UNIVERSITY OF CALIFORNIA, SAN DIEGO

A mechanistic characterization of the regulated degradation of the yeast 3-hydroxy-3-methylglutaryl coenzyme A reductase, Hmg2p

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biology

by

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2000

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DEDICATION

The blossoming of my life and the fruits reaped from its bountiful harvest are most certainly the result of a tender labor by those who persevered and toiled with compassion and selfless dedication, nurturing my growth and maturation. They opened their hearts, homes, lives and souls to comfort and direct my often wayward being. It is those beautiful spirits who rightfully deserve recognition and respect from others, and my deepest admiration, friendship and most heart-felt gratitude for their presence in my life.

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EPIGRAPH

It matters not how strait the gate, How charged with punishments the scroll, I am the master of my fate; I am the captain of my soul.

--William Ernest Henley.

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- **Gardner, R.**, Cronin, S., Leader, B., Rine, J., and Hampton, R. (1998). Sequence determinants for regulated degradation of yeast 3-hydroxy-3methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. Molecular Biology of the Cell 9, 2611-2626.
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ABSTRACT OF THE DISSERTATION

A mechanistic characterization of the regulated degradation of the yeast 3-hydroxy-3-methylglutaryl coenzyme A reductase, Hmg2p

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Proteasomal protein degradation is of critical importance to the normal functioning of the cell. One key role of proteasomal protein degradation is in ERassociated degradation, which is used both to destroy misfolded or mutant proteins as part of a quality control mechanism and to regulate the levels of some normal cellular proteins. To gain a better understanding of ER-associated degradation, we have been studying the degradation characteristics of the protein HMG-CoA reductase, a key enzyme of the mevalonate pathway from which sterols and other important compounds are synthesized. HMG-CoA reductase is an ER resident protein that catalyzes the ratelimiting, irreversible step of the mevalonate pathway and its activity is subject to a variety of feedback control mechanisms including ER-associated, feedback-regulated degradation. Taking advantage of the numerous genetic and biochemical opportunities available in yeast, we have explored the mechanism of degradation for the yeast HMG-CoA reductase isozyme Hmg2p. Through both genetic and pharmacological manipulation of mevalonate pathway enzymes, we have demonstrated that Hmg2p degradation is positively controlled by the downstream product of the mevalonate pathway farnesyl diphosphate (FPP). In addition, a secondary signal derived from the alternate oxysterol pathway acts to enhance the action of the primary FPP-derived signal. By site-specific mutagenesis, we have identified critical regions that are required for normal, regulated degradation within the Hmg2p transmembrane domain, which is both necessary and sufficient to program the mevalonate pathway-regulated, ER-associated degradation of Hmg2p. Specifically, we have discovered two critical lysine residues that function to bring about ubiquitination of Hmg2p. Through chemical cross-linking studies, we have revealed that the ER degradation machinery continually inspects all proteins that it can access for their degradation status. Stable proteins prevent access of the ER ubiquitin conjugating enzyme Ubc7p to any lysine residues. The regulated degradation substrate Hmg2p controls Ubc7p access to its critical lysine residues according to the abundance of downstream mevalonate pathway signals for degradation.

Chapter I: Introduction

It may happen in our daily life that we sometimes give an answer before we have truly and rightly asked the question.

--Martin Foss

Cellular Protein Degradation: Checkpoint Charlie and Quality Control Inspector #5.

The cell is a dynamic chemical reactor, well adapted to function in its environment by producing all the necessary components for assimilation and utilization of the resources that are available. The cell lives, grows, divides and dies by the presence, or absence, of specific protein compositions within its bounds. Many general cellular processes, such as metabolism, division and death, require timely production, and removal, of essential proteins. A lack of production or an inability to remove specific proteins will often lead to disastrous consequences for the cell. The cell avoids such dire situations by exerting rigorous control over protein synthesis through transcriptional and translational mechanisms, which have been well documented. In addition to protein production, the cell also controls protein levels through posttranslational, programmed destruction of proteins. However, most aspects of this regulatory mechanism are not understood even though it is an essential component of many key processes within the cell.

For cellular protein degradation to be a selective, efficient and timely regulatory mechanism in controlling protein levels, the cellular proteases must be readily accessible to their substrates, but have restricted ability to degrade proteins whose stability is desired. In some cases, the cell produces proteases that are specifically designed to cleave only certain protein substrates. This mode is not generally used to degrade proteins, but usually serves to modify a protein's activity or localization through very specific cleavage (YaDeau *et al.*, 1991; Melloni *et al.*, 1992; Branda *et al.*, 1995; Kumar, 1995; Sakai *et al.*, 1996). In other cases, the cell produces general

proteases in order to degrade a variety of very different substrates. However, release of a general protease into the cellular milieu would, in all likelihood, cause the random destruction of proteins not destined for degradation. Thus, the cell confines the activity of most general proteases to specific cellular compartments wherein proteins to be degraded are transported. The lysosome, or vacuole in yeast, serves as a main cellular degradation compartment, housing a variety of general proteases intended to degrade all manner of proteins sent there for destruction. Generally, proteins destined for degradation in the lysosome are marked as such and transported there through very specific mechanisms (Horazdovsky et al., 1995; Klionsky, 1997; Scott and Klionsky, 1998). Proteases that are to function in the lysosome are carefully packaged and transported in vesicles to avoid the mishap of their activity outside the defined compartment (Horazdovsky et al., 1995; Klionsky, 1997; Conibear and Stevens, 1998). In addition to the general proteases in the lysosome, the cell also contains an important general protease, the proteasome, that is distributed throughout the cytosol and nucleus (Peters et al., 1994; Enenkel et al., 1998; Wilkinson et al., 1998; Russell et al., 1999). The proteasome functions in the degradation of specific substrates and damaged or misfolded proteins (Hochstrasser, 1995; Smith et al., 1996; Kopito, 1997). To prevent random degradation of proteins not earmarked for destruction, the proteasome contains a regulatory complex that utilizes a specific targeting mechanism carried out by a hierarchy of auxiliary proteins (Jentsch, 1992; Ciechanover, 1994; Hochstrasser, 1996; Tanaka and Tsurumi, 1997). Protein degradation by the proteasome plays an absolutely vital function in many essential cellular processes, and the emerging importance of its

regulatory role in controlling specific cellular protein levels is beginning to rival those of transcription and translation.

The importance of proteasome-dependent degradation is underscored by the essential nature of the cellular pathways in which it serves a role (Table 1-1). Operation of the cell cycle cannot occur without the precise temporal degradation of the cyclins (Glotzer *et al.*, 1991; Hershko, 1997). The NF-κB-mediated inflammatory response is initiated by the proteasome-dependent degradation of its inhibitory factor IκBα (Scherer *et al.*, 1995). Certain growth-inducing regulatory proteins, such as p53 and c-Jun, are degraded by the proteasome, thereby curbing the growth of the cell and preventing the unchecked growth required for tumorigenesis (Scheffner et al., 1993; Chowdary et al., 1994; Treier et al., 1994; Musti et al., 1996; Scheffner, 1998). During extremely stressful conditions, such as starvation, cancer or infection, free amino acids are mobilized by hormone-induced stimulation of proteasome-dependent muscle protein degradation (Wing and Goldberg, 1993; Medina et al., 1995; Wing et al., 1995; Baracos et al., 1995). In addition, the immune system presents small random peptides on major histocompatability complex (MHC) class I molecules (Gaczynska et al., 1993; Rock, et al., 1994; Gaczynska et al., 1994), peptides which are generated by the slow proteasome-dependent degradation of a sample of most cellular proteins (Michalek et al., 1993; Rock et al., 1994).

Many metabolic pathways employ proteasome-dependent degradation as a feedback mechanism to prevent build-up of pathway products that may be toxic at high levels. Proteasome-dependent degradation of ornithine decarboxylase and spermidine/spermine N1-acetyltransferase occurs in response to increased, and

Substrate	Function	Reference
Mitotic cyclins and regulators	Cell cycle regulation	Glotzer <i>et al.</i> , 1991 Deshaies <i>et al.</i> , 1995 Yaglom <i>et al.</i> , 1995 King <i>et al.</i> , 1996a Willems <i>et al.</i> , 1996 Hershko <i>et al.</i> , 1997 Tokumoto <i>et al.</i> , 1997 Patrick <i>et al.</i> , 1998
p53 MATα2 c-Jun	Tumor Suppressor Transcription factor Transcription factor	Scheffner <i>et al.</i> , 1993,98 Hochstrasser <i>et al.</i> , 1990 Treier <i>et al.</i> , 1994 Musti <i>et al.</i> , 1996
Gcn4 ATF2 E2F ICER IRP2	Transcription factor Transcription factor Transcription factor Transcription inhibitor RNA binding protein	Kornitzer <i>et al.</i> , 1996 Fuchs <i>et al.</i> , 1999 Hateboer <i>et al.</i> , 1996 Folco <i>et al.</i> , 1997 Guo <i>et al.</i> , 1995
Mos	Ser/Thr protein Kinase	Iwai <i>et al.</i> , 1998 Nishizawa <i>et al.</i> , 1993
Gα ΙκΒ	Signal transduction Signal transduction	Madura <i>et al.</i> , 1994 Palombella <i>et al.</i> , 1994 Didonato <i>et al.</i> , 1996
Ornithine decarboxylase	Polyamine synthesis	Murakami <i>et al.</i> , 1992
Spermine/spermidine acetyltransferase Fructose bisphosphatase HMG-CoA reductase	Polyamine synthesis Gluconeogenesis Sterol synthesis	Coleman and Pegg, 1992 Schork <i>et al.</i> , 1995 Nakanishi <i>et al.</i> , 1988 Chun <i>et al.</i> , 1990 Hampton <i>et al.</i> , 1994
Tyrosine amino transferase Apolipoprotein B100	Tyrosine synthesis Lipoprotein synthesis	Gross-Mesilaty,1997 Fisher <i>et al.</i> , 1997 Wu <i>et al.</i> , 1997
Vph1p TCRα	Vacuolar ATPase Immune system surface receptors	Hill <i>et al.</i> , 1994, 1995 Lippincott <i>et al.</i> ,1988 Wileman <i>et al.</i> , 1993 Yu <i>et al.</i> , 1997
CFTR ΔF508	Quality control	Ward <i>et al.</i> , 1995
PiZ α-1 antitrypsin Sec61-2 CPY* Pdr5*	Quality control Quality control Quality control Quality control	Ciccarelli <i>et al.</i> , 1995 Sommer <i>et al.</i> , 1993 Finger <i>et al.</i> , 1993 Plemper <i>et al.</i> , 1998

Table 1-1: Partial list of degradation substrates of the proteasome

potentially toxic, cellular polyamine levels (Murakami *et al.*, 1992; Li and Coffino, 1992; Hayashi *et al.*, 1996; Coleman and Pegg, 1997). Tyrosine amino transferase is degraded by the proteasome to prevent buildup of cellular tyrosine levels (Gross-Mesilaty *et al.*, 1997). Fructose-1,6-bisphosphatase is targeted for proteasomedependent degradation as cellular glucose levels increase (Schork *et al.*, 1995). The cell controls cholesterol synthesis, in part, through the endoplasmic reticulum (ER) associated, and proteasome-dependent, degradation of the rate-limiting enzyme in the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-R) (Edwards *et al.*, 1983; Nakanishi *et al.*, 1988; Hampton and Rine, 1994).

Additionally, the cell utilizes proteasome-dependent degradation as a quality control mechanism, destroying mutant or misfolded proteins, often by an ER-associated mechanism. In mammalian cells, a mutant form of the cystic fibrosis transmembrane conductance regulator (CFTR), CFTR Δ F508 the most common allele of the genetic disease cystic fibrosis (Kerem *et al.*, 1989), is degraded rapidly at the ER by a proteasome-dependent quality control degradation mechanism (Cheng *et al.*, 1990; Ward *et al.*, 1995; Jensen *et al.*, 1995; Sato *et al.*, 1998; Xiong *et al.*, 1999). The Z variant (PiZ) of the hepatic secretory protein α -1 antitrypsin, which is the most abundant serine protease inhibitor in the plasma, is also known to be subject to ERassociated quality control degradation (Ciccarelli *et al.*, 1993; Qu *et al.*, 1996), often resulting in the disease phenotype of panlobular emphysema and cirrhosis (Carlson *et al.*, 1989). In yeast, a mutant form of the translocon pore protein Sec61p, termed Sec61-2p, is subject to rapid proteasome-dependent degradation at the ER (Sommer and Jentsch, 1993; Biederer *et al.*, 1996). Also, a mutant version of the vacuolar carboxypeptidase yscY, named CPY*, is retained in the ER where it is retrotranslocated and degraded by the proteasome (Finger *et al.*, 1993; Hiller *et al.*, 1996; Plemper *et al.*, 1997; Plemper *et al.*, 1999a).

In addition to mutant and misfolded proteins, quality control degradation of unassembled proteins of multi-subunit complexes also occurs in a proteasomedependent and often ER-associated fashion. In mammalian cells, proteasomedependent degradation of newly synthesized T cell receptor (TCR) α chains occurs when abundance of the other subunits is insufficient (Lippincott-Schwartz et al., 1988; Wileman et al., 1993; Yu et al., 1997; Yang et al., 1998). Apolipoprotein B100 (ApoB), the main protein component of lipoprotein particles, is increasingly degraded by the proteasome when there is reduced synthesis of the lipid components, especially triglycerides, of the ApoB-containing lipoprotein particles (Dixon et al., 1991; Sakata et al., 1993; Fisher et al., 1997; Wang et al., 1997a; Wu et al., 1997; Liang et al., 1998). In yeast, Vph1p, a multi-spanning membrane protein that is a subunit of the multimeric, membrane-bound V_{0} complex of the vacuolar membrane ATPase (Manolson *et al.*, 1992), is degraded in a proteasome-dependent manner in the absence of Vma21p (Hill and Stevens, 1994; Hill and Stevens, 1995), a non-V₀ protein required for correct assembly of the V_0 complex in the ER.

From these examples, it is increasingly apparent that the proteasome is involved in controlling the levels of a substantial variety of cellular proteins, thereby allowing the critical function or regulation of many important, and vital, cellular pathways. In fact, loss of proteasome function is lethal to the cell (Hilt and Wolf, 1995). With such encompassing functions, it is not surprising that the proteasome has become an intense focus of numerous studies. Although much has yet to be discovered about the proteasome, its basic mechanism and structure have been elucidated.

The Proteasome: A Barrel of Destruction.

The proteasome is a unique, self-compartmentalized protease that is found in all branches of life (Baumeister et al., 1998). The general architecture of the core proteasome is similar in each branch, but greater complexity is observed with eukaryotic proteasomes (Baumeister et al., 1998). The eukaryotic proteasome is composed of a 20S proteolytic core particle containing 14 different subunits each represented in two copies (Gröll et al., 1997), and a 19S regulatory particle, or PA700 subunit (Chu-Ping et al., 1994; DeMartino et al., 1994), containing at least 18 different subunits (Tanaka and Tsurumi, 1997; Baumeister et al., 1998). The entire 26S proteasome (Fig. 1-1) is arranged as one 20S core particle usually bound with one 19S regulatory particle on one end in an asymmetric fashion or two 19S regulatory particles on each end in a symmetric fashion (Walz et al., 1998). In higher eukaryotes, an 11S particle has sometimes been observed in place of the 19S regulatory particle, but the function of the 11S particle appears to be limited (Chu-Ping et al., 1992; Dubiel et al., 1992; Ma et al., 1992). 26S proteasomes carry cellular localization signals, such as functional nuclear localization signals (Nederlof et al., 1995, Wang et al., 1997b), and in yeast are primarily found in the nucleus and at the surface of the ER (Enenkel et al., 1998; Wilkinson et al., 1998).

The 20S particle contains the primary proteolytic activity of the proteasome. Based upon their sequence similarities to the two subunits of the archael proteasomes,



Figure 1-1: The eukaryotic proteasome.

Cartoon of the 20S proteasome, which can associate with the 19S regulatory particle in an ATP-dependent fashion to form either asymmetric or symmetric 26S proteasomes. The 19S regulatory particle confers ATP-dependent degradation to the 20S proteasome. In the absence of the 19S regulatory particle, the 20S proteasome can degrade proteins in an ATP-independent fashion, but the proteins must first be unfolded through denaturation. the subunits of the eukaryotic 20S particles can be separated into two groups, noncatalytic α -type subunits and catalytic β -type subunits (Tanaka, 1995). The subunits of the yeast 20S core particle associate into heptameric rings of similar subunit type, with four rings stacked to form a barrel complex with a central pore (Gröll et al., 1997; and Fig. 1-1). The two outer rings are comprised of the α -subunits and the two inner rings are comprised of the β -subunits. The subunits assemble into their correct structures while they still possess their propeptide sequences, which are autocatalytically removed only when the entire structure has been formed (Schmidtke et al., 1996; Schmidt et al., 1997; Arendt and Hochstrasser, 1999). The proteolytic activity of the 20S proteasome subunits has been defined as a threonine protease (Brannigan et al., 1995), due to the catalytic nucleophile and primary proton acceptor activity of the N-terminal threonine of the β -type subunits (Löwe *et al.*, 1995; Seemüller *et al.*, 1995). Of the seven β -type subunits in one inner ring, only three are catalytically active; thus there are six total active sites in one 20S particle (Lupas et al., 1994; Fenteany et al., 1995; Arendt and Hochstrasser, 1997; Baumeister *et al.*, 1998). The activities of the β -type subunits are classified as chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolyzing activities that degrade proteins into peptides ranging from 7-9 residues long (Heinemeyer et al., 1993; Enenkel et al., 1994; Arendt and Hochstrasser, 1997). In the 20S particle, a channel leads to a central cavity that contains the catalytic sites (Gröll et al., 1997), but the channel appears able to only accommodate unfolded proteins (Wenzel and Baumeister, 1995; Gröll et al., 1997). Channel access to these catalytic sites is restricted by the N-terminal residues of the α -subunits (Gröll *et al.*, 1997), and

may be regulated by the activity of the 19S particle. However, the 20S proteasome can degrade unfolded peptides in the absence of the 19S regulatory particle *in vitro* (Dick *et al.*, 1994), indicating that the catalytic activity does not require the presence of the 19S regulatory particle.

The 19S regulatory particle does confer ubiquitin-dependent and ATPdependent degradation to the 26S proteasome (Ganoth et al., 1988; Eytan et al., 1989; Driscoll and Goldberg, 1990; Chu-Ping et al., 1994). The 19S regulatory particle of the 26S proteasome is non-covalently linked to the 20S particle in a flexible manner, allowing for considerable movement (Walz et al., 1998). When two 19S regulatory particles are bound to one 20S particle, they face in opposite directions and their movements are independent of one another (Walz et al., 1998). Six of the subunits comprising the 19S regulatory particle are ATPases (Confalonieri and Duguet, 1995; Richmond et al., 1997; Baumeister et al., 1998), but their functions are not clear. These ATPases may be required for recognition of target proteins, dissociation of target proteins from multi-subunit complexes, unfolding of the target proteins, removal of ubiquitin from the target protein, or translocation of the target proteins into the 20S particle. A number of the non-ATPase 19S regulatory particle subunits have been linked to ubiquitin-binding or de-ubiquitination activities (Deveraux *et al.*, 1994; Tsurumi et al., 1996; van Nocker et al., 1996; Kominami et al., 1997; Fu et al., 1998), but the majority of subunits have as yet undefined functions.

Generally, proteins targeted for destruction by the proteasome are covalently linked to the small protein ubiquitin through a collection of ubiquitin conjugating enzymes (Hershko and Ciechanover, 1992). The proteasome can degrade proteins that are mono-ubiquitinated (Shaeffer and Kania, 1995), but seems to prefer multiubiquitinated substrates (Hough et al., 1987; Chau et al., 1989; Hochstrasser et al., 1991; Beal *et al.*, 1996). This is consistent with some proteins in the 19S regulatory particle having a higher affinity for multi-ubiquitin chains compared with monoubiquitin molecules (Deveraux et al., 1994; Kominami et al., 1997; Fu et al., 1998). Multi-ubiquitin chains can have a variety of isopeptide linkages, but the isopeptide linkage utilizing Lys48 within the ubiquitin molecule is the most abundant *in vivo* (van Nocker et al., 1993), implying that this might be the preferred multi-ubiquitin chain for proteasomal degradation. In fact, substitution of Lys48 in ubiquitin with arginine retards the degradation of a variety of proteasome-dependent substrates (Chau et al., 1989; Hochstrasser et al., 1991; Finley et al., 1994; Schork et al., 1995; Hiller et al., 1996). A preference for only one linkage would allow the cell to use alternate-linkage multi-ubiquitin chains for other processes, some of which are beginning to be discovered (Arnason and Ellison, 1994; Spence et al., 1995; Baboshina and Haas, 1996).

Although studies of the proteasome have revealed some of its characteristics, such as its structure, catalytic mechanism, and ubiquitin-utilizing targeting mechanism, little is known about how proteins are actually selected for ubiquitination and delivered to the proteasome for destruction. Ongoing studies with proteasome-dependent degradation substrates should provide insight into how proteins are recognized and processed for delivery to the proteasome.

Protein Degradation at the Endoplasmic Reticulum: Flow and Ebb.

Though the proteasome is located in the cytosol and nucleus, the endoplasmic reticulum (ER) has increasingly been documented as a primary site for proteasome-dependent degradation of both normal proteins and mutant or misfolded proteins. Proteasome-dependent ER degradation substrates can be conveniently classified into two distinct types: soluble, lumenal proteins or membrane-bound proteins. Soluble, lumenal proteins degraded at the ER include the PiZ variant of mammalian α -1 antitrypsin, (Ciccarelli *et al.*, 1993), and a point mutated version of yeast carboxypeptidase yscY CPY* (Finger *et al.*, 1993). Membrane-bound proteins degraded at the ER include mammalian unassembled TCR α chains (Lippincott-Schwartz *et al.*, 1988), Apolipoprotein B100 (Fisher *et al.*, 1997; Wu *et al.*, 1997), the CFTR Δ F508 mutant (Ward *et al.*, 1995), yeast mutant proteins Sec61-2p (Sommer and Jentsch, 1993), and Pdr5* (Plemper *et al.*, 1998), normal Vph1p (Hill and Stevens, 1994, 1995), and both yeast and mammalian HMG-CoA reductase (Edwards *et al.*, 1983; Nakanishi *et al.*, 1988; Hampton and Rine, 1994).

