change, and is thus recognized and degraded by a constitutive quality control pathway. I believe that Hmg2p regulation is just the first example of a regulatory mechanism that may well prove to be as ubiquitous as quality control itself. Should this be the case, I hope my studies on Hmg2p will serve as a useful guide and inspiration for additional elucidation of regulation of this kind.

In the introduction, I mentioned four key issues that my work addressed. They are repeated here with my conclusions, to serve as a starting point for the discussion of possible directions for future research:

1) Whether Hmg2p undergoes a structural transition

Hmg2p undergoes a structural transition that correlates directly with its tendency to be ubiquitinated and degraded in vivo*, as described in chapters two and three.*

2) The identity and action of the degradation signal

Farnesol, which accumulates in cells under conditions when the degradation signal should be abundant, leads to a structural transition of the same kind observed following buildup of in vivo *signal. Though this is not definitive evidence that farnesol is the signal, the evaluation of farnesol and other lipids described in chapter three strongly points toward farnesol as a degradation signal that acts by inducing a conformational change in Hmg2p.*

3) Whether Hmg2p acquires quality control traits

Hmg2p becomes less well folded under conditions that lead to increased proteolytic susceptibility in vitro*, as determined by thermal denaturation assay.*

4) Which chaperones are involved in Hmg2p degradation

Preliminary work on this topic shows that the chaperone requirements for Hmg2p degradation differ substantially from the few other substrates tested to date.

Thus, the present model of Hmg2p degradation is very similar to the hypothesis I began with. In the absence of degradation signal, Hmg2p has the features of a properly folded protein and is ignored by the HRD pathway. When degradation signal is abundant, Hmg2p undergoes a structural change and acquires traits of a misfolded protein. It is then recognized by the constitutive HRD quality-control pathway, ubiquitinated and degraded. Farnesol is probably the degradation signal, though the specific details of its interactions with Hmg2p remain unclear. All the research directions described below originate in this basic model of the mechanism of Hmg2p regulation. Future work will serve to elaborate, explain and possibly change this understanding of Hmg2p regulation specifically, and the regulated degradation of normal proteins by constitutive quality-control pathways generally.

Unraveling the sterol-sensing domain

Mutations in the sterol-sensing domain of Hmg2p frequently lead to disrupted regulation (Gardner and Hampton, 1999). However, conserved residues in this motif are not universally required for proper regulated degradation of Hmg2p, or for the correlated structural response to farnesol treatment *in vitro*. A natural next step in understanding Hmg2p regulation is thus a very specific understanding of its structural basis. Are certain residues important to this change, perhaps indicating the presence of

a farnesol binding pocket? Or do all the known mutations that alter Hmg2p regulation reflect alterations in intraprotein interaction that take away its ability to sense changes in the membrane?

The first step in answering these questions involves examining the other yeast HMGR isozyme, Hmg1p. Hmg1p is not subject to regulated degradation in the manner of Hmg2p, but it shares extensive identity throughout its transmembrane domain. Indeed, part of the mutational analysis of Hmg2p involved swapping in nonidentifical portions of Hmg1p. Only some of these swaps disrupted regulation. Testing Hmg1p via the limited proteolysis and aggregation assays will help guide future work by discerning between two main possibilities. Hmg1p may be structurally responsive to farnesol *in vitro*, suggesting that it simply fails to engage the HRD pathway. Alternately, Hmg1p may not respond *in vitro*, indicating that some of its points of difference with Hmg2p truly matter for the structural response to farnesol and concomitant regulated degradation. Should this latter possibility prove true, comparison of Hmg1p and Hmg2p sequence will be critical in exploring the SSD.