The actual mechanisms for how proteins are delivered from the ER to the cytosol for degradation by the proteasome have yet to be fully elucidated. In the case of membrane proteins, some regions of sequence do reside in the cytosol and could be readily accessible to the cytosolic ubiquitin-proteasome machinery (Fig. 1-2). However, soluble, lumenal proteins are sequestered from the cytosol in the lumen of the ER and must be retro-translocated back across the ER membrane to be accessible to the proteasome and its associated targeting machinery (Fig. 1-2). Thus, a mechanism must



Figure 1-2: ER soluble, lumenal proteins and ER membrane-bound proteins.

Cartoon representing the locations of ER membrane-bound proteins and ER soluble, lumenal proteins. After translocation into the ER, the proteasome has possible direct access to membrane-bound proteins, but not lumenal proteins. It is not clear if this access is sufficient for the membrane-bound proteins to be degraded, or if they must first be retrotranslocated across the ER membrane in a manner similar to lumenal proteins. exist within the lumen of the ER that allows for detection and delivery of such substrates from the lumen to the cytosol. Although membrane proteins may have accessible sequences on the cytosolic side of the ER, it may also be that these proteins are recognized in the lumen of the ER and retro-translocated out of the ER membrane in a manner similar to soluble ER lumenal proteins.

Genetic studies in yeast have revealed that these two different types of ERassociated degradation substrates may actually be targeted and degraded in a very similar fashion. Some genes required for regulated ER degradation of the membranebound protein Hmg2p, termed HRD genes (Hampton et al., 1996a), are identical to genes required for degradation of the soluble lumenal protein CPY*, termed *DER* genes (Hiller et al., 1996; Knop et al., 1996; Bordallo et al., 1998). Both proteins are ubiquitinated by a mechanism that is strongly dependent on the cytosolic Ubc7p (Hampton and Bhakta, 1997; Hiller et al., 1996), and the degradation of both proteins requires the membrane-bound Ubc7p-binding protein Cue1p (Biederer *et al.*, 1997; see Chapter 5). A functional cytosolic 26S proteasome is also essential for the degradation of both proteins (Hampton et al., 1996a, Hiller et al., 1996). The ER-resident, multispanning membrane protein Hrd1p/Der3p, which contains a cytosolic domain homologous to other proteins implicated in ubiquitin ligase function (Skowyra *et al.*, 1999; Bays et al., 2000), is required for degradation of either substrate (Hampton et al., 1996a; Bordallo et al., 1998). In addition, the ER resident, single-spanning membrane protein Hrd3p, which contains a large lumenal domain, is critical for the degradation of both substrates (Hampton et al., 1996a, Plemper et al., 1999b). Thus, there is considerable overlap in the ER degradation machinery for these structurally and



Figure 1-3: The ER-associated degradation machinery.

Cartoon depicting the protein machinery required for the ER-associated, proteasomedependent degradation of the HMG-CoA reductase isozyme Hmg2p and the mutant form of carboxypeptidase yscY CPY* in yeast. The membrane topologies of Cue1p, Hrd1p and Hrd3p are depicted according to the hydropathy plots of their amino acid sequences. Both Hmg2p and CPY* require the presence of Cue1p, Ubc7p, Hrd1p and Hrd3p. However, the mechanism by which these proteins act to program the degradation of CPY* and Hmg2p is unclear. functionally distinct substrates (Fig. 1-3), machinery that is located on both the cytosolic and lumenal sides of the ER membrane.

In addition to these studies, analysis of ER protein degradation in mammalian cells has indicated that some ER-degradation substrates are degraded co-translationally in a proteasome-dependent fashion. Although the actual proteins required for this process have not been revealed, it is increasingly clear that substrates, such as Apolipoprotein B100 and CFTR, are ubiquitinated soon after initiation of translation (Liao *et al.*, 1998; Sato *et al.*, 1998). Thus, ER-associated degradation can occur with proteins completely translated and fully inserted into the ER and with proteins that have only begun to be synthesized. Whether these two processes are identical, similar or divergent remains to be elucidated.

Thus, ER-associated proteasome-dependent degradation provides significant opportunities to study the underlying mechanisms for the recognition of degradation substrates and their delivery to the proteasome. In addition, the mechanisms for degradation of membrane-bound proteins and soluble proteins, including the proteins involved in such processes, can be compared, or contrasted, by such studies.

Regulated Degradation of Mammalian HMG-CoA Reductase (HMG-R): A Heart Smart Mechanism.

A striking example of physiologically regulated proteasome-dependent degradation has been revealed in the study of mammalian HMG-R (Goldstein and Brown, 1990), a key enzyme in the mevalonate pathway from which essential isoprenoids and cholesterol are synthesized (Fig. 1-4). HMG-R is an ER-resident


Figure 1-4: The mevalonate pathway.

Cartoons depicting the structures of the mevalonate pathway products. Enzymes that catalyze the formation of each product are listed on the right in gray.

membrane protein that catalyzes the rate-limiting, irreversible step in the mevalonate pathway, the conversion of HMG-CoA to mevalonate (Goldstein and Brown, 1990). The cell modulates the levels of mevalonate pathway products, in part, through the regulated degradation of HMG-R (Edwards *et al.*, 1983; Nakanishi *et al.*,1988). That is, the rate of HMG-R degradation is accordingly altered by the cell in response to the levels of downstream products of the mevalonate pathway (Chin *et al.*, 1985; Panini *et al.*, 1992; Roitelman *et al.*, 1992; Correll and Edwards, 1994; Meigs *et al.*, 1996). When these products are abundant for the cell's needs, the degradation rate of HMG-R is rapid and its steady-state levels are low (Fig. 1-5). In contrast, when these products are insufficient for the cell's needs, the degradation rate of HMG-R is slow and its steady-state levels are high.

The proteasome-dependent, regulated degradation of mammalian HMG-R occurs without exit from the ER (Chun *et al.*, 1990; Meigs and Simoni, 1992). However, although other proteins are subject to ER-associated proteasome-dependent degradation, including TCR- α chains (Lippincott-Schwartz *et al.*, 1988), normal and mutant forms of CFTR (Ward *et al.*, 1995), PiZ α 1-antitrypsin (Ciccarelli *et al.*, 1993), and MHC class I molecules (Michalek *et al.*, 1993), HMG-R is unique in that its rate of degradation is regulated by physiologically relevant cues from within the cell. Thus, the cell has evolved a signaling pathway that senses mevalonate pathway flux and alters only the rate of ER-associated HMG-R degradation.

The N-terminal transmembrane region of mammalian HMG-R is both necessary and sufficient for its regulated degradation (Gil *et al.*, 1985; Nakanishi *et al.*, 1988). HMG-R is composed of two distinct structural domains (Fig. 1-6), a C-terminal





HMG-R is degraded in a proteasome-dependent fashion. The rate of HMG-R degradation is positively regulated by a feedback mechanism of the mevalonate pathway. When production of downstream pathway products is abundant for the cell's needs, the rate of HMG-R degradation is fast. Conversely, when mevalonate pathway production is insufficient for the cell's requirements, the rate of HMG-R degradation is slow.

catalytic domain connected by a flexible linker region to an N-terminal transmembrane domain (Liscum *et al.*, 1985; Luskey and Stevens, 1985). The catalytic domain is necessary for the essential, enzymatic function of HMG-R, but it is not required for regulated degradation (Gil *et al.*, 1985; Nakanishi *et al.*, 1988). Conversely, the Nterminal transmembrane domain is non-catalytic (Liscum *et al.*, 1985; Luskey and Stevens, 1985), but required for regulated degradation (Gil *et al.*, 1985; Nakanishi *et al.*, 1988). In addition, the N-terminal transmembrane domain can impart regulated degradation to fusion reporter proteins, indicating that it is sufficient for HMG-R regulated degradation (Chun *et al.*, 1990). Thus, the information required for HMG-R degradation is contained within a single domain that can sense mevalonate pathway production and translate it into a corresponding rate for HMG-R degradation.

Regulated Degradation of Yeast HMG-R: Conservation in Eukaryotic Sterol Synthesis Regulation?

In addition to the mammalian studies, a systematic analysis of HMG-R regulated degradation has been launched in the yeast *Saccharomyces cerevisiae* (Hampton *et al.*, 1994; Hampton *et al.*, 1996a; Hampton and Bhakta, 1997). The yeast HMG-R isozyme Hmg2p is subject to regulated degradation that is in many ways similar to the regulated degradation of mammalian HMG-R. These similarities include degradation of Hmg2p without exit from the ER (Hampton and Rine, 1994), regulation of the Hmg2p degradation rate by mevalonate pathway signals (Hampton and Rine, 1994; Hampton and Bhakta, 1997), the necessity and sufficiency of the Hmg2p N-terminal transmembrane domain for regulated degradation (Hampton and Rine, 1994;



Figure 1-6: Topology of HMG-R.

Both mammalian and yeast HMG-R are composed of two domains separated by a flexible linker region. The C-terminal, cytosolic, catalytic domain is required for the conversion of HMG-CoA to mevalonate, but is not required for the regulated degradation of HMG-R. The N-terminal, transmembrane domain is not required for the catalytic function, but is absolutely required for the regulated degradation of HMG-R. Furthermore, the N-terminal transmembrane domain is sufficient to confer mevalonate pathway regulated degradation onto other, normally stable proteins.

Hampton *et al.*, 1996b), and involvement of the proteasome in Hmg2p degradation (Hampton and Rine, 1994; Hampton *et al.*, 1996a).

This conservation of function offers numerous unique opportunities to discover the underlying molecular mechanism of HMG-R regulated degradation in eukaryotes by studying the process in yeast. Genetic studies have already discovered genes required for normal degradation of Hmg2p (Hampton et al., 1996a), and genes required for normal regulation of Hmg2p degradation (Cronin *et al.*, 2000). Although the identities of these genes have been revealed, little is known about their action in Hmg2p regulated degradation. To understand further the mechanism of Hmg2p degradation, more information is required about how these degradatory and regulatory proteins function to bring about Hmg2p degradation. In particular, knowledge of the sequence or structural determinants within Hmg2p that allow it to be recognized for degradation and their role in establishing interactions with the proteins involved in Hmg2p degradation will aid greatly in understanding the specific mechanism of Hmg2p degradation and, possibly, the mechanism of ER-associated degradation in general. The work encompassed herein is a study of the mechanistic characteristics of the degradation of the yeast HMG-R isozyme Hmg2p.

Chapter II: Molecular signals for mevalonate pathway regulation of Hmg2p degradation

The young men showed an impatience to begin to torture, that Rivenoak understood; and as his elder associates manifested no disposition to permit any longer delay, he was compelled to give the signal for the infernal work to proceed.

> The Deerslayer --Nathaniel Hawthorne

For so revolvingly appalling was the White Whale's aspect, and so planetarily swift the ever-contracting circles he made, that he seemed horizontally swooping upon them. And though the other boats, unharmed, still hovered hard by; still they dared not pull into the eddy to strike, lest that should be the signal for the instant destruction of the jeopardized castaways, Ahab and all; nor in that case could they themselves hope to escape.

> Moby Dick --Herman Melville

A. Introduction

The mevalonate pathway is responsible for the biosynthesis of numerous essential molecules including prenyl groups, coenzyme Q, dolichol, and sterols such as cholesterol (Goldstein and Brown, 1990). To maintain the continual, vital production of mevalonate pathway compounds while avoiding potential toxic effects of overaccumulation, the cell exerts tight control over pathway product synthesis. A significant proportion of this control occurs through feedback regulation of the activity of HMG-CoA reductase (HMG-R), which catalyzes the rate-limiting, irreversible conversion of HMG-CoA to mevalonate (Goldstein and Brown, 1990). A main component of HMG-R feedback control is effected through regulation of HMG-R stability (Nakanishi et al., 1988; Chun et al., 1990; Hampton and Rine, 1994). When the cellular amounts of mevalonate pathway products are abundant, HMG-R degradation is rapid and the protein's steady-state levels are correspondingly low. Conversely, when the cellular amounts of pathway products are insufficient for the cell's needs, HMG-R degradation is slow and the steady-state levels of HMG-R are relatively high.

Detailed analyses of HMG-R stability have revealed several characteristics of HMG-R degradation. HMG-R is an integral endoplasmic reticulum (ER) membrane protein and its degradation occurs without exit from the ER (Gil *et al.*, 1985; Lecureux and Wattenberg, 1994; Hampton and Rine, 1994). The non-catalytic, N-terminal transmembrane anchor of HMG-R is both necessary and sufficient for regulated degradation (Gil *et al.*, 1985; Jingami *et al.*, 1987; Skalnik *et al.*, 1988; Hampton and

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Rine, 1994). However, neither the identity of the mevalonate-derived signal nor the mechanism by which this signal is coupled to HMG-R degradation is known.

In order to discover and understand the mechanisms of HMG-R regulated degradation, we have been studying the process in the yeast *Saccharomyces cerevisiae* (Hampton and Rine, 1994; Hampton *et al.*, 1996a; Hampton and Bhakta, 1997). Our earlier work has shown that the yeast HMG-R isozyme Hmg2p is degraded in a regulated manner with many similarities to the analogous process in mammals. Through the use of genetic selections and screens, we have been able to identify genes required for the degradation of Hmg2p, called *HRD* genes (Hampton *et al.*, 1996a), and genes required for normal regulation of Hmg2p degradation, called *COD* genes (Cronin *et al.*, 2000). Further studies have elucidated the functions of some of the *HRD* genes as components of the ubiquitin-proteasome targeting machinery (Hampton *et al.*, 1996a; Hampton and Bhakta, 1997; Bays *et al.*, 2000; Gardner *et al.*, 2000). The first identified *COD* gene appears to be involved in maintenance of ER Ca²⁺ levels (Cronin *et al.*, 2000).

Concurrently with these screens, we have been studying the nature of the mevalonate-derived signals that control Hmg2p stability. Hmg2p degradation is regulated by unknown signals from the mevalonate pathway (Fig. 2-1). Inhibiting early pathway enzymes, such as the HMG-R itself or HMG-CoA synthase, decreases the rate of Hmg2p degradation (Hampton and Rine, 1994). These early pathway blocks decrease the availability of a downstream signal for degradation. Conversely, inhibiting the enzyme squalene synthase, which is downstream of HMG-R, stimulates degradation and ubiquitination of Hmg2p (Hampton and Bhakta, 1997). Furthermore, the



Figure 2-1: The mevalonate pathway.

Representation of the mevalonate pathway indicating key enzymes in yeast and their inhibitors.

degradation-enhancing effect of squalene synthase inhibition is abolished by simultaneous inhibition of HMG-CoA synthase or HMG-R.

These pharmacological studies imply that a signal for Hmg2p degradation is a pathway molecule between mevalonate and squalene (Fig. 2-1). The most reasonable candidate for this signal is farnesyl diphosphate (FPP), or an off-pathway FPP derivative. The idea that FPP, or a derivative, is a positive signal for Hmg2p degradation is particularly interesting since there is accumulating evidence from *in vitro* and *in vivo* studies that farnesol, an FPP-derivative, is a signal for regulation of mammalian HMG-R stability (Bradfute and Simoni, 1994; Correll and Edwards, 1994; Meigs *et al.*, 1996; Lopez *et al.*, 1997; Meigs and Simoni, 1997).

In addition to an FPP-derived signal, mammalian cells also employ an oxysterolderived positive signal for HMG-R degradation (Faust *et al.*, 1982; Chin *et al.*, 1985; Nakanishi *et al.*, 1988; Meigs *et al.*, 1992; Panini *et al.*, 1992; Roitelman *et al.*, 1992). The existence of the oxysterol-derived signal has been proposed by observation of a degradation-enhancing effect as a result of 25-hydroxycholesterol or 24(S),25oxidolanosterol addition to cells (Faust *et al.*, 1982; Chin *et al.*, 1985; Chun *et al.*, 1990; Panini *et al.*, 1992). Endogenous oxysterols may exist in the cell through the action of the alternate oxysterol pathway (Dollis and Schuber, 1994), which branches from the cholesterol biosynthetic pathway at 2,3(S)-oxidosqualene and can synthesize 25hydroxycholesterol by the same enzymes required for normal cholesterol synthesis (Boutaud *et al.*, 1992; Dollis and Schuber, 1994). In fact, inhibition of oxidosqualenelanosterol cyclase in cells, which results in increased production of oxysterols through preferential cyclization of increased levels of 2,3(S);22(S),23-dioxidosqualene (Boutaud *et al.*, 1992; Dollis and Schuber, 1994; Peffley *et al.*, 1998), also results in enhanced HMG-R degradation (Peffley and Gayen, 1997). In mammalian cells, the oxysterolderived signal appears incapable of enhancing HMG-R degradation in the absence of the primary FPP-derived signal (Meigs and Simoni, 1997). It is unknown whether yeast also employ an oxysterol-derived positive signal for HMG-R degradation, or if this is a characteristic of only mammalian HMG-R degradation.

We have tested the hypothesis that FPP provides a molecular signal for control of Hmg2p stability using unique genetic opportunities available in yeast. In conjunction with pharmacological and biochemical approaches, we have constructed yeast strains that allowed either over-expression or down-regulation of specific mevalonate pathway genes. The following results indicated that FPP was indeed the source of a primary signal for Hmg2p degradation in yeast. Furthermore, we demonstrated the existence of a secondary oxysterol product that signals for degradation in addition to, but not in the absence of, FPP. These results further support the striking conservation for this mode of HMG-R regulation amongst eukaryotes.

B. Results

Regulated degradation of the yeast HMG-R isozyme, Hmg2p.

Yeast expresses an isozyme of HMG-R, Hmg2p, that is subject to ERassociated, mevalonate pathway regulated degradation (Hampton and Rine, 1994). The regulated degradation of Hmg2p is clearly observed in a typical degradation assay called the cycloheximide-chase assay. In this assay, cycloheximide is added to cells to inhibit protein synthesis and the stability, or degradation, of the pool of protein made prior to the addition of cycloheximide is followed over an extended period of time. Utilizing this assay to determine the degradation behavior of Hmg2p, it was discovered that Hmg2p was subject to rapid degradation (Hampton and Rine, 1994; and Fig. 2-2a, lane "0" versus lane "4"). Furthermore, the rate of Hmg2p degradation in the cycloheximide chase assay was modulated by the levels of downstream mevalonate pathway products in the cell. This was most readily observed by pharmacological inhibition of the mevalonate pathway. If lovastatin, an inhibitor of HMG-R that acts to block pathway production, was added to the cells at the same time as cycloheximide addition, the rate of Hmg2p was significantly slowed (Hampton and Rine, 1994; and Fig. 2-2a, lane "4" versus "4L").

The regulation of Hmg2p degradation can also be observed as changes in the Hmg2p steady-state level. Because the steady-state level of Hmg2p is an equilibrium state achieved as a balance between its rate of synthesis and its rate of degradation, stabilization of Hmg2p results in an increased steady-state level. Thus, when lovastatin was added to log-phase cells that expressed Hmg2p from the constitutive *TDH3*



Figure 2-2: Degradation behavior of the yeast HMG-R isozyme Hmg2p.

a) Hmg2p degradation is regulated by the mevalonate pathway. Stability of the protein was assessed by cycloheximide-chase assay. Cycloheximide was added to log-phase cells to halt protein synthesis. Lovastatin, an inhibitor of HMG-R, was added to one of the samples at the same time as cycloheximide addition. Lysates were prepared for each indicated time point after cycloheximide addition and samples were immunoblotted to determine the level of the protein. Hmg2p had a single myc epitope tag included in the sequence of the linker region to allow detection with the anti-myc 9E10 antibody. b) Effect of mevalonate pathway drugs on Hmg2p steady-state level. Cells were grown to mid log-phase and either no drug ("-"), lovastatin ("Lov"), or zaragozic acid ("ZA", an inhibitor of squalene synthase) was added. Cells were incubated for an additional 4 hours after addition of drugs. Lysates were prepared and samples were immunoblotted to determine the level of Hmg2p. In all experiments in all figures, all HMG-R proteins were expressed from the constitutive *TDH3* promoter (a.k.a. GAPDH promoter, Bitter and Egan, 1984) to eliminate any possible transcriptional regulation incurred by changes in mevalonate pathway production.

promoter (a.k.a. GAPDH promoter, Bitter and Egan, 1984), a time-dependent increase in the steady-state level of Hmg2p was observed (Hampton and Rine, 1994; Fig. 2-2b, "-" versus "Lov"), and this was solely to due to a decrease in the Hmg2p degradation rate. Interestingly, when the drug zaragozic acid, an inhibitor of squalene synthase (Bergstrom *et al.*, 1993), was added to cells growing in log-phase, a time-dependent decrease in the steady-state levels of Hmg2p was observed (Hampton and Bhakta, 1997; and Fig. 2-2b, "ZA"). Thus, it appeared that farnesyl diphosphate (FPP), the substrate for squalene synthase, acted as a positive signal for Hmg2p degradation. Reduced FPP levels, through inhibition of HMG-R by addition of lovastatin, stabilized Hmg2p, whereas increased FPP levels, by inhibition of squalene synthase, hastened Hmg2p degradation.

Zaragozic acid increased Hmg2p degradation by squalene synthase inhibition.

Squalene synthase inhibition would be expected to cause a build-up of FPP, the enzyme's substrate, implicating FPP as a source of the positive signal for Hmg2p degradation. However, it was possible that the effect of zaragozic acid was the result of some other action of the drug. Therefore, we tested the effect of zaragozic acid addition on Hmg2p degradation in a strain that over-expressed squalene synthase. If zaragozic acid was inducing Hmg2p degradation through squalene synthase inhibition, then increased levels of squalene synthase in the cell should require increased amounts of zaragozic acid to cause the same degree of degradation.

To allow over-expression, the squalene synthase gene (*ERG9*) was placed under control of the constitutive *TDH3* promoter, P_{TDH3} (a.k.a. GAPDH promoter, Bitter and

Egan, 1984), and the P_{TDH3} -*ERG9* construct was transformed into yeast cells. P_{TDH3} -*ERG9* was present as a single integrated copy through targeted insertion of an integrating vector. The strain expressing a single, integrated copy of P_{TDH3} -*ERG9* was tested for over-expression of squalene synthase and was found to be 8-fold more resistant to the growth-slowing effect of zaragozic acid than a wild-type strain (Fig. 2-3a, "Int" versus "wt"). This was consistent with approximately 8-fold higher squalene synthase levels in the P_{TDH3} -*ERG9* strain than the wild-type strain, as measured by western blot of HSV-tagged versions of squalene synthase (Fig. 2-3b, "*TDH3*" versus "wt"). Additional increased expression of squalene synthase by the presence of the P_{TDH3} -*ERG9* allele on multi-copy vectors, such as an ARS-CEN vector or a 2 micron vector, further increased the strain resistance to the growth-slowing effect of zaragozic acid (Fig. 2-3a, "ARS-CEN" and "2 μ ").

We then tested if the Hmg2p degradation-enhancing effect of zaragozic acid required higher doses upon squalene synthase over-expression. The yeast strains used above also co-expressed two versions of Hmg2p: 1myc-Hmg2p and the fluorescence reporter protein Hmg2p-GFP, which have identical degradation behaviors as normal Hmg2p (Hampton and Bhakta, 1997; Cronin *et al.*, 2000). Cells that contained a single, integrated copy of P_{TDH3} -*ERG9* required 8-fold more zaragozic acid to decrease 1myc-Hmg2p steady-state levels and Hmg2p-GFP steady-state levels as was required for wild-type cells (Fig. 2-4a & b, 40 µg/ml for " P_{TDH3} -*ERG9*" versus 5 µg/ml for "wt"), consistent with the 8-fold over-expression of squalene synthase. These results suggested that the mechanism for zaragozic acid-induced degradation of Hmg2p was



Figure 2-3: Squalene synthase over-expression increased resistance to growthslowing effect of zaragozic acid.

a) Zaragozic acid growth curves of strains expressing squalene synthase from its wildtype promoter ("wt") or from the *TDH3* promoter (P_{TDH3} -*ERG9*) on an integrating vector ("INT"), ARS-CEN vector ("ARS-CEN"), or 2micron vector ("2 μ "). Cultures were grown at 30 °C in minimal media with the indicated concentration of zaragozic acid. Absorbance (OD₆₀₀) was measured after 24 hours of growth. b) Western blot of HSVtagged squalene synthase expressed from its wild-type promoter ("wt") or from the *TDH3* promoter ("P_{TDH3}") on an integrating vector. Cells were grown to mid-log phase in minimal media and lysed. Numbers on the bottom indicate the fold dilution of the original cellular lysate.



Figure 2-4: Squalene synthase over-expression increased the concentrations of zaragozic acid required to stimulate Hmg2p degradation.

a) Effect of zaragozic acid (ZA), which hastens Hmg2p degradation, on 1myc-Hmg2p steady-state levels in a wild-type strain ("wt") or in an otherwise isogenic strain carrying a single integrant of the P_{TDH3} -*ERG9* allele ("P_{TDH3}-*ERG9*"). Strains were treated for four hours with the indicated concentrations of ZA, then subjected to immunoblotting to determine 1myc-Hmg2p steady-state levels. b) Effect of zaragozic acid on Hmg2p-GFP steady-state levels in a wild-type strain ("wt") or in the strain carrying a single integrant of the P_{TDH3}-*ERG9* allele ("P_{TDH3}-*ERG9*"). Cells were grown 4 hours in the presence of the indicated concentrations of zaragozic acid, then analyzed by flow cytometry.

through squalene synthase inhibition.

Squalene synthase levels determined the degree of Hmg2p degradation.

The above results indicated that zaragozic acid altered Hmg2p degradation by decreasing squalene synthase activity. In that case, genetic down-regulation of the squalene synthase gene (*ERG9*) should also increase Hmg2p degradation. Because mevalonate pathway enzymes are essential for yeast viability, a null allele of *ERG9* in yeast results in cell death (Fegueur et al., 1991; Jennings et al., 1991). Therefore, a conditional allele of squalene synthase was made by placing the wild-type ERG9 gene under control of the *MET3* promoter (Cherest *et al.*, 1985), which is repressed by the presence of high extracellular concentrations (>0.5mM) of methionine (Mountain et al., 1991). A "promoter-switch" plasmid that contained a truncated version of erg9 placed behind the MET3 promoter was constructed and targeted integration of this plasmid into the ERG9 locus resulted in the creation of a single, functional copy of ERG9 under control of the regulated MET3 promoter (P_{MET3}) (Fig. 2-5). This plasmid was used to transform a methionine prototroph yeast strain to allow growth in any concentration of methionine. The strain also co-expressed 1myc-Hmg2p and Hmg2p-GFP allowing a complete characterization of Hmg2p degradation. When grown in the absence of methionine, normal regulated Hmg2p degradation was observed in the yeast strain that expressed squalene synthase from the MET3 promoter (P_{MET3} -ERG9) (Fig. 2-6a, " P_{MET3} -*ERG9*" compared to "wt").

Genetic down-regulation of squalene synthase enhanced Hmg2p degradation in a manner identical to inhibition with zaragozic acid. After 15 hour growth in 2mM



Figure 2-5: Construction of a *MET3* promoter-regulated allele by "promoter switching".

A mevalonate pathway gene, *ERGX*, was placed under control of the *MET3* promoter through targeted integration of a "promoter–switch" plasmid at the *ERGX* genomic locus. The plasmid consisted of a non-functional, 5' portion of the *ERGX* gene placed behind the *MET3* promoter (P_{MET3}). Integration resulted in a functional copy of the *ERGX* gene placed under control of the *MET3* promoter and a non-functional, deleted copy behind the native promoter.

methionine, Hmg2p degradation in the P_{MET3} -*ERG9* strain was increased. This was indicated by a lower steady-state level of Hmg2p-GFP and 1myc-Hmg2p in the P_{MET3} -*ERG9* strain compared to the wild-type strain (Fig. 2-6b&c, "P_{MET3}-*ERG9*" versus "wt"). The effect of down-regulation was similar to that in the wild-type strain after 15 hour incubation in the presence of zaragozic acid ("P_{MET3}-*ERG9*" compared with "wt, +ZA"). Hmg2p-GFP in the P_{MET3}-*ERG9* was stabilized by the addition of lovastatin to a similar degree as the lovastatin stabilization of Hmg2p-GFP in the wild-type strain incubated with zaragozic acid ("P_{MET3}-*ERG9*, +Lov" compared with "wt, +ZA+Lov), indicating that enhanced Hmg2p degradation caused by squalene synthase down-regulation or zaragozic acid addition was still regulated by the mevalonate pathway

The enhanced Hmg2p degradation by zaragozic acid addition or squalene synthase down-regulation required the *HRD* gene-encoded proteins. Enhanced degradation by squalene synthase or zaragozic acid addition was completely eliminated by the presence of the *hrd1* Δ allele (Fig. 2-6b, "P_{MET3}-*ERG9*, *hrd1* Δ " and "wt +ZA, *hrd1* Δ "), which normally stabilizes Hmg2p (Hampton *et al.*, 1996a). This indicated that the degradation-enhancing effect of squalene synthase down-regulation or zaragozic acid addition required a functional *HRD* pathway and was not due to aberrant degradation by an alternate pathway. Furthermore, identical steady-state levels of 1myc-Hmg2p in the wild-type *ERG9*, *hrd1* Δ strain and the P_{MET3}-*ERG9*, *hrd1* Δ strain indicated that the lower steady-state levels of Hmg2p in the P_{MET3}-*ERG9* strain were due only to enhanced degradation, not reduced translation efficiency.



Figure 2-6: Squalene synthase down regulation decreased Hmg2p levels.

a) Cells expressing squalene synthase from the MET3 promoter (" P_{MET3} -ERG9") had normal regulated Hmg2p degradation in the absence of exogenous methionine. Cycloheximide-chase assay of strains expressing squalene synthase from the wild-type promoter ("wt") or the MET3 promoter. Lovastatin (25 µg/ml) was added to the indicated samples at the same time as cycloheximide addition. After addition of cycloheximide, lysates were prepared at the indicated times and immunoblotted to determine Hmg2p levels. b) Effect of squalene synthase down-regulation on Hmg2p-GFP steady-state levels. Cells expressing squalene synthase from either the wild-type promoter ("wt") or the MET3 promoter ("P_{MET3}-ERG9") were grown for 15 hours at 30°C in the presence of 2mM methionine. Zaragozic acid ("+ZA", 10µg/ml final concentration) or lovastatin ("+Lov", 25µg/ml final concentration) was added to the appropriate cultures and all cultures were grown an additional 4 hours at 30°C. Hmg2p-GFP fluorescence was analyzed by flow cytometry. c) Increased 1myc-Hmg2p degradation as a result of squalene synthase down-regulation was *HRD1* dependent. Cycloheximide-chase assay of strains expressing squalene synthase from the wild-type promoter ("wt") or from the MET3 promoter ("P_{MET3}-ERG9") in the presence of a normal *HRD1* gene or the *hrd1* Δ allele ("P_{MET3}-*ERG9*, *hrd1* Δ " and "wt, *hrd1* Δ ").

It was possible that enhanced Hmg2p degradation was the result of cell death inadvertently caused by squalene synthase down-regulation rather than build-up of a positive signal for degradation. Cells containing the P_{MET3} -*ERG9* allele ceased to grow after 15 hour incubation in 2mM methionine (6 doublings) (see below, Fig. 2-12a). However, when the P_{MET3} -*ERG9* cells from this 15 hour time point were transferred to media without methionine, they retained the same plating efficiency and growth curve as identically treated wild-type *ERG9* cells (see below, Fig. 2-12b), indicating that these cells were still viable.

Lastly, we also observed that over-expression of squalene synthase stabilized Hmg2p. In the strains previously described in figure 2-3, which over-expressed squalene synthase by the presence of the P_{TDH3} -*ERG9* allele, both versions of Hmg2p were significantly stabilized. This was observed as both an increase in the steady-state level of 1myc-Hmg2p (Fig. 2-7a, " P_{TDH3} -*ERG9*" versus "wt") and Hmg2p-GFP (Fig. 2-7b, " P_{TDH3} -*ERG9*" versus "wt") and as a decrease in 1myc-Hmg2p degradation when squalene synthase was over-expressed (Fig. 2-7a, " P_{TDH3} -*ERG9*" versus "wt"). Thus, squalene synthase over-expression had the opposite effect on Hmg2p degradation as squalene synthase down-regulation. Together, these results indicated that the levels of squalene synthase activity in the cell determined the degree of Hmg2p degradation, most likely by controlling the cellular levels of FPP.



Figure 2-7: Squalene synthase over-expression decreased Hmg2p degradation.

a) Effect of squalene synthase over-expression on 1myc-Hmg2p degradation. Otherwise identical strains expressing squalene synthase from the wild-type promoter ("wt") or from a single, integrated allele with the *TDH3* promoter ("P_{*TDH3*}-*ERG9*") were subjected to a cycloheximide-chase assay. Lysates for each indicated time point after addition of cycloheximide were made and immunoblotted to determine Hmg2p levels. b) Effect of squalene synthase over-expression on Hmg2p-GFP steady-state levels. Otherwise identical strains expressing squalene synthase from the wild-type promoter ("wt") or from a single, integrated allele with the *TDH3* promoter ("P_{*TDH3*}-*ERG9*") were analyzed by flow cytometry to determine Hmg2p-GFP levels.

Down-regulation of farnesyl diphosphate synthase stabilized Hmg2p.

The above studies implicated the substrate of squalene synthase, FPP, as a central molecule in the regulation of Hmg2p stability. Manipulations of squalene synthase predicted to increase FPP levels hastened Hmg2p degradation, whereas manipulations of squalene synthase predicted to decrease FPP levels slowed Hmg2p degradation. In order to test further the hypothesis that FPP was the source of the positive signal for Hmg2p degradation, a way to directly eliminate FPP production was required. Inhibition of farnesyl diphosphate synthase (FPP synthase), which generates FPP as a product, is one possible method. Unfortunately, no drugs are currently available that inhibit yeast FPP synthase in vivo. Therefore, a genetic approach was employed to lower FPP synthase production. As with the other enzymes of the mevalonate pathway, yeast cells that contain a null allele of FPP synthase are not viable (Chambon et al., 1990). Thus, a conditional allele of the FPP synthase coding region (ERG20) was constructed by placing the wild-type ERG20 gene under control of the MET3 promoter, similar to ERG9 described above. When grown in the absence of methionine, normal regulated Hmg2p degradation was observed in the yeast strain that expressed FPP synthase from the MET3 promoter (P_{MET3}-ERG20) (Fig. 2-8a, "P_{MET3}-ERG20" compared to "wt").

In contrast to enhanced Hmg2p degradation caused by squalene synthase down-regulation, FPP synthase down-regulation resulted in stabilization of Hmg2p. When the P_{MET3} -ERG20 strain was grown 15 hours in 2mM methionine, Hmg2p was exceedingly



Figure 2-8: FPP synthase down-regulation decreased Hmg2p degradation.

a) Cells expressing FPP synthase from the MET3 promoter ("P_{MET3}-ERG20") had normal regulated Hmg2p degradation in the absence of exogenous methionine. Cycloheximide-chase assay of strains expressing FPP synthase from the wild-type promoter ("wt") or from the MET3 promoter. Lovastatin ($25 \mu g/ml$) was added to the indicated samples at the same time as cycloheximide addition (50 μ g/ml). Lysates were prepared at the indicated times and immunoblotted to determine Hmg2p levels. b) Effect of FPP synthase down-regulation on 1myc-Hmg2p degradation. Otherwise identical strains expressing FPP synthase from the wild-type promoter ("wt") or from the MET3 promoter (" P_{MET3} -ERG20") were grown 15 hours at 30°C in the presence of 2mM methionine, then subjected to a cycloheximide-chase assay. Zaragozic acid $(10\mu g/ml \text{ final concentration})$ was added to the indicated sample ("4ZA") at the same time as addition of cycloheximide. Lovastatin ($50\mu g/ml$ final concentration) was added to the indicated wild-type strain ("wt, +Lov") at the same time as addition of methionine. c) Effect of FPP synthase down-regulation on Hmg2p-GFP steady-state levels. Cells expressing FPP synthase from either the wild-type promoter ("wt") or the MET3 promoter ("P_{MET3}-ERG20") were grown for 15 hours at 30°C in the presence of 2mM methionine. Zaragozic acid ("+ZA", $10\mu g/ml$ final concentration) or lovastatin ("+Lov", 25µg/ml final concentration) was added to the appropriate cultures, which were grown an additional 4 hours at 30°C. Hmg2p-GFP fluorescence was analyzed by flow cytometry.

stable, as indicated by both a higher steady-state level and decreased degradation of 1myc-Hmg2p in the P_{MET3}-ERG20 strain compared to the wild type strain (Fig. 2-8b, "P_{MET3}-ERG20" versus "wt"). The effect in the P_{MET3}-ERG20 strain was similar to the effect of lovastatin addition to the wild-type ERG20 strain ("P_{MET3}-ERG20" compared with "wt, +Lov"). Similarly, Hmg2p-GFP steady state-levels were increased dramatically in the PMET3-ERG20 strain (Fig. 2-8c, "PMET3-ERG20" versus "wt"), and this effect was also mimicked by growth of the wild-type strain in the presence of lovastatin ("P_{MET3}-ERG20" compared with "wt, +Lov"). The effect of FPP synthase down-regulation was reversed by the presence of zaragozic acid in the degradation assay (Fig. 2-8b, "P_{MET3}-ERG20", 4ZA lane), and in Hmg2p-GFP steady-state fluorescence (Fig. 2-8c, "P_{MET3}-ERG20, +ZA" compared with "wt, +ZA"), indicating that regulated degradation of Hmg2p was still operative. Although the methionine treatment halted the growth of the P_{MET3}-ERG20 strain after 15 hours (see below, Fig. 2-12a), if these cells were then transferred to media that did not contain methionine, they retained a similar plating efficiency and growth curve as the wild-type ERG20 control cells (see below, Fig. 2-12b), indicating that Hmg2p stabilization was not a result of inadvertent cell death.

Thus, FPP synthase down-regulation resulted in stabilization of Hmg2p similar to the addition of lovastatin to cells. This further strengthened the model that FPP was the source of a positive signal for Hmg2p degradation.

FPP synthase down-regulation also blocked Hmg2p ubiquitination.

The covalent attachment of ubiquitin is a critical and regulated step in Hmg2p degradation (Hampton and Bhakta, 1997). Because FPP synthase down-regulation stabilized Hmg2p, we also determined its effect on Hmg2p ubiquitination. To assay Hmg2p ubiquitination, strains containing the appropriate wild-type or regulated alleles of the FPP synthase gene were transformed with plasmids that expressed HA epitope-tagged ubiquitin. Hmg2p ubiquitination was assayed by immunoprecipitation of 1myc-Hmg2p, followed by anti-HA immunoblotting to detect covalently attached HA-Ub. Regulation of Hmg2p ubiquitination was assessed by performing each assay in the presence of lovastatin ("Lov"), which decreases ubiquitination, or zaragozic acid ("ZA"), which increases ubiquitination (Hampton and Bhakta, 1997; and Fig. 2-9, "wt").

Down-regulation of FPP synthase caused a drastic decrease in the level of Hmg2p ubiquitination (Fig. 2-9, "P_{MET3}-ERG20" versus "wt", no drug lanes). This effect was similar to the addition of lovastatin to the wild-type strain during the ubiquitination assay ("wt", Lov lane). Furthermore, the addition of ZA for 10 min during the ubiquitination assay, which normally increases Hmg2p ubiquitination, had no effect on Hmg2p ubiquitination in the FPP synthase down-regulated strain ("wt" ZA lane versus "P_{MET3}-ERG20", ZA10 lane). This effect of FPP synthase down-regulation on ZA action was identical to the effect of lovastatin addition to the wild-type cells treated with zaragozic acid ("P_{MET3}-ERG20", ZA10 lane compared with "wt", ZA+Lov lane). However, addition of ZA for 1 hour did increase Hmg2p ubiquitination in the FPP synthase down-regulated strain ("P_{MET3}-ERG20",



Figure 2-9: FPP synthase down-regulation decreased Hmg2p ubiquitination.

Regulated ubiquitination of 1myc-Hmg2p in cells expressing FPP synthase from the wild-type promoter ("wt") or the *MET3* promoter ("P_{*MET3*}-*ERG20*"). Cells were grown 15 hours at 30°C in the presence of 2mM methionine. Ubiquitination assays were performed in the presence of no drug ("-"), $25\mu g/ml$ lovastatin ("Lov"), or $10\mu g/ml$ zaragozic acid ("ZA"). For the wild-type strain, lovastatin was added 30 min prior to cell lysis and ZA was added 10 min prior to cell lysis. For the P_{*MET3*}-*ERG20* strain, zaragozic acid was added either 10 min ("ZA10") or 60 min ("ZA60") prior to cell lysis. Upper panels are the result of anti-HA (" α -HA") immunoblotting for covalently linked HA-tagged Ub. Lower panels are the result of parallel immunoblotting an aliquot (1/8 total volume) of the same immunoprecipitates with the 9E10 anti-myc antibody (" α -myc") to assess total immunoprecipitated Hmg2p.

ZA60 lane compared with "wt" ZA lane), demonstrating that enhanced ubiquitination could be induced when sufficient time was allowed for FPP accumulation. Thus, pharmacological and genetic manipulations that altered the cellular levels of FPP correspondingly altered Hmg2p ubiquitination in a manner consistent with the role of FPP as a positive degradation signal.

Genetic manipulations did not alter general ER degradation.

The above genetic experiments were consistent with FPP being the source of a positive signal for Hmg2p degradation. However, the results could also be explained by effects on the general machinery for ER degradation, rather than on the signal controlling Hmg2p degradation. To test this, the effect of these genetic manipulations on the degradation of 6myc-Hmg2p, a previously described unregulated mutant of Hmg2p (Hampton *et al.*, 1996a), was examined. Degradation of 6myc-Hmg2p requires the same degradation pathway as normal Hmg2p (Hampton *et al.*, 1996a), but 6myc-Hmg2p degradation is not regulated by signals from the mevalonate pathway. Thus, if down-regulation of mevalonate pathway enzymes altered only the regulatory signal for Hmg2p degradation, then 6myc-Hmg2p degradation should be unaffected.

The effect of squalene synthase or FPP synthase down-regulation on 6myc-Hmg2p degradation was determined in a strain that was isogenic to the wild-type strain described above, except that unregulated 6myc-Hmg2p and 6myc-Hmg2p-GFP were co-expressed rather than 1myc-Hmg2p and Hmg2p-GFP. As shown in the top left panel of figure 2-10, 6myc-Hmg2p-GFP steady-state levels were unaffected by addition of drugs, such as lovastatin or zaragozic acid, which normally altered the steady-state levels of Hmg2p-GFP (top right panel). Similarly, down-regulation of squalene synthase or FPP synthase had no effect on the steady-state levels of the unregulated 6myc-Hmg2p-GFP (bottom left panel), whereas these perturbations appropriately altered the steady-state levels of the regulated Hmg2p-GFP (bottom right panel). The co-expressed 6myc-Hmg2p was similarly unresponsive to the same pharmacological or genetic manipulations that affected 1myc-Hmg2p or the optical Hmg2p-GFP reporter (data not shown). These results indicated that squalene synthase and FPP synthase down-regulation affected only the regulation of Hmg2p stability, and not the general process of ER degradation.

Squalene epoxidase down-regulation did not alter Hmg2p degradation.

The previous experiments provided strong evidence that FPP levels within the cell controlled the rate of Hmg2p degradation. However, it was not clear if this feature was unique to FPP or if other downstream products could also affect Hmg2p degradation. Squalene epoxidase is the next enzyme after squalene synthase in the pathway and is responsible for the formation of squalene epoxide from squalene (Fig. 2-1). To determine if alteration of squalene epoxidase levels had any effect on Hmg2p degradation, a *MET3*-regulated allele of the squalene epoxidase gene (*ERG1*) was constructed, similar to the *ERG9* and *ERG20* alleles described above. When grown in the absence of methionine, normal regulated Hmg2p degradation was observed in the yeast strain that expressed squalene epoxidase from the *MET3* promoter (P_{MET3} -*ERG1*" compared to "wt").