In-depth evaluation of the role of the SSD in Hmg2p regulation depends on an understanding of both distantly and closely related proteins and subsequent testing of hypotheses based on that understanding. As mentioned in the preceding paragraph, if Hmg1p does not undergo a farnesol-dependent structural change *in vitro*, it will serve as a basic guide to which regions to explore for key residues. The Hmg2p sequence

swaps and changes that have already been tested further narrow the search space, ruling out broad portions of the protein. Additional guidance may also be found by comparing closely and distantly related HMGR sequences. As described in chapter 3, the nonresponder mutant NR1 appears to involve a change in a consensus sequence conserved across HMGRs from organisms as diverse as fungi and mammals. That consensus sequence may be generally required for the kind of structural change I have observed in the Hmg2p SSD and may be present in all SSDs. This is distinct from residues required for interaction with a specific small molecule signal such as farnesol. The two types of residues can potentially be separated by comparing identities between Hmg2p and distant relatives, such as mammalian HMGR and identities present only between Hmg2p and close relatives, such as other fungal HMGRs. The first class might represent residues critical to the general SSD structural response, and the second, residues required to bind or detect farnesol. In this way, a survey of the full range of HMGRs should provide useful, testable hypotheses to evaluate *in vivo* and *in vitro* concerning the structural requirements for general and specific SSD action.

It has been suggested that changes in mammalian HMGR degradation rate are due to changes in its oligomerization state (Cheng et al., 1999). Though the experiments used to evaluate this did not adequately test this possibility, it represents a reasonable model for how changes in the structure of an SSD protein might translate into altered degradation rates. This is testable for Hmg2p, using an adaptation of a

crosslinking assay previously applied to observing changes in the interaction between Hmg2p and proteins involved in its degradation (Gardner et al., 2001).

Structural changes in other SSD proteins

My results with Hmg2p mark the second instance of structural change in an SSD protein in response to a lipid. As discussed in chapter three, the sterol regulatory protein SCAP undergoes a structural change to reveal a trypsin site when in the presence of its *in vivo* signal molecule, cholesterol (Brown et al., 2002). That chapter also describes other SSD proteins which respond to a range of lipids in a number of organisms. Given these two known cases of a significant role for structural change in SSD protein function, it may be that a similar mechanism is at work in other proteins with SSD motifs.

This general model of SSD action through structural response can be tested using the limited proteolysis assay. A lumenally tagged version of mammalian HMGR already exists, as do membrane fractionation protocols for mammalian cells. In this and other systems, membranes containing tagged HMGR could be prepared, treated with small molecules and evaluated by limited proteolysis, quite similarly to the procedure described in chapter three. If the model suggested above is correct, one would expect to see HMGR from a given organism undergo structural change in the presence of its

specific signaling molecule, be it cholesterol for mammalian HMGR or phosphatidylethanolamine for drosophila SCAP (Brown et al., 2002; Dobrosotskaya et al., 2002).

Farnesol and its mechanism of action

The work described in chapter three strongly implies that farnesol is the *in vivo* degradation signal and that it acts by promoting a structural change in Hmg2p, one that results in the acquisition of traits of a quality control substrate. This part of the story is not concrete, however. Farnesol is abundant *in vivo* when degradation is rapid, and structural change *in vitro* is both pronounced and very specific to farnesol, but this molecule has not been shown to interact directly with Hmg2p *in vivo* or *in vitro*.

Testing for an effect of farnesol *in vivo* is not as straightforward as one might hope. In the yeast *Saccharomyces cerevisiae*, exogenous farnesol is toxic at relatively modest doses (Machida et al., 1999). This toxicity pathway is mitochondrial, and appears to be independent of the potential role of farnesol in regulation of Hmg2p in the ER (Machida and Tanaka, 1999). Indeed, the presence of abundant farnesol in cells and media when degradation is rapid suggests that yeast farnesol derived from endogenous FPP is present in the ER and then trafficked out of the cell, never interacting with the cell's mitochondria. The problems that occur with exogenous

addition of farnesol probably are a consequence of an unnatural spread of farnesol through all the membranes of the cell.