Figure 2-10: Conditional alleles of squalene synthase and FPP synthase altered regulation of Hmg2p degradation, not general ER degradation.

Upper panels: Cells expressing either unregulated 6myc-Hmg2p-GFP or normally regulated Hmg2p-GFP were treated with either no drug, 10μ g/ml zaragozic acid ("ZA") or 25μ g/ml lovastatin ("Lov") and grown for 4 hours at 30°C. **Bottom panels:** Cells from the upper panel expressing either squalene synthase from the *MET3* promoter ("P_{MET3}-ERG9"), FPP synthase from the *MET3* promoter ("P_{MET3}-ERG20"), or both from their respective wild-type promoters ("wt") were grown for 15 hours at 30°C in the presence of 2mM methionine. 6myc-Hmg2p-GFP or Hmg2p-GFP steady-state fluorescence was analyzed by flow cytometry.

Unlike the other manipulations above, squalene epoxidase down-regulation had no effect on Hmg2p degradation. When grown in methionine, the P_{MET3} -ERG1 strain and the wild-type strain had identical Hmg2p-GFP steady-state levels (Fig. 2-11b, " P_{MET3} -ERG1" compared with "wt"), and 1myc-Hmg2p degradation (data not shown). This was in contrast to squalene synthase down-regulation (see above and Fig. 2-11b, " P_{MET3} -ERG1" versus " P_{MET3} -ERG9"), which had the expected effects in the same experiment.

It was possible that squalene epoxidase down-regulation had no effect on Hmg2p degradation because the conditional allele was not correctly functioning. To confirm further that a block at squalene epoxidase did not affect Hmg2p degradation, the wild-type strain was treated with terbinafine, a specific inhibitor of fungal squalene epoxidase (Ryder, 1989; Jandrositz *et al.*, 1991; Ryder, 1992; Sakakibara *et al.*, 1995). No concentration of terbinafine had any observable effect on Hmg2p-GFP steady-state levels (Fig. 2-12a, "+Tb" compared with "no drug"), or 1myc-Hmg2p degradation (data not shown), despite using lethal doses of the drug. Furthermore, cells carrying the ineffective P_{MET3} -*ERG1* allele showed identical methionine-dependent growth retardation as the other alleles that affected degradation (Fig. 2-12b). Thus, the conditional allele of squalene epoxidase was correctly integrated and down-regulated. Together these results indicated that FPP was the primary source of the positive signal for Hmg2p degradation and that there was no requirement for a downstream molecule in regulation of Hmg2p degradation.



Figure 2-11: Squalene epoxidase down-regulation of inhibition had no effect on Hmg2p-GFP steady-state levels.

a) Cells expressing squalene epoxidase from the *MET3* promoter ("P_{*MET3*}-*ERG1*") had normal regulated Hmg2p degradation in the absence of exogenous methionine. Cycloheximide-chase assay of strains expressing squalene epoxidase from the wild-type promoter ("wt") or from the *MET3* promoter grown for 15 hours at 30°C in minimal media. Lovastatin (25 μ g/ml) was added to the indicated samples at the same time as addition of cycloheximide (50 μ g/ml). After addition of cycloheximide, lysates were prepared at the indicated times and immunoblotted to determine Hmg2p levels. b) Effect of squalene epoxidase down-regulation on Hmg2p steady-state levels. Cells expressing squalene epoxidase from either the wild-type promoter ("wt") or the *MET3* promoter ("P_{*MET3*}-*ERG1*") or squalene synthase from the *MET3* promoter ("P_{*MET3*}-*ERG9*") were grown for 15 hours at 30°C in the presence of 2mM methionine. Hmg2p-GFP fluorescence was analyzed by flow cytometry.



Figure 2-12: Conditional squalene epoxidase allele was functioning correctly.

a) Effect of terbinafine, a squalene epoxidase inhibitor, on Hmg2p-GFP steady-state levels. Cells were treated with either no drug, 40μ g/ml terbinafine ("Tb"), or 10μ g/ml ZA ("ZA") and grown for 4 hours at 30°C. Hmg2p-GFP steady-state fluorescence was analyzed by flow cytometry. **b) Left panel:** Cells containing either the conditional alleles of squalene synthase ("P_{MET3}-ERG9"), FPP synthase ("P_{MET3}-ERG20"), or squalene epoxidase ("P_{MET3}-ERG1") were grown to mid-log phase (OD₆₀₀ approximately 0.5) in media that did not contain methionine. New cultures containing 2mM methionine were inoculated from these previously grown cultures to an initial OD₆₀₀ of 0.01, then incubated for 32 hours at 30°C. The new OD₆₀₀ for these cultures was measured at the indicated time points and plotted versus time. Growth of each strain was compared to the wild-type strain ("wt"). **Right Panel:** New cultures, which contained no methionine, were inoculated from the previous 15 hour methionine-grown cultures to an initial OD₆₀₀ of 0.01, then incubated for 32 hours at 30°C. The new OD₆₀₀ strain methionine-grown cultures to an initial OD₆₀₀ of 0.01, then incubated from the previous 15 hour methionine-grown cultures to an initial OD₆₀₀ of 0.01, then incubated for 32 hours at 30°C. The new OD₆₀₀ strain methionine-grown cultures to an initial OD₆₀₀ of 0.01, then incubated from the previous 15 hour methionine-grown cultures to an initial OD₆₀₀ of 0.01, then incubated for 32 hours at 30°C. The new OD₆₀₀ for these cultures was measured at the indicated time points and plotted versus time.

2,3 oxidosqualene-lanosterol cyclase down-regulation or inhibition enhanced Hmg2p degradation.

In mammalian cells, an oxysterol-derived molecule acts to hasten HMG-R degradation in conjunction with a non-sterol signal derived from FPP (Meigs and Simoni, 1997). The existence of such an oxysterol-derived signal has been proposed from the observations that HMG-R degradation is enhanced as a result of either 24(S),25-oxidolanosterol or 25-hydroxycholesterol addition to cells (Faust *et al.*, 1982; Chin et al., 1985; Chun et al., 1990; Panini et al., 1992), or when oxidosqualenelanosterol cyclase is inhibited in cells (Peffley and Gayen, 1997). This oxysterolderived signal does not effect increased HMG-R degradation alone, but requires the presence of a non-sterol, positive signal derived from FPP to enhance HMG-R degradation (Meigs and Simoni, 1997). It is important to note that these studies only demonstrate that exogenous addition of molecules alters HMG-R degradation; they do not verify that endogenous levels of similar molecules effect the rate of HMG-R degradation. They do strongly suggest that the exogenously added molecules may mimic endogenously produced ones. In our following examination of oxysterol-derived signals for yeast HMG-R degradation, we utilized both genetic and pharmacological manipulations to address this issue.

Oxysterols can be produced in the cell from the alternate oxysterol pathway (Dollis and Schuber, 1994; Fig. 2-18), which branches from the sterol biosynthetic pathway at 2,3(S)-oxidosqualene. Oxidosqualene-lanosterol cyclase plays a critical role in flux through the oxysterol pathway by indirect and direct means. By catalyzing the cyclization of 2,3(S)-oxidosqualene to lanosterol (Dollis and Schuber, 1994),
oxidosqualene-lanosterol cyclase indirectly controls the cellular levels of 2,3(S)oxidosqualene available for further epoxidation to 2,3(S):22(S),23-dioxidosqualene. Through preferential cyclization of the 2,3(S):22(S),23-dioxidosqualene (Boutaud *et al.*, 1992), oxidosqualene-lanosterol cyclase directly controls the production of 24(S),25oxidolanosterol. Accordingly, the cellular levels of oxidosqualene-lanosterol cyclase activity may be an important factor in determining oxysterol production. Thus, pharmacological or genetic manipulations of oxidosqualene-lanosterol cyclase served as a useful starting point to examine the effect of oxysterol production on yeast HMG-R degradation.

To determine if HMG-R degradation in yeast could be hastened by an oxysterolderived product, we examined the effect of altering cellular oxidosqualene-lanosterol cyclase activity on the degradation of the yeast HMG-R isozyme Hmg2p. We first examined the effect of decreased cellular oxidosqualene-lanosterol cyclase activity on Hmg2p degradation by addition of the drug Ro48-8071, which is a specific inhibitor of oxidosqualene-lanosterol cyclase (Morand *et al.*, 1997), to cells. Addition of Ro48-8071 to cells co-expressing both versions of Hmg2p caused an increase in Hmg2p degradation in a dose-dependent manner, as seen by the reduced steady-state levels of both 1myc-Hmg2p and Hmg2p-GFP (Fig. 2a&b, "+Ro48", respectively). However, the maximal degradation-enhancing effect of Ro48-8071 was not as great as that caused by the addition of zaragozic acid ("+Ro48" versus "+ZA"). The degradation-enhancing effect of Ro48-8071 addition was blocked by simultaneous addition of lovastatin, as observed by the lack of effect of Ro48-8071 addition on the stabilized Hmg2p-GFP steady-state levels in lovastatin treated cells (Fig. 2b, "+Ro48,+Lov" versus "+Lov").



Figure 2-13: Inhibition of oxidosqualene-lanosterol cyclase enhanced Hmg2p degradation.

a) Effect of Ro48-8071 on 1myc-Hmg2p steady-state levels. Log-phase cultures were treated for 4 hours with either no drug ("-"), 40 μ g/ml Ro48-8071 ("+Ro48"), or 10 μ g/ml zaragozic acid ("+ZA"), then subjected to immunoblotting with anti-myc 9E10 antibody to determine 1myc-Hmg2p steady-state levels. b) Effect of Ro48-8071 on Hmg2p-GFP steady-state levels. Log-phase cultures were treated for four hours with either no drug ("-"), 40 μ g/ml Ro48-8071 ("Ro48"), 25 μ g/ml lovastatin ("+Lov"), 10 μ g/ml zaragozic acid ("+ZA"), or 80 μ g/ml terbinafine ("+Tb"), then analyzed by flow cytometry to determine Hmg2p-GFP steady-state fluorescence.

Furthermore, the degradation-enhancing effect of Ro48-8071 addition was suppressed by addition of terbinafine, a potent, fungal squalene epoxidase inhibitor (Ryder, 1989; Jandrositz *et al.*, 1991; Ryder, 1992), to the cells ("+Ro48, +Tb" versus "+Ro48"). This implicated the substrate of oxidosqualene-lanosterol cyclase, 2,3(S)-oxidosqualene, as a source of the signal for enhanced Hmg2p degradation by Ro48-8071 addition.

It was possible that the Hmg2p degradation-enhancing effect of Ro48-8071 addition to cells was through an action other than oxidosqualene-lanosterol cyclase inhibition. Accordingly, we tested the effect of reducing cellular oxidosqualene-lanosterol cyclase activity on Hmg2p degradation by genetic down-regulation of oxidosqualene-lanosterol cyclase. To do so, we constructed a "promoter-switch" plasmid containing a truncated version of the oxidosqualene-lanosterol cyclase gene, *ERG7*, placed behind the *MET3* promoter, similar to our previously described conditional alleles of the other mevalonate pathway enzymes described above. Targeted integration of this plasmid into the *ERG7* locus resulted in the creation of a single, functional copy of the oxidosqualene-lanosterol cyclase gene under control of the regulated *MET3* promoter (P_{MET3}). The plasmid was used to transform the 1myc-Hmg2p and Hmg2p-GFP co-expressing, methionine prototroph yeast strain described above. When grown in the absence of methionine, normal regulated Hmg2p degradation was observed in the P_{MET3} -*ERG7* yeast strain (Fig. 2-14a).

Down-regulation of oxidosqualene-lanosterol cyclase, by growth of the P_{MET3}^{-} ERG7 strain in 2mM methionine, resulted in increased degradation of Hmg2p as seen by reduced Hmg2p-GFP steady-state levels (Fig. 2-14b, " P_{MET3}^{-} -ERG7" versus "wt"). The effect was similar to the effect of squalene synthase down-regulation (" P_{MET3} -

ERG7" versus "P_{*MET3*}-*ERG9*"), but was not as drastic. These observations were identical to the effects seen by inhibition of each respective enzyme with Ro48-8071 or zaragozic acid shown above (Fig. 2-13b). Furthermore, Hmg2p-GFP in the P_{*MET3*}-*ERG7* strain was stabilized by the addition of lovastatin to a similar degree as the stabilization of Hmg2p-GFP by lovastatin addition in the wild-type strain (Fig. 2-14b, "P_{*MET3*}-*ERG9*, +Lov" compared with "wt, +Lov"), indicating that enhanced Hmg2p degradation caused by oxidosqualene-lanosterol cyclase down-regulation was still regulated by mevalonate pathway flux. Thus, the effect of Ro48-8071 addition on Hmg2p degradation.

It was possible, however, that the effect of Ro48-8071 addition on Hmg2p steady-state levels was not due to enhanced degradation, but occurred through decreased transcription of *HMG2* mRNA or decreased Hmg2p synthesis through diminished translation of the mRNA itself. We eliminated transcriptional regulation in the cells used for the above analyses by expression of 1myc-Hmg2p and Hmg2p-GFP from the constitutive *TDH3* promoter. In mammalian cells, the 5' untranslated region of HMG-R mRNA has been implicated in regulation of HMG-R synthesis (Gayen and Peffley, 1995). The role of the 5' untranslated region of *HMG2* mRNA in regulation of the mg2p synthesis in yeast is unknown. Construction of the P_{*TDH3*}-*1mycHMG2* and P_{*TDH3*}-*hmg2::GFP* alleles resulted in replacement of the native *HMG2* 5' untranslated region with that from the *TDH3* gene, thereby eliminating possible transcriptional regulation from this region of *HMG2* mRNA. However, it was still possible that the effect of



Figure 2-14: Down-regulation of oxidosqualene-lanosterol cyclase enhanced Hmg2p degradation.

a) Cells expressing oxidosqualene-lanosterol cyclase from the *MET3* promoter ("P_{*MET3*}-*ERG7*") had normal regulated Hmg2p degradation in the absence of exogenous methionine. Cycloheximide-chase assay of strains expressing squalene synthase from the wild-type promoter ("wt") or from the *MET3* promoter. Lovastatin (25 μ g/ml) was added to the indicated samples at the same time as addition of cycloheximide. After addition of cycloheximide, lysates were prepared at the indicated times and immunoblotted to determine Hmg2p levels. b) Effect of oxidosqualene lanosterol cyclase down-regulation on Hmg2p-GFP steady-state levels. Otherwise identical strains expressing oxidosqualene lanosterol cyclase from either the wild-type promoter ("wt") or the *MET3* promoter ("P_{*MET3*}-*ERG7*"), or squalene synthase from the *MET3* promoter ("P_{*MET3*}-*ERG9*") were grown for 15 hours at 30°C in the presence of 2mM methionine. 25 μ g/ml lovastatin was added to the indicated cultures after 11 hours incubation. Hmg2p-GFP steady-state fluorescence was analyzed by flow cytometry. Ro48-8071 on the Hmg2p steady-state levels was through alteration of Hmg2p synthesis due to some regulatory element in the coding region of the *HMG2* mRNA.

To rule out the possibility that oxidosqualene-lanosterol cyclase inhibition resulted in altered translation and decreased Hmg2p synthesis, we examined the effect of Ro48-8071 addition on Hmg2p-GFP steady-state levels in cells that were deficient for Hmg2p degradation through either in-cis or in-trans stabilization. The effect of Ro48-8071 addition was first examined with an in-cis stabilized Hmg2p reporter protein K6R-Hmg2p-GFP. Introduction of the K6R mutation in both 1myc-Hmg2p and Hmg2p-GFP results in complete stabilization (see Chapter 3). When Ro48-8071 was added to cells that expressed the completely stable K6R-Hmg2p-GFP, no decrease in the steady-state levels of K6R-Hmg2p-GFP was observed (Fig. 2-15, left panel). Next, the effect of Ro48-8071 addition was examined with normal Hmg2p-GFP expressed in cells deficient for ER degradation by the presence of a hrdl null allele, which results in the in-trans stabilization of Hmg2p (Hampton et al., 1996a). The gene product of HRD1, Hrd1p, is an ER-associated ubiquitin ligase essential for ubiquitin-dependent, Hmg2p degradation (Hampton et al., 1996a; Bays et al., 2000). Similar to the in-cis stabilization by introduction of the K6R mutation, no decrease in the steady-state levels of normal Hmg2p-GFP in cells carrying the $hrd1\Delta$ allele was observed after Ro48-8071 addition (Fig. 2-15, middle panel). In fact, for each stabilized version of Hmg2p-GFP, whether in-*cis* or in-*trans*, there appeared to be a slight increase in the steady-state levels, which was also observed with the steady-state levels of the normally stable Hmg1p-GFP (Fig. 2-15, right panel). Thus, the decrease in 1myc-Hmg2p and



Figure 2-15: Oxidosqualene-lanosterol cyclase inhibition did not lower steady-state levels of degradation-deficient Hmg2p-GFP.

Otherwise identical strains expressing K6R-Hmg2p-GFP (left panel), Hmg2p-GFP in the presence of the $hrd1\Delta$ allele (middle panel), or Hmg1p-GFP (right panel) were grown to log-phase and then treated with 40μ g/ml Ro48-8071 ("Ro48") or no drug ("no drug") for 4 hours at 30°C. Cultures were analyzed by flow cytometry to determine cellular steady-state Hmg2p-GFP or Hmg1p-GFP fluorescence.

Hmg2p-GFP steady-state levels as a result of Ro48-8071 addition to cells was even more striking, and was the result of enhanced degradation, not decreased Hmg2p synthesis.

Oxidosqualene-lanosterol cyclase inhibition did not affect general ER degradation.

It was possible that the degradation stimulating effect of oxidosqualenelanosterol cyclase inhibition on Hmg2p degradation occurred through enhancement of ER degradation in general, rather than through the specific action of mevalonate pathway regulation on Hmg2p degradation. To test this, we examined if Ro48-8071 addition to cells altered the degradation of 6myc-Hmg2p, a previously described mutant of Hmg2p that is degraded in a similar *HRD*-dependent, ER-associated manner as normal Hmg2p (Hampton *et al.*, 1996a), but its degradation is not regulated by signals from the mevalonate pathway (Hampton *et al.*, 1996a, and Fig. 2-10). When Ro48-8071 was added to cells co-expressing 6myc-Hmg2p and 6myc-Hmg2p-GFP in place of 1myc-Hmg2p and Hmg2p-GFP, no decrease in the steady-state levels of 6myc-Hmg2p-GFP was observed (Fig. 2-16). Thus, inhibition of oxidosqualene-lanosterol cyclase enhanced only the mevalonate pathway regulated degradation of Hmg2p, not general ER-associated, *HRD*-dependent degradation.

Oxidosqualene-lanosterol cyclase inhibition enhanced Hmg2p ubiquitination.

In figure 2-9, we demonstrated that the upstream FPP-derived signal enhanced Hmg2p degradation by acting to increase Hmg2p ubiquitination. Because inhibition or down-regulation of oxidosqualene-lanosterol cyclase resulted in increased degradation



Figure 2-16: Inhibition of oxidosqualene-lanosterol cyclase did not affect general ER degradation.

Otherwise identical strains expressing either the mevalonate pathway-regulated, degradation substrate Hmg2p-GFP or the constitutive degradation substrate 6myc-Hmg2p-GFP were grown to log-phase and then treated with 40 μ g/ml Ro48-8071 ("Ro48") or no drug ("no drug") for 4 hours at 30°C. Cultures were analyzed by flow cytometry to determine cellular steady-state Hmg2p-GFP or 6myc-Hmg2p-GFP fluorescence.

of Hmg2p, we examined if this effect was through enhanced Hmg2p ubiquitination by a similar assay as in figure 2-9.

When the cells co-expressing 1myc-Hmg2p and 3HA-Ub were treated with Ro48-8071, Hmg2p ubiquitination was increased (Fig. 2-17, "R"), but the degree of enhanced ubiquitination was slightly lower than that observed when cells were incubated with zaragozic acid (compare "R" with "ZA"). The effect of Ro48-8071 on Hmg2p ubiquitination was blocked by addition of lovastatin at the same time as Ro48-8071 and was identical to the effect of only lovastatin addition (compare "R + L" with "L"), indicating that the Ro48-8071 effect on Hmg2p ubiquitination was regulated by the mevalonate pathway. Thus, increased, regulated Hmg2p ubiquitination by addition of Ro48-8071 strongly supported the observation that reduced Hmg2p steady-state levels by oxidosqualene-lanosterol cyclase inhibition was the result of enhanced Hmg2p degradation.

Complete inhibition of oxidosqualene-lanosterol cyclase did not affect Hmg2p degradation.

Oxysterols produced in the cell can be generated from the alternate oxysterol pathway (Dollis and Schuber, 1994), which branches from sterol biosynthetic pathway at 2,3(S)-oxidosqualene (Fig. 2-18). If 2,3(S)-oxidosqualene is not readily converted to lanosterol by oxidosqualene-lanosterol cyclase, the remaining pool of 2,3(S)-oxidosqualene is available for further epoxidation to 2,3(S):22(S),23-dioxidosqualene, which occurs by squalene epoxidase (Field and Holmlund, 1977; Bai *et al.*, 1992). Reduced oxidosqualene-lanosterol cyclase activity in yeast may result in the build-up



Figure 2-17: Oxidosqualene-lanosterol cyclase inhibition increased Hmg2p ubiquitination.

Cells expressing 1myc-Hmg2p and 3HA-Ubiquitin were grown to log-phase. Ubiquitination assays were performed in the presence of no drug ("-"), 25 μ g/ml lovastatin ("L"), 40 μ g/ml Ro48-8071 ("R"), or 10 μ g/ml zaragozic acid ("ZA"). Lovastatin and Ro48-8071 were added 3 hours prior to cell lysis and ZA was added 10 min prior to cell lysis. Upper panels are the result of anti-HA (" α -HA") immunoblotting for covalently linked HA-tagged Ub. Lower panels are the result of parallel immunoblotting an aliquot (1/8 total volume) of the same immunoprecipitates with the 9E10 anti-myc antibody (" α -myc") to assess total immunoprecipitated Hmg2p.



Figure 2-18: The alternate pathway.

Cartoon representation of the mevalonate pathway and the alternate oxysterol pathway. Enzymes responsible for each step and their yeast inhibitors are marked accordingly. Figure was adapted from Goldstein and Brown (1990) and Dollis and Schuber (1994). of its substrate 2,3(S)-oxidosqualene, which can be further epoxidized by squalene epoxidase. However, if sufficient oxidosqualene-lanosterol cyclase activity remains, 2,3(S):22(S),23-dioxidosqualene can be further converted to 24(S),25-oxidolanosterol (Field and Holmlund, 1977). Oxidosqualene-lanosterol cyclase has a higher affinity for the di-oxidosqualene than for the mono-oxidosqualene (Boutaud *et al.*, 1992), and thus the cyclization of the di-oxidosqualene is the favored reaction when both substrates are present.

A consequence of the higher affinity for di-oxidosqualene is that, in mammalian cells, moderate inhibition of oxidosqualene-lanosterol cyclase increases production of molecules in the alternate oxysterol pathway, but decreases production of molecules in the sterol pathway (Mark et al., 1996; Peffley et al., 1998). Complete inhibition of oxidosqualene-lanosterol cyclase blocks production of molecules in both the sterol and oxysterol pathways (Mark et al., 1996; Peffley et al., 1998). Thus, the different degrees of oxidosqualene-lanosterol cyclase inhibition, partial versus complete, provide a strict criterion to test if HMG-R degradation is being regulated by products of the oxysterol pathway. If HMG-R degradation were being regulated by a sterol pathway product, then partial or complete inhibition of oxidosqualene-lanosterol cyclase should result in reduced HMG-R degradation. If HMG-R degradation were being regulated by an oxysterol pathway product, then partial inhibition of oxidosqualene-lanosterol cyclase should result in enhanced degradation, whereas complete inhibition should have the opposite effect. Indeed, moderate inhibition of oxidosqualene-lanosterol cyclase in mammalian cells resulted in a decrease in HMG-R activity, whereas complete inhibition resulted in an increase in HMG-R activity (Mark et al., 1996). However, whether this

effect was the result of alteration of only HMG-R degradation or all forms of HMG-R regulation was not examined.

In our above experiments, reduced cellular oxidosqualene-lanosterol cyclase activity, by inhibition or down regulation, resulted in enhanced Hmg2p degradation, suggesting that a product of the oxysterol pathway, and not the sterol pathway, was acting to positively regulate Hmg2p degradation. If this were the case, then it would be expected that moderate inhibition of oxidosqualene-lanosterol cyclase in yeast would increase Hmg2p degradation, but complete inhibition would have no effect. To test this hypothesis, increasing concentrations of Ro48-8071 were first added to cells, but high concentrations (>100 μ g/ml) rapidly killed the cells and Hmg2p degradation could not be assessed (data not shown). Therefore, we employed a more sophisticated way to acutely inhibit all of the available oxidosqualene-lanosterol cyclase in the cell while avoiding the extremely lethal, high concentrations of Ro48-8071. Specifically, production of oxidosqualene-lanosterol cyclase was first down-regulated by addition of 2mM methionine to the P_{MET3} -ERG7 cells. This resulted in the expected decrease in Hmg2p-GFP steady-state levels (Fig. 2-19a, left panel: "P_{MET3}-ERG7, no drug" versus "wt, no drug"), and the decrease was similar to the decrease observed in the wild-type ERG7 strain after addition of 20 µg/ml Ro48-8071 (left panel: "P_{MFT3}-ERG7" compared with right panel: "wt, $+40\mu$ M Ro48"). The low levels of oxidosqualene-lanosterol cyclase in the P_{MET3}-ERG7 strain under these down-regulated conditions should be completely inhibited by addition of relatively low concentrations of Ro48-8071. In fact, when just $5\mu g/ml$ Ro48-8071 was added to these cells, Hmg2p-GFP degradation was



Figure 2-19: Complete inhibition of the oxysterol pathway did not enhance Hmg2p degradation.

a) Partial inhibition of oxidosqualene-lanosterol cyclase enhanced Hmg2p degradation, whereas complete inhibition had no effect. Otherwise identical strains expressing oxidosqualene lanosterol cyclase from either the wild-type promoter ("wt") or the *MET3* promoter ("P_{MET3}-*ERG7*") were grown for 13 hours at 30°C in the presence of 2mM methionine. Ro48-8071 (left panel: 10 μ M, "+Ro48"; right panel: 40 μ M, "+40 μ MRo48" or 50 μ M, "+50 μ MRo48") was added to the appropriate cultures and the cells were incubated for an additional 4 hours at 30°C. Hmg2p-GFP fluorescence was analyzed by flow cytometry. b) Similar experiment in (a), except that the strains were allowed to grow 17-24 hours in 2mM methionine and no drug was added.

noticeably decreased in the P_{MET3} -*ERG7* cells (Fig. 2-19a, left panel: " P_{MET3} -*ERG7*, +Ro48" versus " P_{MET3} -*ERG7*, no drug"), and the marked increase in Hmg2p-GFP steady-state fluorescence resulted in a similar Hmg2p-GFP steady-state level as that in wild-type *ERG7* cells without drug treatment (" P_{MET3} -*ERG7*, +Ro48" compared with "wt, no drug"). In contrast, the same incremental addition of 5 µg/ml Ro48-8071 to wild-type cells did not alter the Hmg2p degradation-enhancing effect observed after incubation of cells with 20 mg/ml Ro48-8071 (right panel: "wt, +25µg/ml Ro48" compared with "wt, +20µg/ml Ro48").

In addition to complete inhibition of oxidosqualene-lanosterol cyclase by the combination of pharmacological manipulation and genetic down-regulation, we tested if down-regulation for further extended periods of time, which should result in almost complete loss of oxidosqualene-lanosterol cyclase activity in the cell, would have the same effect. Indeed, continued incubation of the P_{MET3} -*ERG7* strain in 2mM methionine after the maximal Hmg2p-GFP degradation-enhancing effect was observed at 17 hours resulted in a complete reversal by 24 hours (Fig. 2-19b, left panel), similar to the effect of Ro48-8071 addition. This effect was not seen for the wild-type *ERG7* strain (right panel).

Thus, moderate reduction in the activity of oxidosqualene-lanosterol cyclase, either by down-regulation or pharmacological inhibition, resulted in hastened Hmg2p degradation. In contrast, complete inhibition of oxidosqualene-lanosterol cyclase did not result in hastened Hmg2p degradation, indicating that a product of the oxysterol pathway acted as a positive signal for HMG-R degradation in yeast.

Yeast contained endogenous levels of oxysterols that enhanced Hmg2p degradation.

Squalene epoxidase can act to convert its product 2,3(S)-oxidosqualene to 2,3(S):22(S),23-dioxidosqualene. If under normal growth conditions there is sufficient accumulation of 2,3(S)-oxidosqualene to allow it to become a substrate for squalene epoxidase, then it is quite possible that yeast would contain endogenous levels of oxysterols that could act to enhance Hmg2p degradation. Accordingly, increased expression of oxidosqualene-lanosterol cyclase should result in the hastened conversion of 2,3(S)-oxidosqualene to lanosterol, thereby reducing the levels of 2,3(S)-oxidosqualene to lanosterol, thereby reducing the levels of an oxysterol pathway molecule was acting as a positive signal for Hmg2p degradation, then such over-expression of oxidosqualene-lanosterol cyclase would result in reduced Hmg2p degradation.

To examine the effect of increased oxidosqualene-lanosterol cyclase expression, we placed the *ERG7* gene behind the strong, constitutive *TDH3* promoter and transformed the P_{TDH3} -*ERG7* gene into yeast cells expressing 1myc-Hmg2p and Hmg2p-GFP. Increased expression of oxidosqualene-lanosterol cyclase did indeed have a stabilizing effect on Hmg2p degradation, as seen by the rightward shift in the Hmg2p-GFP fluorescence histogram of the P_{TDH3} -*ERG7* cells compare to wild-type *ERG7* cells (Fig. 2-20a, left panel: " P_{TDH3} -*ERG7*" versus "wt"). This result was similar to that previously reported for over-expression of squalene synthase (see above, and Fig. 2-20b, right panel), which stabilizes Hmg2p through reduced FPP levels by enhanced conversion to squalene. Therefore, it appeared that enhanced conversion of 2,3(S)



Figure 2-20: Endogenous levels of oxysterol pathway products contributed to normal Hmg2p degradation.

a) Over-expression of oxidosqualene-lanosterol cyclase stabilized Hmg2p. Otherwise identical strains expressing either oxidosqualene-lanosterol cyclase from the *TDH3* promoter ("P_{*TDH3*}-*ERG7*"), squalene synthase from the *TDH3* promoter ("P_{*TDH3*}-*ERG9*"), or all from the native promoters ("wt") were grown to mid log-phase. b) Down-regulation of squalene epoxidase modestly stabilized Hmg2p. Otherwise identical strains expressing either squalene epoxidase from the *MET3* promoter ("P_{*MET3*}-*ERG1*"), FPP synthase from the *MET3* promoter ("P_{*MET3*}-*ERG20*"), or both from their respective native promoters ("wt") were grown for 15 hours at 30°C in the presence of 8mM methionine. c) Chronic treatment with terbinafine modestly stabilized Hmg2p. Cells were initially grown to log-phase (OD₆₀₀=0.5). Cells were then diluted back to an initial OD₆₀₀=0.01 in media that contained either a lethal dose of terbinafine ("+Tb", 80µg/ml), a lethal dose of lovastatin ("+Lov", 300µg/ml), or no adenine ("-Ade") and incubated for 18 hours at 30°C. In all cases, Hmg2p-GFP steady-state fluorescence was analyzed by flow cytometry.

oxidosqualene to lanosterol in the cell also resulted in stabilization of Hmg2p, further implicating a molecule of the oxysterol pathway as a positive signal for Hmg2p degradation. These results additionally demonstrated that endogenous levels of oxysterols were acting to increase Hmg2p degradation under normal growth conditions.

If endogenous levels of oxysterols were acting to program a specific rate of Hmg2p degradation, then it might be expected that inhibition of squalene epoxidase would have a stabilizing effect on Hmg2p degradation. In figure 2-12, we had shown that acute treatment of cells with lethal doses of terbinafine, a fungal squalene epoxidase inhibitor (Ryder, 1989; Jandrositz *et al.*, 1991; Ryder, 1992), had no effect on Hmg2p-GFP steady-state levels. However, it was possible that the limited, acute treatment had little effect on the pre-existing pool of oxysterols present as a result of oxysterol pathway production prior to inhibition of squalene epoxidase. To reduce flux through the oxysterol pathway more stringently and reduce the pool of potential accumulated oxysterols, we examined if either down-regulation or chronic inhibition of squalene epoxidase resulted in stabilization of Hmg2p degradation.

Although previously we had shown that down-regulation of squalene epoxidase had little effect on Hmg2p degradation under the conditions tested (Fig. 2-11), we explored whether incubation of cells expressing squalene epoxidase from the *MET3* promoter (P_{MET3} -*ERG1*) in different concentrations of methionine and/or with different incubation periods after methionine addition would reveal if squalene epoxidase downregulation had any stabilizing effect on Hmg2p degradation. In fact, we were able to observe a modest stabilizing effect on Hmg2p-GFP degradation due to squalene epoxidase down-regulation, as cells carrying the P_{MET3} -*ERG1* gene had higher steadystate levels of Hmg2p-GFP after incubation with 8mM methionine, rather than 2mM, for 15 hours compared to wild-type cells with the same treatment (Fig. 2-20b, "P_{MET3}-*ERG1*" versus "wt"). However, down-regulation of squalene epoxidase did not have nearly the same stabilizing effect as down-regulation of the upstream mevalonate pathway enzyme farnesyl diphosphate synthase (see above and Fig. 2-20b, "P_{MET3}-*ERG1*" versus "P_{MET3}-*ERG20*"), which results in complete stabilization of Hmg2p by reduced FPP production within the cell.

Modest stabilization by down-regulation of squalene epoxidase suggested that the acute treatment of cells with terbinafine was most likely not stringent enough to see the limited stabilization. Accordingly, we tested if chronic treatment of cells with terbinafine would have a similar stabilizing effect on Hmg2p degradation as downregulation of squalene epoxidase. When cells were incubated with lethal doses of terbinafine for increasing periods of time past the inhibition of cell growth, modest stabilization of Hmg2p degradation was observed, as seen by the increased Hmg2p-GFP steady-state levels compared to wild-type cells that were similarly growth compromised by removal of adenine from the growth media (Fig. 2-20c, "+Tb" versus "-Ade"). However, chronic treatment of cells with lethal doses of lovastatin resulted in much greater stabilization of Hmg2p, which lead to greatly increased Hmg2p-GFP steadystate levels ("+Lov"). Thus, the Hmg2p-stabilizing effect of reduced oxysterol production was observable, but limited in its ability to alter Hmg2p degradation. The stabilizing effect of squalene epoxidase down-regulation or chronic inhibition did not it have an equivalent stabilizing effect as oxidosqualene-lanosterol cyclase overexpression, possibly due to the increased cellular FPP levels as a result of the growthretarding manipulations. However, together the genetic and pharmacological manipulations of both oxidosqualene-lanosterol cyclase and squalene epoxidase activity revealed that a product of the oxysterol pathway acted to positively regulate Hmg2p degradation. Furthermore, it appeared that endogenous levels of the oxysterol-derived molecule were acting to program the specific rate of Hmg2p degradation under normal growth conditions.

The oxysterol-derived signal functioned only when sufficient levels of the primary FPP-derived signal were present.

The above results suggested that the oxysterol-derived molecule was an additional signal for Hmg2p degradation and not a primary signal. In mammalian cells, the secondary oxysterol-derived signal cannot work independently of the primary FPP-derived signal (Nakanishi *et al.*, 1988; Roitelman and Simoni, 1992; Meigs and Simoni, 1997). To test if this was similar for Hmg2p degradation, we required a means to reduce FPP levels that still allowed production of downstream mevalonate pathway molecules. Previously, we constructed strains that over-expressed squalene synthase from the *TDH3* promoter (Fig. 2-7), which resulted in stabilization of Hmg2p through reduced FPP levels (see also Fig. 2-20). In these strains, mevalonate pathway production was still fully operational because FPP levels were reduced by increased conversion to squalene, not by inhibition of FPP production. When Ro48-8071 was added to cells that over-expressed squalene synthase, no effect on Hmg2p degradation was observed (Fig. 2-21a, left panel: P_{TDH3} -*ERG9*, "Ro48" compared with "no drug"), even though we used up to 4-fold higher concentrations of Ro48-8071 than were



Figure 2-21: The oxysterol-derived signal for Hmg2p degradation required sufficient amounts of the FPP-derived signal.

a) Squalene synthase over-expression blocked degradation enhancing effect of oxidosqualene-lanosterol cyclase inhibition. Otherwise identical strains expressing squalene synthase from a single, integrated allele with the *TDH3* promoter (" P_{TDH3} -*ERG9*") or from the wild-type promoter ("wt") were grown to log-phase and treated with either no drug, 40 µg/ml Ro48-8071 ("+Ro48"), or 10 µg/ml zaragozic acid ("+ZA") for 4 hours at 30°C. Cells were analyzed by flow cytometry to determine Hmg2p-GFP levels. **b**) Oxidosqualene-lanosterol cyclase over-expression did not block the degradation enhancing effect of squalene synthase inhibition. Otherwise identical strains expressing oxidosqualene-lanosterol cyclase as a single, integrated allele from the *TDH3* promoter (" P_{TDH3} -*ERG7*") or from the wild-type promoter ("wt") were grown to log-phase and treated with either no drug, 40 µg/ml Ro48-8071 ("+Ro48"), or 10 µg/ml zaragozic acid ("+ZA") for 4 hours at 30°C. Cells were analyzed by flow cytometry to determine Hmg2p-GFP levels.

sufficient to enhance degradation in a wild-type squalene synthase strain (right panel: wt, "Ro48" compared with "no drug"). Addition of zaragozic acid, which inhibits squalene synthase, did result in enhanced Hmg2p-GFP degradation in the strain over-expressing squalene synthase as well as the wild-type strain (both panels: "ZA"). Thus, the action of the oxysterol-derived positive signal for HMG-R degradation in yeast required a sufficient level of the primary FPP-derived positive signal.

The secondary oxysterol-derived signal acted to enhance Hmg2p degradation, but was not an essential requirement.

Previous studies delineating the regulatory signals required for mammalian HMG-R degradation demonstrated that the primary FPP-derived signal also did not function to enhance HMG-R degradation in the absence of sufficient levels of the oxysterol-derived signal (Correll and Edwards, 1994; Meigs *et al.*, 1996, Meigs and Simoni, 1997). In one study, the obligatory nature of the oxysterol-derived signal was examined by observing HMG-R degradation after inhibition of squalene epoxidase (Correll and Edwards, 1994). The other two studies examined HMG-R degradation in cell lines that were deficient for squalene synthase activity (Meigs *et al.*, 1996; Meigs and Simoni, 1997). In both cases, sterol synthesis was severely reduced and the evidence supported a co-dependent nature for the HMG-R degradation enhancing effect of the FPP-derived signal and the oxysterol-derived signal.

To examine further the requirement for the oxysterol-derived signal in yeast, we tested if over-expression of oxidosqualene-lanosterol cyclase prevented the ability of

FPP accumulation to enhance Hmg2p degradation. We expected that if the oxysterolderived signal were obligatory, then the hastened conversion of 2,3(S)-oxidosqualene to lanosterol would result in a blunted effect of zaragozic acid addition to cells overexpressing oxidosqualene-lanosterol cyclase. However, addition of zaragozic acid to the cells over-expressing oxidosqualene-lanosterol cyclase had an equivalent effect on Hmg2p-GFP steady-state fluorescence as zaragozic acid addition to wild-type cells (Fig. 2-21b, compare "P_{TDH3}-*ERG7*" with "wt"), at all concentrations tested (data not shown). Addition of Ro48-8071 to the P_{TDH3}-*ERG7* cells did result in enhanced Hmg2p-GFP degradation ("+Ro48"), but it required 4-fold higher concentrations than those needed to enhance degradation in the wild-type cells, as would be expected for increased expression of the drug's target enzyme oxidosqualene-lanosterol cyclase. Thus, it appeared that the upstream FPP-derived signal could work in the absence of the downstream oxysterol-derived signal.

These combined results implied that the function of the oxysterol-derived signal was to enhance FPP-dependent degradation of Hmg2p, rather than serve as an essential signal. If this were the case, then it would be expected that the oxysterol-derived signal would act synergistically with the FPP-derived signal. To examine this model, we treated cells expressing Hmg2p-GFP with concentrations of zaragozic acid that normally did not enhance Hmg2p-degradation combined with increasing concentrations of Ro48-8071 that also normally did not enhance Hmg2p degradation. When we treated the cells in this manner we observed that the oxysterol-derived signal did indeed work synergistically with the FPP-derived signal, as a greater than additive decrease in Hmg2p-GFP steady-state levels was observed (Fig. 2-22, left panel: "+ZA,+Ro48"

versus "+ZA" and "+Ro48"), with the maximal synergistic effect produced by addition of 5 μ g/ml Ro48-8071 (Fig. 2-22, right panel). However, sufficiently high levels of the FPP-derived signal in the cell, accomplished by inhibition of squalene synthase, did allow the fastest rate of Hmg2p degradation even with genetic or pharmacological manipulated decreases in the oxysterol-derived signal (Fig. 2-21), suggesting the independence of the FPP-derived signal. Thus, the function of the oxysterol-derived signal in yeast was to enhance Hmg2p degradation beyond the limited capacity of low levels of the FPP-derived signal.



Figure 2-22: The oxysterol-derived signal worked synergistically with the FPPderived signal to promote Hmg2p degradation.

Cells were grown to log-phase and treated with a dose of zaragozic acid that did not elicit a degradation enhancing effect (2.5 μ g/ml, left panel: "+ZA"). Increasing concentrations of Ro48-8071, which did not promote Hmg2p degradation alone, were added to these zaragozic acid-treated cells. All cultures were incubated at 30°C for 4 hours after drug addition. Cultures were analyzed by flow cytometry to determine Hmg2p-GFP steady-state fluorescence. The maximal effect of Ro48-8071 was observed with a final concentration of 5.0 μ g/ml (right panel, and left panel: "+ZA, +Ro48" versus "+Ro48" and "+ZA").

C. Discussion

HMG-R degradation is regulated by signals generated downstream in the mevalonate pathway. The identity of these signals and the mechanism by which they control HMG-R degradation is currently not known. To understand better the nature of this regulatory mechanism, we have examined the regulated degradation of the yeast HMG-R isozyme Hmg2p through genetic and pharmacological manipulation of the mevalonate pathway.

The results detailed here strongly implicated the mevalonate pathway product FPP as the source of the positive, primary signal for Hmg2p degradation. ZA addition or squalene synthase down-regulation, both predicted to increase FPP levels in the cell, increased Hmg2p degradation. Conversely, addition of lovastatin, FPP synthase downregulation, or squalene synthase over-expression, all predicted to decrease FPP levels in the cell, stabilized Hmg2p. Identical Hmg2p steady-state levels in degradation-deficient strains containing either normal or down-regulated squalene synthase levels indicated that FPP levels affected only Hmg2p degradation.

Additionally, our results also implicated a product of the alternate oxysterol pathway as a source of a positive, secondary signal for Hmg2p degradation in yeast. Moderate inhibition or genetic down-regulation of oxidosqualene-lanosterol cyclase increased Hmg2p degradation, whereas complete inhibition had no effect. These manipulations were consistent with increased oxysterol production by moderate oxidosqualene-lanosterol cyclase inhibition previously demonstrated in mammalian cells (Mark *et al.*, 1996; Peffley *et al.*, 1998). Furthermore, Hmg2p was stabilized by

increased expression of oxidosqualene-lanosterol cyclase, which should act to decrease production of oxysterols by hastening conversion of 2,3(S)-oxidosqualene to lanosterol thereby reducing the amount of 2,3(S)-oxidosqualene that could be further epoxidized. Thus, all manipulations predicted to increase oxysterols in the cell resulted in increased Hmg2p degradation, whereas all manipulations predicted to decrease oxysterols in the cell resulted in stabilization of Hmg2p.

FPP as the source of the signal for Hmg2p degradation.