An understanding of this toxicity suggests a number of additional *in vivo* farnesol assays that might circumvent its lethal effects on cells. First, farnesol could be added to mitochondrially nonfunctional *rho*$^-$ petite cells. The absence of mitochondrial function in these cells might similarly limit the generalized toxicity of farnesol. It may also be possible to express Hmg2p and its degradation machinery in a closely related organism such as another yeast, where toxicity may not be an issue. Finally, crosslinking could be used to show how farnesol addition alters *in vivo* relationships pertinent to Hmg2p regulation. Prior work in the lab has shown that under conditions when degradation signal is present, more Ubc7p crosslinks with Hmg2p. A pilot assay I carried out adding farnesol to living cells for a brief period prior to crosslinking suggested that it, too may lead to increased Hmg2p-Ubc7p crosslinking in the manner of *in vivo* degradation signal. However, given the generalized toxicity of endogenous farnesol, a number of controls must be tested to ensure that this outcome is specific to Hmg2p and not a consequence of disruption of normal metabolism or membrane coherence. Though the accident of mitochondrial farnesol toxicity makes evaluation of farnesol's *in vivo* role difficult due to the impossibility of successful incubation with farnesol, it should still be possible to tease out additional information using the methods described above.

How the presence of farnesol leads to structural change in Hmg2p is still an open question. A cholesterol-binding pocket has been proposed to exist in mammalian HMGR (Xu and Simoni, 2003). More recently, the cholesterol-sensing protein SCAP has been demonstrated to bind directly to cholesterol (Radhakrishnan et al., 2004). Given that SCAP undergoes a structural response to cholesterol, this suggests that Hmg2p, too, may bind directly to its signal molecule. This possibility can be tested via a number of methods. Photocrosslinkable farnesyl moieities exist, and could be added directly to microsomes to evaluate Hmg2p binding (Omer et al., 1993). Binding assays can also be attempted with radiolabeled farnesol in detergent-solubilized Hmg2p or in renatured Hmg2p on blots. Regardless of the method used, evidence of direct farnesol binding would be extremely helpful in understanding its action in Hmg2p regulation. This would be especially true in combination with the structural study of the SSD described above.

The role of chaperones in Hmg2p ERAD

The preliminary work described in chapter four makes it clear that our understanding of the role of chaperones in ERAD is far from complete. Increased comprehension of the effects of chaperones on Hmg2p synthesis, stability and degradation not only increases understanding of Hmg2p regulated degradation, it also

adds to the knowledge of chaperone requirements in ERAD generally. Indeed, Hmg2p could be an ideal tool to explore this part of the living cell.

Additional basic testing is required to develop a complete picture of chaperone requirements in Hmg2p degradation. More putative chaperones must be evaluated, and all chaperones with an effect on Hmg2p steady-state levels should be subject to additional testing to determine whether they are required for synthesis, stability or degradation.

Once an understanding of chaperone requirements for the ERAD of wild type Hmg2p has been reached, the whole range of Hmg2p mutants previously developed in the lab can be tested, with an eye toward exploring whether or not chaperone requirements actually change depending on the location of mutations in the substrate. The direct effects of chaperones on Hmg2p structure can also be evaluated by limited proteolysis, as was done with *NSG1* in chapter four. The tools that continue to be so useful for comprehending Hmg2p regulation should serve just as well in exploring the role of chaperones in ERAD.

The future, broadly

In the introduction, I mentioned my belief that the regulated degradation of specific proteins by constitutive quality control pathways will prove to be a general

biological principle that will turn up repeatedly in years to come. There is now a reasonable mechanism, supported by experimental evidence, for how this occurs in the case of Hmg2p. The potential studies discussed above would expand our understanding of Hmg2p and other SSD proteins. Beyond this, I hope that the combination of regulation and quality control will merit consideration in other cases of regulated degradation, especially when the specific details of that degradation are unclear. Perhaps a number of other examples of regulated degradation via quality control pathways are already out there, partially explored yet still unexplained.

Any advance in biological understanding has medical ramifications. Narrowly, if the regulated degradation of human HMGR occurs through a mechanism similar to that of Hmg2p, a whole new class of inhibitors of HMGR activity could be developed. Broadly, understanding how a protein can be made subject to a quality control pathway in a regulated fashion has implications for any condition that depends on the relative abundance of a protein. Given the plethora of human diseases that share that dependence, it is clear that each step toward such a general understanding is useful, as I hope this step has been.

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