Manipulation of FPP levels by both pharmacological and genetic means resulted in the expected changes to Hmg2p degradation consistent with FPP, or a derivative, as a positive primary signal for Hmg2p degradation. The idea that intracellular levels of FPP, or a derivative, serve to modulate Hmg2p degradation in yeast paralleled similar observations in mammalian cells. It has been proposed that degradation of mammalian HMG-R is regulated by the intracellular levels of farnesol, a derivative of FPP (Bradfute and Simoni, 1994; Correll and Edwards, 1994; Meigs *et al.*, 1996; Lopez *et al.*, 1997; Meigs and Simoni, 1997). It may also be the case that farnesol is the positive signal for Hmg2p degradation in yeast, and we are currently examining whether FPP or farnesol regulates Hmg2p degradation.

One way to distinguish between FPP and farnesol as the regulatory signal would be by elimination of the pyrophosphatase activity required to convert FPP to farnesol (Meigs and Simoni, 1997). Recently, two pyrophosphatases, *LPP1* and *DPP1*, that appear to convert FPP to farnesol *in vitro* have been described (Faulkner *et al.*, 1999). Null alleles of the genes encoding these enzymes have been made in yeast and the cells are viable. We examined Hmg2p degradation in these strains and found that the absence of Lpp1p and Dpp1p had no effect on Hmg2p regulated degradation (data not shown). This could mean that either FPP, not farnesol, is the signal for HMG-R degradation in yeast or that these enzymes are not solely responsible for the conversion of FPP to farnesol *in vivo*. In either case, it is apparent that FPP serves as the source of a signal for HMG-R degradation in both mammals and yeast.

In mammalian cells, an additional oxysterol-derived signal appears to modulate HMG-R degradation. The addition of oxysterols to mammalian cells results in increased degradation of HMG-R (Nakanishi et al., 1988; Chun et al., 1990). However, this downstream oxysterol-derived signal does not accelerate HMG-R degradation in the absence of the upstream FPP-derived signal (Nakanishi et al., 1988; Meigs and Simoni, 1997), indicating that oxysterols provide an additional positive signal for HMG-R degradation that works only in conjunction with the FPP-derived signal. In this study, our results demonstrated that no other pathway product downstream of FPP was required for Hmg2p degradation in yeast. That is, inhibition of squalene epoxidase activity or down-regulation of squalene epoxidase had only a modest stabilizing effect on Hmg2p degradation. However, inhibition or down-regulation of oxidosqualene lanosterol cyclase did result in an increase in Hmg2p degradation, but only in the presence of sufficient quantities of FPP. This indicated that an additional oxysterolderived signal generated from downstream products of the mevalonate pathway acted in conjunction with FPP to hasten HMG-R degradation in yeast.

Hmg2p degradation required the FPP-derived signal, but not the oxysterol-derived signal.

It has been previously demonstrated in mammalian cells that the FPP-derived signal and the oxysterol-derived signal were both required for HMG-R degradation (Correll and Edwards, 1994; Meigs et al., 1996, Meigs and Simoni, 1997). The evidence for this dual requirement was obtained by pharmacological manipulation of squalene epoxidase (Correll et al., 1994), and by genetic manipulation of squalene synthase (Meigs et al., 1996; Meigs and Simoni, 1997). We examined if the oxysterolderived signal in yeast was similarly required for Hmg2p degradation by pharmacological inhibition of squalene epoxidase and by increased expression of oxidosqualene-lanosterol cyclase. In all cases, we could not establish an essential requirement of the oxysterol-derived signal for Hmg2p degradation. However, it is possible that these manipulations did not effectively remove all the oxysterols present within the cell and the residual oxysterols acted to allow Hmg2p degradation. Despite this possibility, we are convinced that the lack of effect seen with our manipulations is indicative of the non-essential nature of the oxysterol-derived signal for Hmg2p degradation.

We did demonstrate that the oxysterol-derived signal required a sufficient level of the FPP-derived signal to promote Hmg2p degradation. Similar manipulations of the oxysterol pathway that enhanced Hmg2p degradation in normal cells no longer promoted Hmg2p degradation in cells with decreased levels of FPP as a result of squalene synthase over-expression. In the squalene synthase over-expressing cells, Hmg2p is stabilized due to the low cellular levels of FPP by the hastened conversion of

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FPP to squalene. Although Hmg2p degradation was stimulated by sufficient squalene synthase inhibition in these cells, Hmg2p degradation could not be stimulated by inhibition of oxidosqualene-lanosterol cyclase. Consistent with this, we have clearly demonstrated that the Hmg2p degradation-enhancing function of the oxysterol-derived signal was through a synergistic effect with the FPP-derived signal. Pharmacological inhibition of squalene synthase and oxidosqualene-lanosterol cyclase, at levels that did not effect Hmg2p degradation when each enzyme was inhibited separately, did result in a greater than additive enhancement of Hmg2p degradation when the enzymes were inhibited together. Thus, the levels of the FPP-derived signal programmed a particular rate of Hmg2p degradation, which could be accordingly hastened by increased oxysterol production.

Mechanisms by which FPP regulates Hmg2p degradation.

The identification of FPP, or a derivative, as the positive primary signal for HMG-R degradation in yeast leads to several models for regulation of Hmg2p degradation. The simplest model for the regulatory mechanism is that the FPP-derived signal acts as an allosteric regulator and physically binds to the transmembrane domain of Hmg2p, altering its susceptibility to degradation. Alternatively, the FPP-derived molecule could modify or interact with a separate effector protein that alters Hmg2p stability. The effector protein could act to promote or prevent Hmg2p degradation, as either of these possibilities have precedent in cellular degradation (Hayashi *et al.*, 1996; Johnson *et al.*, 1998). It is also possible that the FPP-derived signal directly affects the structure of the ER membrane. By this model, the Hmg2p transmembrane domain would respond to the altered membrane by becoming increasingly susceptible to degradation.

Mechanisms by which the oxysterol-derived signal enhances Hmg2p degradation.

The transmembrane domain of the yeast HMG-R isozyme Hmg2p shares homology with the transmembrane domains of both mammalian HMG-R and the mammalian protein SCAP (SREBP cleavage-activating protein), both of which are postulated to function as sterol-sensing domains (Kumagai et al., 1995; Hua et al., 1996). It was previously not clear if the Hmg2p transmembrane domain had any sterolsensing ability, as it was unknown if any sterol-derived signal stimulated Hmg2p degradation. Because we have established the existence of an oxysterol-derived signal for Hmg2p degradation, it may be likely that the Hmg2p transmembrane domain does possess a sterol-sensing capability similar to its mammalian counterpart. Based upon this proposed function of the transmembrane domain, it is possible that the oxysterolderived signal in yeast acts to alter the structure of the Hmg2p transmembrane domain to achieve a greater, degradation-promoting sensitivity towards lower cellular levels of the FPP-derived signal. In the absence of the oxysterol-derived signal, the Hmg2p transmembrane domain would have a reduced sensitivity to the action of the FPPderived signal, which could be overcome only by sufficient accumulation of the FPPderived signal. The degradation-enhancing action of the oxysterol-derived signal could be effected through similar means as those described for the FPP signal above, including alteration of Hmg2p transmembrane domain structure by direct allosteric

interaction, by modulation of an effector protein or by changes in ER membrane structure.

The action of the oxysterol-derived signal may be similar in mammalian HMG-R degradation. However, because mammalian HMG-R requires the oxysterol-derived signal for degradation, it is possible that the sterol-sensing, transmembrane domain of mammalian HMG-R is completely insensitive to any cellular concentration of the FPPderived signal in the absence of the oxysterol-derived signal. By this model, only a sufficient cellular concentration of the oxysterol-derived signal would allow the transmembrane domain to become sensitive to the FPP-derived signal and effect enhanced HMG-R degradation. Further molecular studies will be required for both yeast and mammalian HMG-R degradation in order to elucidate the actual mechanism of both the oxysterol-derived signal and the FPP-derived signal.

Eukaryotic conservation of the signals for HMG-R degradation.

The fact that we have demonstrated the existence of both an FPP-derived signal and an oxysterol-derived signal for yeast HMG-R degradation further strengthens the similarities between mammalian HMG-R degradation and yeast HMG-R degradation already established. Although yeast and mammals may regulate sterol production for different purposes, it is becoming increasingly clear that the mechanism of HMG-R degradation as a means to control sterol production is highly conserved between the two organisms. These similarities include degradation without exit from the ER (Gil *et al.*, 1985; Lecureux and Wattenberg, 1994; Hampton and Rine, 1994), degradation dependent upon both the proteasome and ubiquitination (Hampton *et al.*, 1996a; McGee *et al.*, 1996; Hampton and Bhakta, 1997; Ravid *et al.*, 2000), a well-defined N-terminal transmembrane domain that is both necessary and sufficient for regulated degradation (Gil *et al.*, 1985; Jingami *et al.*, 1987; Skalnik *et al.*, 1988; Hampton and Rine, 1994), the use of both an FPP-derived positive signal and an oxysterol-derived positive signal to effect degradation (Nakanishi *et al.*, 1988; Chun *et al.*, 1990; Meigs and Simoni, 1997; this work), and a role for ER Ca2+ in the regulatory signal's action (Roitelman *et al.*, 1991; Cronin *et al.*, 2000).

In mammalian cells and yeast, FPP serves as a source for a primary positive signal for HMG-R degradation (Bradfute and Simoni, 1994; Correll and Edwards, 1994; Meigs *et al.*, 1996; Hampton and Bhakta, 1997; Lopez *et al.*, 1997; Meigs and Simoni, 1997; this work). FPP occupies a key branch point in the mevalonate pathway (Goldstein and Brown, 1990, and see Fig. 2-18), from which numerous other essential molecules and cellular modifications are derived. The cellular levels of FPP would be contingent on the demands from each branch pathway. If the demands of each pathway are minimal in relation to FPP production, FPP would most likely accumulate. Conversely, if the demands of each pathway are maximal in relation to FPP production, FPP would most likely be scarce. Thus it is not surprising that both yeast and mammalian cells utilize this key branch point as a sensor to optimally control mevalonate pathway production.

Oxysterols also serve as a source for a positive signal for HMG-R degradation in both mammalian cells (Faust *et al.*, 1982; Chin *et al.*, 1985; Nakanishi *et al.*, 1988; Chun *et al.*, 1990; Meigs and Simoni, 1992; Panini *et al.*, 1992; Roitelman and Simoni, 1992), and yeast (this work). The alternate pathway for oxysterol production branches from the mevalonate pathway at 2,3(S)-oxidosqualene (Fig. 2-18). Formation of oxysterols would be an effective measure of sterol content within the cell. Insufficient levels of sterols in the cell may bias the conversion of 2,3(S)-oxidosqualene to lanosterol, and thus decrease the availability of 2,3(S)-oxidosqualene for further epoxidation and subsequent oxysterol production. Conversely, abundant levels of sterols may allow increased levels of 2,3(S)-oxidosqualene that would be readily available for further epoxidation by squalene epoxidase and greater production of oxysterols. Such regulation of 2,3(S)-oxidosqualene levels could be effected by regulation of oxidosqualene-lanosterol cyclase activity by sterol abundance in the cell. Whatever the cellular mechanism may be to allow increased or decreased oxysterol production, it is clear that both yeast and mammals utilize this sensor, most likely as a means to prevent the detrimental build-up of sterols. However, both organisms also have vital interest in sufficient FPP production and appear to engage a feedback regulation process for HMG-R degradation that is critically dependent upon FPP production within the cell.

The use of an FPP-derived signal and an oxysterol-derived signal for HMG-R degradation bolsters the idea that yeast and mammalian cells effect regulated degradation of HMG-R degradation by a highly conserved mechanism, thus allowing for rigorous experimentation and comparison between two highly divergent species.

Lovastatin and the proteasome.

A final and important note should be made regarding a recent study on a fascinating side effect of the HMG-R inhibitor lovastatin. It has been demonstrated that

the closed β -lactone ring form of this particular statin can directly inhibit the proteasome (Rao et al., 1999). At first thought, it might be reasonable to conclude that this action is responsible for the stabilizing effects of lovastatin on Hmg2p, which is degraded by the proteasome (Hampton et al., 1996a). However, several observations show quite clearly that all actions of lovastatin we have observed were due to altered signal production and not proteasome inhibition. Degradation of 6myc-Hmg2p, which is proteasome dependent, is unaffected by doses of lovastatin that slow Hmg2p degradation (Hampton et al., 1996a). Furthermore, the pathway inhibitor L659-699, which inhibits HMG-CoA synthase but contains no lactone moiety, is equally effective at stabilizing Hmg2p as lovastatin (Hampton and Bhakta, 1997). Thirdly, genetic manipulations of HMG-CoA synthase (Hampton and Rine, 1994), or FPP synthase, which slow production of the degradation signals but do not involve drugs, have identical actions to lovastatin on Hmg2p degradation, but not 6myc-Hmg2p degradation. Finally, lovastatin decreases ubiquitination of Hmg2p (Hampton and Rine, 1994; and this study), an effect opposite of that observed when the proteasome is compromised or inhibited (Hampton and Rine, 1994). Although proteasome inhibition by the closed-ring form of lovastatin is interesting and perhaps important clinically, it plays no obvious or important role in the actions of this drug on regulating Hmg2p stability in yeast and is most likely not involved in mammalian HMG-R degradation, for similar reasons.
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Chapter III: In-*cis* determinants required for Hmg2p degradation

How that might change his nature, there's the question.

Julius Caesar --William Shakespeare

In the brief existence of Ellison, I fancy that I have seen refuted the dogma, that in man's very nature lies some hidden principle, the antagonist of bliss.

The Domain of Arnheim --Edgar Allen Poe

A. Introduction

Cellular degradation pathways target and destroy specific proteins for purposes of physiological regulation and quality control (Hochstrasser, 1995; Kopito 1997). In both circumstances, high specificity is essential and observed. However, these two roles for protein degradation have very different requirements for substrate selection. Regulated degradation occurs by specific recognition of a single protein. Quality control involves recognition of different proteins with common structural features. The value in understanding these distinct modes of recognition is underscored by the importance of the cellular processes controlled or affected by each. For example, cell division is coordinated by the temporally regulated, targeted destruction of specific proteins (King *et al.*, 1996a; Hershko, 1997). On the other hand, the etiology of numerous clinical syndromes, including cystic fibrosis and neurodegenerative disorders, have molecular aspects best viewed as examples of protein quality control (Ward *et al.*, 1995; Johnston *et al.*, 1998).

Selective degradation of a protein for regulation requires its accurate identification. Often, a small region of a protein's sequence provides information that targets the protein for degradation. Such regions are known as "degrons" (Varshavsky, 1991), and some examples include: the Deg1 sequence of the MAT α 2 transcriptional regulator in yeast (Hochstrasser and Varshavsky, 1990; Johnson *et al.*, 1998), the destruction box of mitotic cyclins (Glotzer *et al.*, 1991; King *et al.*, 1996b; Hershko, 1997), the degradation motif of IkB (Whiteside *et al.*, 1995), the stability-regulating region of cMOS (Nishizawa *et al.*, 1992), and the N-terminal residue of some proteins

(Bachmair *et al.*, 1989; Varshavsky, 1996). In most instances, a degron functions autonomously when included in the sequence of a heterologous protein, bringing about degradation of the fusion protein by the same mechanisms that operate in the original degradation substrate (Hochstrasser and Varshavsky, 1990; Glotzer *et al.*, 1991; King *et al.*, 1996b).

Degron recognition is modulated in several simple ways to effect cellular regulation of protein stability. These include masking of the degron by binding to a protein or a non-protein effector (Johnson *et al.*, 1998), covalent modification of the degron itself (Baldi *et al.*, 1996; DiDonato *et al.*, 1996; Desterro *et al.*, 1998), and altered production of receptors that bind the degron (Murikami *et al.*, 1992). Although the number of precisely described degrons is currently small, it is likely that this is a common strategy of targeted proteolysis due its high specificity and ease of control.

Selection of quality control substrates for degradation demands a very different strategy from targeting of a specific protein. For quality control, the cell must recognize common structural hallmarks of damage or misfolding in a diverse group of proteins that may share no primary sequence homology. As in regulated degradation, quality control recognition must be quite accurate, yet it appears to be based on structural features rather than specific identifying sequences.

The study of protein degradation associated with the endoplasmic reticulum (ER) provides an opportunity for direct comparison of these two modes of recognition. In both yeast and mammals, the ER is a major site of cellular protein degradation that functions in both regulated degradation of normal proteins and in quality control degradation of mutant or misfolded proteins. The normal, ER-resident, integral

membrane protein HMG-CoA reductase (HMG-R) undergoes regulated degradation by ER-associated processes that target it to the proteasome for destruction (Inoue *et al.*, 1991; Meigs and Simoni, 1992; Hampton *et al.*, 1996a). HMG-R is a key enzyme in the mevalonate pathway, from which sterols are synthesized, and is subject to feedback regulated degradation as part of the cellular control of sterol synthesis (Edwards *et al.*, 1983; Nakanishi *et al.*, 1988; Chun *et al.*, 1990; Roitelman and Simoni, 1992; Hampton and Rine, 1994). At the same time, misfolded and mutant ER proteins, such as the membrane proteins CFTR Δ F508 and Sec61-2p (Ward *et al.*, 1995; Biederer *et al.*, 1996) or the soluble lumenal proteins α -antitrypsin and CPY* (Ciccarelli *et al.*, 1993; Finger *et al.*, 1993), are recognized by a quality control mechanism and degraded by ER-associated processes that also target them to the proteasome.

This dual role for ER degradation has been analyzed by parallel genetic approaches in yeast. Some genes required for regulated ER degradation of the HMG-R isozyme Hmg2p, termed *HRD* genes (Hampton *et al.*, 1996a), are identical to genes required for degradation of the mutant protein CPY*, termed *DER* genes (Hiller *et al.*, 1996; Knop *et al.*, 1996; Bordallo *et al.*, 1998). Degradation of either protein requires ubiquitination that is strongly dependent on Ubc7p (Hampton and Bhakta, 1997; Hiller *et al.*, 1996), and a functional 26S proteasome (Hampton *et al.*, 1996a, Hiller *et al.*, 1996). Furthermore, at least two ER-resident membrane proteins, Hrd1p/Der3p and Hrd3p, are required for degradation of either substrate (Hampton *et al.*, 1996a; Bordallo *et al.*, 1998; Plemper *et al.*, 1999b). Thus, the ER degradation pathways for these structurally and functionally distinct substrates employ common machinery. The shared use of the ER degradation machinery for both quality control and regulated degradation substrates implies that a similar mode of recognition may be used for each type of substrate. However, only the degradation of Hmg2p (and related reporter proteins) is subject to regulation by the mevalonate pathway (Hampton and Rine, 1994; Hampton *et al.*, 1996a&b). Thus, the question arises as to how Hmg2p is distinguished from quality control substrates. Is Hmg2p a typical quality control substrate for which presentation to the structure-scanning mechanism is regulated by the mevalonate pathway? Or, is Hmg2p a typical substrate for regulated degradation that, by virtue of specific characteristic sequences, recruits proteins to deliver it to the shared ER degradation machinery?

To understand better how Hmg2p is recognized for degradation, we have studied the sequence features of Hmg2p important for this process. Specifically, we have performed an encompassing analysis of the 523 residue N-terminal transmembrane domain of Hmg2p, which is necessary and sufficient for regulated degradation and is capable of transferring regulated degradation to other stable proteins when included as a heterologous sequence (Hampton and Rine, 1994, Hampton *et al.*, 1996b). We were particularly interested in the degree to which Hmg2p regulated degradation depended on discrete, modular determinants such as degrons, which often underlie regulated protein degradation, or on structural features, which are important in recognition of quality control substrates. The following results detail the sequence requirements for Hmg2p degradation and demonstrate that Hmg2p degradation depends more upon a defined structure of the N-terminal transmembrane domain rather than specific, discrete modular determinants.

B. Results

The yeast HMG-R isozymes have very different degradation behaviors.

Yeast expresses two isozymes of HMG-R: Hmg1p and Hmg2p encoded by the *HMG1* and *HMG2* genes, respectively (Basson *et al.*, 1986). The two proteins are approximately 50% identical in the N-terminal transmembrane domain and approximately 93% identical in the C-terminal catalytic domain (Fig. 3-1). Furthermore, the amino acid sequences of the two isozymes have nearly super-imposable hydropathy plots (Basson *et al.*, 1988). The two are functionally similar in that each can synthesize adequate amounts of mevalonate (Basson *et al.*, 1986), and both appear to be residents of the ER (Koning *et al.*, 1996).

In spite of these similarities, the dynamic behavior of the two proteins is strikingly different. This is most clearly demonstrated in the cycloheximide-chase assay, in which cycloheximide is added to cells to inhibit protein synthesis and the stability, or degradation, of the pool of protein made prior to cycloheximide addition is followed over an extended period of time. Using this assay to assess the degradation behavior of the two HMG-R isozymes, it was discovered that Hmg1p is extremely stable, whereas Hmg2p is subject to regulated degradation (Hampton and Rine, 1994; and Fig. 3-2a). Not only is Hmg2p subject to degradation, but the rate of Hmg2p degradation is modulated by the levels of downstream mevalonate pathway products in the cell, as was previously discussed in chapter 2. If lovastatin, an inhibitor of HMG-R, is added to the cells at the same time as cycloheximide addition, the rate of Hmg2p is significantly slowed (Hampton and Rine, 1994; and Fig. 3-2b, lane "4" versus "4L").



Figure 3-1: Amino acid sequences of Hmg1p and Hmg2p.

Identical residues are highlighted in green and gaps are highlighted in yellow. The highly homologous catalytic domains are boxed. The transmembrane spans are indicated by a line above the corresponding sequence and do not exactly match those previously published. This figure was adapted from Basson *et al.*, 1988.



Figure 3-2: Degradation behavior of the two yeast HMG-R isozymes.

a) Hmg1p is stable, whereas Hmg2p is degraded. Stability of each protein was assessed by cycloheximide-chase assay. Cycloheximide was added to log-phase cells to halt protein synthesis. Lysates were prepared for each indicated time point after cycloheximide addition and samples were immunoblotted to determine the level of the protein. Both HMG-R isozymes had a single myc epitope tag included in the sequence of the linker region to allow detection with the anti-myc 9E10 antibody. b) Hmg2p degradation is regulated by the mevalonate pathway. Same experiment as in (a), except that lovastatin, an inhibitor of HMG-R, was added to one of the samples at the same time as cycloheximide. c) Effect of mevalonate pathway drugs on Hmg2p steady-state level. Cells were grown to mid log-phase and either no drug ("-"), lovastatin ("Lov"), or zaragozic acid ("ZA", an inhibitor of squalene synthase) was added. Cells were incubated for an additional 4 hours after addition of drugs. Lysates were prepared and samples were immunoblotted to determine the level of Hmg2p. In all experiments in all figures, all HMG-R proteins were expressed from the constitutive TDH3 promoter (a.k.a. GAPDH promoter, Bitter and Egan, 1984) to eliminate any possible transcriptional regulation incurred by changes in mevalonate pathway production.

The regulation of Hmg2p degradation is also observed as changes in the Hmg2p steady-state level. Because the Hmg2p steady-state level is an equilibrium state achieved as a balance between its rate of synthesis and its rate of degradation, stabilization of Hmg2p results in an increased steady-state level. Thus, when lovastatin is added to log-phase cells that express Hmg2p from the constitutive TDH3 promoter, a time-dependent increase in the steady-state level of Hmg2p is observed (Hampton and Rine, 1994; Fig. 3-2c, "-" versus "Lov"), and this is solely to due to a decrease in the Hmg2p degradation rate. In contrast, when the drug zaragozic acid, an inhibitor of squalene synthase (Bergstrom et al., 1993), is added to cells growing in log-phase, a time-dependent decrease in the steady-state levels of Hmg2p is observed (Hampton and Bhakta, 1997; and Fig. 3-2c, "ZA"). This decrease in the steady-state levels of Hmg2p is due to an increased rate of Hmg2p degradation caused by build-up of the squalene synthase substrate FPP (see Chapter 2). Thus, from the two types of assays described, the cycloheximide-chase assay and the steady-state assay, the stability of Hmg1p and the regulated degradation of Hmg2p are readily distinguishable.

Sequence determinants required for Hmg2p regulated degradation were distributed throughout the N-terminal transmembrane domain.

To reveal completely the specific degradation and regulation determinants of Hmg2p, two different mutagenic strategies were employed. The first strategy entailed constructing chimeric mutants between Hmg2p and Hmg1p. Studies with fusions between the two HMG-R sequences and reporter genes indicated that the isozyme-specific differences in degradative behavior are imparted by the homologous (50%

identical), non-catalytic N-terminal transmembrane domains (Hampton and Rine, 1994; Hampton et al., 1996b). Because these domains are homologous and structurally similar, reciprocal chimeras between the two proteins would allow mapping of stability and degradation determinants that differ between the two isozymes without creating drastic topological alterations. A similar approach has been successfully employed to map the features of the N-terminal transmembrane domain that allow the Hmg1pspecific formation of the stacked nuclear structures known as karmellae (Parrish *et al.*, 1995). Thus, small sequences of the HMG2 transmembrane domain coding region were replaced with the corresponding sequences from the HMG1 transmembrane domain coding region (Fig. 3-3a). The second mutagenic strategy entailed making conservative changes to small stretches of the Hmg2p transmembrane domain sequence that were not altered by the first strategy (Fig. 3-3b). The second method was employed to ensure that no critical regions of Hmg2p would be left undiscovered. This was a riskier strategy in the sense that, although only conservative changes were made, the topology or structure of Hmg2p may have suffered serious alterations and may be recognized for degradation in a very different fashion than normal Hmg2p.

Together, these mutagenic strategies revealed the regions and residues of Hmg2p that were required for normal regulated degradation. The mutagenesis that resulted in small replacement of Hmg2p sequences with the corresponding sequences from Hmg1p yielded ten mutants of Hmg2p that acquired partial or full stability (Fig. 3-4a) and nine mutants of Hmg2p that were hypomorphs for regulation of degradation (Fig. 3-4b). The mutagenesis that resulted in small scale, conservative replacement of



Figure 3-3: Strategies to mutagenize Hmg2p.

a) Small regions of Hmg2p that were different from Hmg1p were altered by replacement of the native Hmg2p sequence with a PCR fragment encoding the corresponding sequence from Hmg1p.
b) Small regions of Hmg2p that were identical to Hmg1p were altered by replacement of the native Hmg2p sequence with a PCR fragment encoding the Hmg2p sequence with corresponding conservative substitutions.



Figure 3-4: Regulated degradation phenotypes of chimeric Hmg2p/Hmg1p mutants.

(hrs)

a) Partial or complete stabilization of a subset of Hmg2p/Hmg1p chimeric mutants. Otherwise identical strains expressing the indicated Hmg2p variant were subject to a cycloheximide-chase assay. Lysates for each indicated time point were made and immunoblotted to determine the level of protein. In all experiments in all figures, each Hmg2p variant had a single myc epitope tag included in the sequence of the linker region to allow detection with the anti-myc 9E10 antibody. b) Reduced regulation of degradation of a subset of Hmg2p/Hmg1p chimeric mutants. Cycloheximide-chase assay was performed as in (a) except that $25\mu g/ml$ lovastatin was added to the appropriate sample at the same time as cycloheximide addition ("4L").



Figure 3-5: Regulated degradation phenotypes of conservative replacement Hmg2p mutants.

a) Partial or complete stabilization of a subset of conservative replacement Hmg2p mutants. Otherwise identical strains expressing the indicated Hmg2p variant were subject to a cycloheximide-chase assay. Lysates for each indicated time point were made and immunoblotted to determine the level of protein. b) Reduced regulation of degradation of a subset of conservative replacement Hmg2p mutants. Cycloheximide-chase assay was performed as in (a) except that $25\mu g/ml$ lovastatin was added to the appropriate sample at the same time as cycloheximide addition ("4L"). Asterisks denote mutants that were completely unregulated in the cycloheximide-chase assay.

the Hmg2p residues left unaltered by the exchange mutagenesis yielded ten stable mutants of Hmg2p (Fig. 3-5a) and eleven mutants of Hmg2p that were hypomorphs for regulation of degradation (Fig. 3-5b). In both cases, the critical regions for Hmg2p regulated degradation were scattered throughout the entire transmembrane domain and were distributed on both sides of the ER membrane (Fig. 3-6). None of these critical regions shared any similarity to previously characterized degradation determinants from other proteins.

To determine if the effect of a small replacement was attributable to a single residue that was required for normal regulated degradation of Hmg2p, a more detailed analysis of each critical region was performed. In each case where an altered degradation phenotype was observed, individual changes in the replacement were evaluated by producing the appropriate point mutations. These subsequent analyses revealed that the Hmg2p transmembrane domain contained two singular sequence requirements and one singular sequence restriction for normal Hmg2p degradation. No singular sequence requirements for regulation of Hmg2p degradation were discovered, however some detailed structural requirements for degradation and its regulation were revealed. These requirements are discussed in detail below.

Analysis of the 1-26 replacement revealed that Lysine 6 was necessary for degradation of Hmg2p.

The above mutagenesis studies showed that the first 26 residues of Hmg2p were required for normal degradation. Accordingly, a detailed analysis of this region was



Figure 3-6: Sequence determinants of Hmg2p regulated degradation were distributed throughout the N-terminal transmembrane domain.

Topological cartoon of the Hmg2p transmembrane domain. Regions required for Hmg2p degradation are marked with black lines. Regions required for normal regulation of Hmg2p degradation are marked with red lines. Numbers indicate the residue positions for the start and end of each transmembrane span. Note that regions required for both Hmg2p degradation and its normal regulation are present on both sides of the ER membrane.



Figure 3-7: Lys6 was required for Hmg2p degradation.

a) Sequence of the first 26 residues of Hmg2p. b) Degradation of normal Hmg2p ("WT"), Hmg2p with residues 2-13 deleted (" Δ 2-13"), Hmg2p with Lys6 replaced with arginine ("K6R"), Hmg2p with Lys13 replaced with arginine ("K13R"), or Hmg2p with Lys13 replaced with alanine ("K13A"). Otherwise identical strains expressing the indicated Hmg2p variant were subject to a cycloheximide-chase assay. Lysates for each indicated time point were made and immunoblotted to determine the level of protein. c) Effect of zaragozic acid (ZA), which hastens Hmg2p degradation, on steady-state levels of Hmg2p ("WT") or K6R-Hmg2p ("K6R"). Strains were treated for 4 hours with 10 μ g/ml zaragozic acid, then subjected to immunoblotting to determine steady-state level of Hmg2p.

performed. When residues 2-13 were deleted from Hmg2p, the resulting mutant was stable (Fig. 3-7b, " Δ 2-13"), indicating that this portion of Hmg2p contained a positive determinant of degradation. Lysines often serve as ubiquitination sites for proteasome-dependent degradation substrates (Finley and Chau, 1991; Jentsch, 1992), and the Δ 2-13 deletion removed the only two lysines (K6 and K13) in the 26 residue tract (Fig. 3-7a). Accordingly, each lysine was separately mutated to assess its role in degradation. Substitution of Lys6 with arginine completely stabilized Hmg2p (Fig. 3-7b, "K6R"), despite the presence of nearby Lys13. In contrast, substitution of Lys13 with arginine (Fig. 3-7b, "K13R") or even alanine ("K13A"), had no effect on Hmg2p degradation. Furthermore, similar site-specific alterations revealed that no other residue in this region was required for Hmg2p degradation (data not shown). Therefore, it appeared that Lys6 was the only residue essential for Hmg2p degradation in this 1-26 region, whereas all other residues, including Lys13, were completely dispensable.

Hmg2p degradation is hastened by addition of the squalene synthase inhibitor zaragozic acid to cells (Hampton and Bhakta, 1997; and Fig. 3-2c). Zaragozic acid causes the build-up of a mevalonate-derived molecule that stimulates Hmg2p ubiquitination and degradation (see Chapter 2). Because the presence of the K6R mutation stabilized Hmg2p in the above assay, K6R-Hmg2p stability after zaragozic acid addition was also evaluated. Addition of zaragozic acid to cells expressing normal Hmg2p caused the expected decrease in the Hmg2p steady-state level due to stimulated degradation (Fig. 3-7c, "WT"). In contrast, addition of zaragozic acid to cells expressing K6R-Hmg2p had no effect on the K6R-Hmg2p steady-state level (Fig. 3-7c, "K6R"). Thus, the single K6R substitution rendered Hmg2p stable even when physiological signals for degradation were maximal.

The optical reporter Hmg2p-GFP was used to study further the effect of the K6R replacement. This reporter is a chimeric protein that contains the entire GFP coding sequence fused in frame to the C-terminus of the Hmg2p N-terminal transmembrane domain (Hampton *et al.*, 1996b), resulting in replacement of the Hmg2p catalytic domain with GFP. The GFP reporter facilitates quantitative examination of degradation through analysis of steady-state Hmg2p-GFP fluorescence. Alterations in Hmg2p stability brought about by physiological or genetic means are correctly reported by changes in Hmg2p-GFP steady-state fluorescence (Hampton *et al.*, 1996b; Cronin and Hampton, 1999). That is, stabilization of Hmg2p-GFP is observed as a rightward shift in the fluorescence histogram, whereas increased degradation of Hmg2p-GFP is observed as a leftward shift in the fluorescence histogram. When K6R was introduced into Hmg2p-GFP, the resulting K6R-Hmg2p-GFP was stable as measured by an optical cycloheximide-chase assay (Fig. 3-8a, "K6R" versus "wt").

Cells expressing the stable K6R-Hmg2p-GFP had significantly increased fluorescence compared to cells expressing normal Hmg2p-GFP (Fig. 3-8b, "K6R" versus "wt"), indicated by the rightward shift in the fluorescence histogram. The stabilizing effect of the in-*cis* K6R mutation was directly compared to the stabilizing effect of the in-*trans hrd1-1* mutation, which completely blocks normal Hmg2p-GFP degradation (Cronin and Hampton, 1999). The histogram of a *HRD1* strain expressing K6R-Hmg2p-GFP was super-imposable with the histogram of a *hrd1-1* strain expressing normal Hmg2p-GFP (Fig. 3-8b, "K6R" versus "wt, *hrd1-1*"). Furthermore,



Figure 3-8: Stabilization by the in-*cis* K6R mutation was identical to stabilization by the in-*trans hrd1-1* allele.

a) K6R-Hmg2p-GFP was stable. Strains expressing the indicated Hmg2p variant were analyzed by flow cytometry to determine the level of fluorescence in each strain after 4 hour incubation in the absence ("-CHX") or presence ("+CHX") of cycloheximide. **b)** Effect of the in-*cis* K6R mutation or the in-*trans hrd1-1* mutation on Hmg2p-GFP. Flow cytometry analysis of *HRD1* or *hrd1-1* strains expressing normal Hmg2p-GFP ("wt") or K6R-Hgm2p-GFP ("K6R"). In all flow cytometric analyses, histograms are representative of 10,000 cells. A shift of the histogram to the right indicates increased degradation and a shift of the histogram to the left indicates stabilization.

presence of the *hrd1-1* allele had no added effect on the fluorescence of a strain expressing K6R-Hmg2p-GFP (Fig. 3-8b, "K6R" versus "K6R, *hrd1-1*"). These results indicated that the effect of the in-*cis* K6R replacement was entirely due to an inability of K6R-Hmg2p-GFP to undergo *HRD*-dependent degradation.

Lys6 function had a singular sequence restriction.

Replacement of the Hmg2p N-terminal 26 residues with the corresponding tract from Hmg1p resulted in a stable protein (Fig. 3-4a, "1-26"). However, Lys6 was preserved in this stable chimera (Fig. 3-9a), indicating that some feature of the local context was also important. The stable chimera had 16 residues different from normal Hmg2p (Fig. 3-9a), including 8 non-conservative replacements. One of these was the replacement of valine 9 with a third lysine (Fig. 3-9a, large star). This single lysine replacement was entirely responsible for the stabilizing effect of the 1-26 exchange. If only Lys9 in the stable chimeric protein was reverted to valine, the resulting protein was once again degraded (Fig. 3-9b, "K9V"). Furthermore, if only residue 9 of normal Hmg2p was changed to lysine, the resulting mutant was stable ("V9K"). Surprisingly, the stabilizing effect of the V9K replacement was highly specific for lysine. Replacement of position 9 in Hmg2p with an arginine, despite it also having a positive charge, had no effect on degradation ("V9R"). Replacement of residue 9 with aspartate or leucine also had no effect on Hmg2p degradation ("V9D" and "V9L", respectively), indicating that stability was only caused by specific substitution of lysine in that position.

The stabilizing effect of the lysine replacement was also highly specific for



Figure 3-9: Position 9 could not be a lysine.

a) Sequence comparison of the first 26 residues of Hmg1p (top row) and Hmg2p (bottom row). Asterisks indicate conserved residues and position 9 is indicated with a large star. b) and c) Degradation of the indicated Hmg2p mutants was directly compared by cycloheximide-chase assay. Cylindrical portion represents only the first 26 residues of Hmg2p (in magnifying glass in (a)). Blue color represents Hmg2p native sequence. The green cylinder (second from left) represents the first 26 residues of Hmg1p present in the replacement, with the critical Lys9 and conserved Lys6 shown. Other cartoons represent Hmg2p with the indicated point mutations and Lys6 as shown.

position 9. Replacement of residue 3, which was a similar distance from Lys6 as position 9, had no effect on degradation (Fig. 3-9c, "L3K"), nor did the replacement of residues 8, 7 or 2 with lysine ("I8K", data not shown, and "S2K", respectively). Therefore, Hmg2p degradation was specifically disrupted by the presence of a lysine at position 9, and neither proximity nor charge appeared to explain the stabilizing effect. One interpretation of this was that the presence of a lysine in both of these positions resulted in a highly specific, inhibitory interaction with the degradative machinery, whereas the presence of only one lysine allowed degradation. If this were the case, then the presence of only a single lysine at position 9 should allow degradation. However, Hmg2p with only a lysine at position 9 was stable (Fig. 3-9c, "K6A, V9K"). Thus, a lysine at position 9 could not, by itself, substitute for the essential degradative function of the normal lysine at position 6, but could only inhibit degradation.

Thus, the above studies revealed a highly specific role for Lys6 in degradation. However, except for the no-lysine restriction on position 9, Lys6 function was quite insensitive to changes in its surrounding primary sequence. Again, each residue in the tract has been individually altered, and no single residue was specifically required for regulated degradation except Lys6 (data not shown).

Distance from the ER membrane was critical for Lys6 function.

Lysine 6 was critical for Hmg2p degradation and a lysine at position 9 instead of position 6 did not support degradation. This implied that the critical lysine must be a particular distance from the ER membrane in order to function in degradation. Accordingly, residues were added or removed between Lys6 and the first trans-

membrane span to determine if an "increased" or "decreased" distance between Lys6 and the ER membrane would have a stabilizing effect.

All mutations that altered this distance had marked effects on Hmg2p degradation. Addition of 3 alanines between residues 18 and 19 of Hmg2p caused significant stabilization (Fig. 3-10, "+3A"). In contrast, replacement of residues 17 and 18 in Hmg2p with alanines, which created a similar four-alanine tract but did not lengthen the N-terminus, had no effect on degradation ("C17A, T18A"). Addition of even a *single* alanine between residues 18 and 19 had a strong stabilizing effect on Hmg2p ("+1A"). Moreover, removal of endogenous Ala16, thus shortening the Nterminal region by one residue, also had a strong stabilizing effect ("-1A"). However, addition of an alanine between residues 18 and 19 and simultaneous removal of Ala16, which preserved the distance between Lys6 and the ER membrane but included *both* separate stabilizing mutations, allowed normal Hmg2p degradation ("+/-1A"). Thus, it appeared that the function of Lys6 depended upon its position from the ER membrane. This was not the exclusive interpretation of these results, and alternate explanations were tested. For instance, it was possible that the effects were due to a change in the overall length of the N-terminal region rather than alteration of the Lys6 position relative to the ER membrane. However, addition of 1 or 3 alanines N-terminal to Lys6, between residues 1 and 2, had no effect on degradation (Fig. 3-10, "+1A-N" and "+3A-N", respectively). Alternatively, it was possible that the distance between Lys6 and some critical region after the first transmembrane span was affected and that was the cause of stability. However, addition of three alanines between residues 58 and



Figure 3-10: A ruler for degradation: distance from the ER membrane was critical for Lys6 function.

Strains expressing each indicated variant of Hmg2p, in which alanines were either inserted ("+3A", "+1A", "+1A-N", "+3A-N", "+3A-tm", "+/-1A") and/or removed ("-1A", "+/-1A") from the Hmg2p primary sequence, were assayed for Hmg2p degradation by cycloheximide-chase assay. Again, cylinders represent the tract of N-terminal residues that is normally 26 residues long. The distance between Lys6 and the ER membrane is depicted schematically by the "Ruler" and the dotted line.

59, located immediately after the first transmembrane span, had no effect on Hmg2p degradation ("+3A-tm). Thus, Hmg2p degradation required a precise distance between the critical Lys6 and the start of the first transmembrane span at the surface of the ER membrane.

Hydropathy of the N-terminal 26 residues was important for normal, regulated Hmg2p degradation.

Residues 1-18 of Hmg2p, which contain Lys6, are predicted to adopt an amphipathic α -helical conformation (Basson *et al.*, 1988). When these residues were plotted onto a model α -helix, Lys6 was on the hydrophilic face of the predicted helix (Fig. 3-11a, blue-shaded side-chains). The importance of the amphipathic character of this region in Hmg2p degradation was evaluated by altering the region's hydrophobicity/hydrophilicity. When the helical region was made completely hydrophilic by replacing all hydrophobic residues with hydrophilic ones (L3E, L5N, I8N, V9E, L11Q, V12T, F15Y, A16S), the resulting protein was subject to rapid, and unregulated, degradation. Addition of lovastatin, which strongly stabilizes normal Hmg2p (Fig. 3-11b, "WT", lane "4" versus "4L"), had no effect on the degradation of the hydrophilic mutant (Fig. 3-11b, "hydrophilic" lane "4" versus "4L"). Surprisingly, when the region was made entirely hydrophobic by replacing all of the hydrophilic residues (except Lys6) with hydrophobic ones (S2A, T7G, H10Y, K13M, T18V), the resulting protein was still subject to normal, regulated degradation (Fig. 3-11b, "hydrophobic").

These results suggested that the hydrophobic face of the helical region, but not



Figure 3-11: Hydrophobicity of the Lys6-containing α -helix was important, not amphipathicity.

a) α -helical model of the region surrounding Lys6, residues 1-18. The hydrophobic side chains are colored green and the hydrophilic side chains are colored blue. The arrow points to Lys6. b) Effect of hydrophobicity/hydrophilicity alterations on regulated Hmg2p degradation. Each Hmg2p mutant was tested for regulated degradation by cycloheximide-chase assay in the presence or absence of $25\mu g/ml$ lovastatin ("L"), which was added at the same time as the cycloheximide. The Hmg2p mutants tested include normal ("WT"), all hydrophobic residues replaced with alternate hydrophobic residues ("amphipathic"), all hydrophobic residues replaced with hydrophilic ones ("hydrophilic"), and all hydrophilic residues, except Lys6, replaced with hydrophobic ones ("hydrophobic").

the amphipathic character, was important for regulated degradation. When the natural hydrophobic residues were replaced with alternate hydrophobic residues (L5F, I8L, V9I, L11M, V12A, F15I, A16V, C17A), Hmg2p regulated degradation was normal (Fig. 3-11b, "amphipathic"). Thus, the hydrophobicity of the face, rather than any specific sequence, was important for regulated degradation.

Analysis of replacement 355-362 revealed that Lysine 357 was also critical for Hmg2p degradation.

From the mutagenic analyses described above (Fig. 3-4a), it was additionally discovered that residues 355-362 of the transmembrane domain were required for Hmg2p degradation. Further characterization of this region, by making the individual substitutions encompassed by the replacement, revealed that only one residue was required for Hmg2p degradation. Stabilization of Hmg2p by replacement of the 355-362 region with the corresponding region from Hmg1p was recapitulated by the singular substitution of Lys357 in Hmg2p with arginine (Fig. 3-12b, "K357R"). Furthermore, K357R-Hmg2p was exceedingly stable even in the presence of increased signals for degradation caused by addition of zaragozic acid (Fig. 3-12c, "K357R"). Thus, an additional lysine, distant from position 6, was also essential for Hmg2p degradation.

The K357R replacement was introduced into Hmg2p-GFP to allow a more detailed analysis of its effects. K357R-Hmg2p-GFP behaved similar, but not identical, to K6R-Hmg2p-GFP. The fluorescence of a strain expressing K357R-Hmg2p-GFP was significantly brighter than an identical strain expressing Hmg2p-GFP (Fig. 3-13a,
"K357R" versus "wt"). Furthermore, the brightness of a *HRD1* strain expressing K357R-Hmg2p-GFP was nearly the same as that of a *hrd1-1* strain expressing Hmg2p-GFP ("K357R" versus "wt, *hrd1-1*"). However, unlike the K6R mutant, the K357R mutant did show very slow degradation, such that a strain expressing K357R-Hmg2p-GFP was made slightly brighter (~1.25 fold) by the presence of the *hrd1-1* allele ("K357R" versus "K357R, *hrd1-1*"). The residual degradation was not blocked by addition of lovastatin (data not shown), and thus was not regulated by the mevalonate pathway. In addition, when the in-*cis* K6R mutation was introduced into K357R-Hmg2p-GFP, the fluorescence of the strain expressing the double mutant was identical to that of a strain expressing K6R-Hmg2p-GFP or a strain expressing K357R-Hmg2p-GFP with the *hrd1-1* allele (Fig. 3-13b, "K6R, K357R" compared with "K6R" or "K357R, *hrd1-1*"). Thus, the residual degradation in K357R-Hmg2p was dependent on the presence of Lys6 and a functional *HRD* pathway.

Lysine 357 function was also permissive of changes in local sequence that did not alter length, as was observed for Lys6 function. Within the collection of mutants, each residue in the neighborhood of Lys357 (position 340-399) has been altered without effect on degradation (data not shown). Thus, no other single residue in the region of Lys357 was specifically required for Hmg2p degradation. Since the only specific contextual requirement for Lys6 function was the no-lysine restriction at position 9, similar mutations in the "sequence neighborhood" of Lys357 were tested. However, substitution of lysines at positions +3 or –3 to Lys357 were without effect on Hmg2p regulated degradation (Fig. 3-14, "L354K" and "I360K", respectively).

In contrast, Lys357 function was sensitive to changes in distance between



Figure 3-12: Lys357 was also required for degradation.

a) Sequence of the 355-362 exchange (dark blue cylinders in magnifying glass) with a cartoon of the relative location of Lys357 in Hmg2p. Identical residues are colored in blue and non-identical residues are colored in green. Lys357 is colored with red and marked with an asterisk. b) Strains expressing either normal Hmg2p ("WT") or K357R-Hmg2p ("K357R") were assayed for Hmg2p degradation by cycloheximide-chase assay. c) Effect of zaragozic acid, which stimulates Hmg2p degradation, on steady-state levels of each variant. Strains expressing either form of Hmg2p were incubated 4 hours in the presence of $10\mu g/ml$ zaragozic acid.



Figure 3-13: Stabilization by the in-*cis* K357R mutation was not as complete as stabilization by the in-*trans hrd1-1* allele or the in-*cis* K6R mutation.

Effect of the in-*cis* K357R mutation, the in-*cis* K6R mutation, the in-*cis* K6R, K357R double mutation, or the in-*trans hrd1-1* mutation on Hmg2p-GFP steady-state fluorescence. **a**) Strains expressing either normal Hmg2p-GFP ("wt") or K357R-Hmg2p-GFP ("K357R") were compared by flow cytometry. Each protein was expressed in both *HRD1* and *hrd1-1* strains. **b**) Strains expressing either K357R-Hmg2p-GFP ("K357R"), K6R-Hmg2p-GFP ("K6R") or K6R, K357R-Hmg2p-GFP ("K6R, K357R") were compared by flow cytometry.



Figure 3-14: Lys357 does not have a no-lysine sequence restriction.

Lysine substitutions were placed either -3 residues ("L354K") or +3 residues ("I360K") from Lys357 in the Hmg2p sequence. Strains expressing the indicated Hmg2p variant were assayed for Hmg2p degradation by cycloheximide-chase assay.

Lys357 and the nearest transmembrane span, similar to that seen for Lys6. Insertion of 1 or 3 alanines between residues 351 and 352 on the N-terminal side of Lys357, which resulted in a change in the distance between the nearest predicted transmembrane span and Lys357, significantly stabilized Hmg2p (Fig. 3-15a, "+1A" and "+3A", respectively). Similar insertions between residues 360 and 361 on the C-terminal side of Lys357 did not effect Hmg2p degradation ("+1A-C" and "+3A-C", respectively), indicating that the distance between Lys357 and the nearest transmembrane span was important for degradation. However, the stabilized mutants did show some slow degradation (half-life approximately 3.5 hours). This residual degradation was not slowed by addition of lovastatin and, thus, was not subject to regulation by the mevalonate pathway (Fig. 3-15b). The slow, residual degradation was most likely a result of concomitant structural alteration caused by the central position of the insertion site (see below).

Lys6/Lys357 function depended on the structure of the entire transmembrane region.

Lysines 6 and 357 are separated by 350 residues in the linear sequence of Hmg2p. Both lysines were required for Hmg2p degradation, and loss of either by conservative replacement resulted in strong stabilization. Unlike typical degrons, the sequence regions containing Lys6 and Lys357 did not contribute independently to degradation. When these sequence regions were introduced into the stable HMG-R isozyme Hmg1p, they did not result in degradation of the chimeric protein (Fig. 3-16, "1-26" and "355-362", respectively), even when both were included ("1-26 + 355-



Figure 3-15: A second ruler for degradation: correct distance from the ER membrane was required for Lys357 function.

a) Strains expressing each indicated variant of Hmg2p, in which alanines were either inserted ("+3A", "+1A", "+3A-C", "+1A-C") or removed ("-1A"), were assayed for Hmg2p degradation by cycloheximide-chase assay. Cylinders represent the normal residues surrounding Lys357. Distance of Lys357 from the ER membrane is depicted schematically by the "Ruler" and the dotted line. Top of the diagram is the C-terminal side of Lys357, and the bottom is the N-terminal side. The bottom of each blue cylinder is the predicted juxtamembrane residue, Asp342. b) Residual degradation of the insertion/deletion mutants was not regulated. Each Hmg2p mutant was tested for regulated degradation by cycloheximide-chase assay in the presence or absence of $25\mu g/ml$ lovastatin ("L"), which was added at the same time as the cycloheximide.

362"). In contrast, the well-defined degron of the yeast MAT $\alpha 2$ transcriptional regulator, Deg1 (Hochstrasser and Varshavsky, 1990; Johnson *et al.*, 1998), was able to program the degradation of Hmg1p when included as a heterologous sequence (Fig. 3-16, "Deg1"). Thus, these regions were necessary for Hmg2p degradation, but they were not sufficient to confer degradation to a stable protein.

In fact, the mutagenic analyses conducted above, which either altered the position of Lys6/Lys357 relative to the ER membrane or the hydrophobicity of the α -helix containing Lys6, indicated that structure, not specific sequences, was the important determinant for the function of the two critical lysines. Furthermore, in the search for sequence determinants of Hmg2p degradation, numerous replacement mutations that stabilized Hmg2p but did not disturb the local sequence context around Lys6 and Lys357 were discovered. Degradation experiments of these stabilizing mutants are shown in figures 3-4a and 3-5a. Despite the preservation of each critical lysine and its local sequence, these mutants showed strong stabilization. Thus, normal function of Lys6 and Lys357 was not solely determined through autonomous action of their local sequences and structure, but also required sequence and structural information from many parts of the Hmg2p transmembrane domain.

Except for Lys6 and Lys357, no other single residue was required for Hmg2p degradation. In all other small replacements where an altered degradation phenotype was observed, except for the 1-26 and 355-362 replacements, substitution of 3-6 residues was required to observe either of the two classes of effects, slowed degradation or poor regulation. The number and wide distribution of these sensitive regions was consistent with the idea that the entire transmembrane domain, including information



Figure 3-16: Small regions of Hmg2p necessary for normal degradation were not sufficient to confer degradation to Hmg1p.

Region 1-26 of Hmg2p, which contains Lys6, and region 355-362 of Hmg2p, which contains Lys357, were tested for their ability to independently program the degradation of the normally stable HMG-R isozyme, Hmg1p. These regions of Hmg2p were used to replace the corresponding regions in Hmg1p by the previously described mutagenesis. Strains expressing each indicated variant of Hmg1p were assayed for degradation by cycloheximide-chase assay.

from both sides of the ER membrane, was involved in regulated degradation of Hmg2p. Since no single residue change mimicked the altered regulated degradation phenotype of the small region mutations, it appeared that these 3-6 residue changes exerted their effect through alteration of the Hmg2p tertiary structure, rather then recognition of specific sequences.

Lys6/Lys357 functioned only in degradation that was physiologically regulated.

A different series of mutants indicated that Lys6 and Lys357 functioned only in Hmg2p degradation that was subject to physiological regulation. Many mutants of Hmg2p are still degraded in a *HRD*-gene dependent manner, but are no longer regulated by the mevalonate pathway. Examples include 6myc-Hmg2p (Hampton *et al.*, 1996a), Δ40-Hmg2p (deletion of residues 146-186, data not shown), and hydrophilic-Hmg2p (Fig. 3-11b). Because these unregulated mutants were still subject to *HRD*-gene dependent degradation (data not shown), the dependency of their degradation on Lys6 or Lys357 was evaluated. In no case was there any effect of changing either or both lysines to arginine (Fig. 3-17b). In each of these diverse structural mutants, loss of regulation and loss of Lys6/Lys357 dependence coincided. Furthermore, the *HRD*gene dependent degradation of these mutants also did not require other regions of the Hmg2p sequence necessary for normal regulated degradation, such as region 421-497 (Fig. 3-17b). Analysis of a number of other unregulated mutants demonstrated a similar inability to uncouple regulation from Lys6/Lys357 dependence (data not shown).

Although the unregulated mutants of Hmg2p showed no dependence on Lys6 or Lys357, Hmg2p mutants that were hypomorphs for regulation did require the presence



Figure 3-17: Hmg2p mutants subject to unregulated degradation no longer required Lys6 or Lys357.

a) Cartoons depict the effects of each mutation, all of which result in unregulated degradation, and their location relative to each lysine. **b**) The structurally perturbed mutants were tested for Lys6 and Lys357 dependence by introducing the appropriate K6R or K357R replacement into their sequences and assessing degradation by cycloheximide-chase assay.

of Lys6 and Lys357. These regulation-hypomorph Hmg2p mutants are seen in figures 3-4b and 3-5b. Their characteristic phenotype was observed in the typical cycloheximide-chase degradation assay. After addition of lovastatin, the degradation of these mutants was slowed only slightly. To examine this phenotype in more detail, the poorly regulated degradation of the 27-54 mutant, which contained a replacement of the first transmembrane span of Hmg2p with that from Hmg1p, was further characterized (Fig. 3-18a).

Regulation of 27-54 degradation was examined by observing the change in the steady-state level of 27-54 in the presence of various drugs that inhibit mevalonate pathway enzymes. Addition of zaragozic acid to strains expressing normal Hmg2p or 27-54 resulted in a decrease in either protein's steady-state level (Fig. 3-18b). Furthermore, addition of increasing doses of lovastatin caused increases in the steady-state levels of both normal Hmg2p and 27-54. However, while the level of the normal Hmg2p was maximally increased by the addition of 0.2 μ g/ml lovastatin, the amount of lovastatin required to maximally increase the level of 27-54 was 1 μ g/ml (Fig. 3-18b). In addition, the maximal steady-state level of 27-54 after complete lovastatin stabilization was still approximately 3-fold less than the steady-state level of normal Hmg2p.

One difficulty in comparing the effects of lovastatin on the degradation of normal Hmg2p with the degradation of the poorly regulated 27-54 is that the two proteins are present at different levels in the two strains due to their different rates of degradation. Thus, the associated HMG-CoA reductase activity, and therefore the sensitivity to lovastatin, was different between the strains being examined. To compare



Figure 3-18: Characterization of an Hmg2p mutant that is hypomorphic for regulation of degradation.

a) Cartoon depicting the 27-54 chimeric Hmg2p/Hmg1p mutant and the position of the replacement relative to each critical lysine. **b)** Effect of drug treatments on the logphase, steady-state levels of normal Hmg2p or the 27-54 chimeric mutant. Log-phase cultures were grown 4 hours in the presence of the indicated doses of lovastatin ("Lov"), or 25 μ g/ml zaragozic acid ("ZA"), and levels of the proteins were determined by immunoblotting. **c)** Effect of indicated doses of lovastatin on the log-phase, steady-state levels of Hmg2p-GFP or 27-54-GFP in strains with identical HMG-R activity. Log-phase cultures were grown in the indicated dose of lovastatin (μ g/ml) for 3 hours, then analyzed by flow cytometry to determine the levels of the fluorescent reporter protein.

the effects of lovastatin on the stability of Hmg2p and 27-54 at identical levels of HMG-R activity, either Hmg2p-GFP or 27-54-GFP were expressed in a strain that coexpressed the enzymatically active, stable, soluble Hmg2p catalytic domain from the constitutive TDH3 promoter as the sole source of essential HMG-R catalytic activity (Hampton et al., 1996b). In this way, Hmg2p-GFP or 27-54-GFP were expressed in strains with constant and identical HMG-R activity that was independent of their degradation. Flow cytometric analysis of the two strains revealed that the steady-state level of 27-54-GFP was 3 to 4-fold lower than that of Hmg2p-GFP (Fig. 3-18c), as was the case between 27-54 and Hmg2p examined above. However, the stabilizing effect on 27-54-GFP required significantly more lovastatin. A 2-fold shift for 27-54-GFP required 0.8 μ g/ml lovastatin, whereas the same shift for Hmg2p-GFP required only 0.1 μ g/ml (Fig. 3-18c). Similarly, saturation of the effect of lovastatin occurred at 3.2 μ g/ml for 27-54-GFP, whereas saturation for Hmg2p-GFP occurred at only 0.4 μ g/ml. In addition, these amounts of lovastatin caused the steady-state level of Hmg2p-GFP to increase 5-fold, whereas the 27-54-GFP level increased only 3-fold. Because these strains had identical HMG-R catalytic activity, this analysis confirmed that 27-54 was still regulated by alterations in the mevalonate pathway, but required significantly stronger inhibition of the pathway to be stabilized.

It was possible that 27-54 had a lower steady-state level because it was degraded through a different pathway or was translated at a different rate than that of normal Hmg2p. To test these possibilities, the steady-state levels of 27-54 and normal Hmg2p were compared in *HRD*-pathway deficient strains. To do so, strains were constructed that contained either the mutant *hrd1-1* or wild-type *HRD1* alleles, and co-expressed the

appropriate GFP reporter with the free Hmg2p catalytic domain to normalize HMG-R activity. In strains with the normal *HRD1* gene, the steady-state level of 27-54-GFP was 3 to 4-fold lower than that of Hmg2p-GFP (Fig. 3-19, "27-54" versus "wt"). In contrast, the steady-state levels of 27-54-GFP and Hmg2p-GFP were identical to each other in the strains with the *hrd1-1* allele, such that the fluorescent histograms were virtually super-imposable (Fig. 3-19, "27-54, *hrd1-1*" compared with "wt, *hrd1-1*"). This implied that the different steady-state levels of the two proteins in *HRD1* strains were due solely to differences in *HRD-*gene dependent degradation, and that *HRD-*gene dependent degradation was the only, or at least primary, mechanism by which the levels of the two proteins differed. Furthermore, it indicated that the different rates of *HRD-*gene dependent degradation, not different rates of translation.

If residues 27-54 of Hmg2p were truly involved in the regulation of *HRD*-gene dependent Hmg2p degradation, then any in-*cis* mutation that stabilized Hmg2p should similarly stabilize 27-54. Previously, it was demonstrated that replacement of the first 26 residues of Hmg2p with those from Hmg1p resulted in stabilization of Hmg2p (see above, Fig. 3-4a). When the first 54 residues of Hmg2p were replaced with those from Hmg1p, which contained *both* the stabilizing 1-26 mutation and the 27-54 regulation-hypomorph mutation, the resulting mutant Hmg2p, 1-54, was stable (Fig. 3-20, "1-54" versus "27-54"). More importantly, 27-54 degradation was absolutely dependent upon presence of Lys6 or Lys357. Alteration of either lysine resulted in complete stabilization of the poorly regulated 27-54 (Fig. 3-20, "K6R" and "K357R"), unlike the previously described mutants that were subject to unregulated degradation. Thus, these



Figure 3-19: Degradation of the poorly regulated 27-54 was HRD1 dependent.

Fluorescence histograms of strains expressing the GFP versions of normal Hmg2p or 27-54 in strains with the *HRD1* or *hrd1-1* allele. All strains had identical HMG-R catalytic activity supplied by expression of the Hmg2p catalytic domain. Strains were analyzed by flow cytometry directly from early log-phase cultures.



Figure 3-20: Degradation of 27-54 required the presence of Lys6 and Lys357.

The 27-54 Hmg2p/Hmg1p chimeric mutant was tested for Lys6 and Lys357 dependence by introducing the appropriate K6R or K357R replacement into the sequence and assessing degradation by cycloheximide-chase assay.

results indicated that Lys6/Lys357 were required only when Hmg2p degradation was regulated.

C. Discussion

The ER-associated degradation of Hmg2p is tightly regulated and isozymespecific, yet occurs by the action of cellular machinery responsible for destruction of a diverse array of misfolded proteins. In order to understand this seemingly dichotomous aspect of the process, the sequence features of Hmg2p important for degradation were explored by mutagenic analyses.

Two lysine residues, Lys6 and Lys357, located at distant points along the linear sequence, were each critical for degradation. Lysines 6 and 357 did not independently contribute to the regulated degradation of Hmg2p. Both were essential and their degradative function required a correct structural context. Their participation in regulated Hmg2p degradation was dependent on precise local structural information. Alterations in the distance of both lysine residues from the ER membrane and the hydrophobicity of the helix containing Lys6 resulted in dramatic alteration of normal Hmg2p degradation. The function of both lysine residues also required information globally distributed throughout the 523 residue transmembrane. Numerous alterations of Hmg2p in regions distant from Lys6 and Lys357 strongly stabilized Hmg2p (Fig. 3-6, black patches), indicating that Lys6 and Lys357 did not function autonomously.

Additionally, these lysine residues were intimately involved in the regulation of Hmg2p degradation. A variety of structurally compromised Hmg2p mutants were subject to unregulated degradation in a *HRD*-gene dependent fashion that no longer required either lysine residue. In all cases tested, degradation that was not regulated by the mevalonate pathway was also not dependent on Lys6/Lys357. However, Hmg2p

mutants that were hypomorphic for regulation of degradation still required the presence of Lys6 and Lys357. Thus, these lysine residues worked together in a synergistic manner within a defined structure of Hmg2p to allow regulated degradation. From these analyses, we propose that Hmg2p presents specific information for degradation within a "distributed" degron, in which the structure of the entire transmembrane domain is required to present the sequence information that allows the cell to uniquely control Hmg2p stability.

The distributed degron of Hmg2p.

The action of the Hmg2p distributed degron combines aspects of regulation and quality control. Like more traditional degrons, the Hmg2p distributed degron is recognized in a highly specific manner for purposes of physiological regulation and is transferable to other proteins (Hampton and Rine, 1994; Hampton *et al.*, 1996b). However, recognition of the Hmg2p distributed degron depends upon structural aspects of the entire transmembrane domain, a feature consistent with degradation of quality control substrates.

The Hmg2p distributed degron is different from previously described degrons in that it requires structural information distributed over an entire protein domain of 523 residues. These individual determinants are not autonomous and cannot program the degradation of stable proteins when included as heterologous sequences. In contrast, typical degrons are composed of single, short sequences that can act autonomously and program the degradation of other stable proteins when included as a heterologous sequences as is the case for the well-defined degron of the yeast MAT α 2 transcriptional

regulator, Deg1 (Hochstrasser and Varshavsky, 1990; Johnson *et al.*, 1998; Mayer *et al.* 1998). Within the Deg1 sequence there are specific sequence requirements and structural features critical for degradative function (Johnson *et al.*, 1998), but they are restricted to a small, independently folding region.

Some degrons act by binding molecules that protect them from recognition by the degradation machinery or that actively promote degradation (Murakami *et al.* 1992; Johnson *et al.*, 1998). The Hmg2p distributed degron contained regions necessary for Hmg2p degradation that are present on both sides of the ER membrane. This suggests that there may be two, or more, binding regions within the distributed degron for proteins or molecules involved in Hmg2p degradation. Proteins known to be required for Hmg2p degradation are located in both the lumen, Hrd3p (Hampton *et al.*, 1996a; Saito *et al.*, 1999), and the cytosol, Ubc7p, Cue1p, Hrd1p and Hrd2p (Hampton *et al.*, 1996a; Hampton and Bhakta, 1997; Bays *et al.*, 2000; Biederer *et al.*, 1997). It is possible that these regions required for Hmg2p degradation form distinct lumenal or cytosolic binding sites for the respective proteins to interact with Hmg2p. Perhaps, the dispersed elements of the distributed degron allow the formation of an emergent structure, such as a protein surface, that is recognized by the degradation or regulatory apparatus and is not reducible to small, autonomous, sequence determinants.

Alterations in Hmg2p structure resulted in a variety of degradative phenotypes including stability, poorer recognition of degradative signals, or enhanced recognition of degradative signals. The sensitivity of Hmg2p degradation to structural changes may be important in the regulation of stability. It is quite possible that physiological control of Hmg2p degradation is brought about by induced changes in the structure of the

Hmg2p distributed degron of a similar magnitude to those we have introduced by mutation.

Lys6/Lys357: gateway to the *HRD* pathway?

How do Lys6 and Lys357 participate in the function of the distributed degron? One possibility is that these two lysine residues are required for establishment of a tertiary structure required for Hmg2p regulated degradation. Perhaps they interact with a regulatory molecule resulting in an alteration of the Hmg2p structure from one that is stable to one that is recognized as a quality control substrate. Alternatively, since lysine residues are the usual sites for addition of ubiquitin to proteins (Finley and Chau, 1991; Jentsch, 1992), it may be that Lys6 and/or Lys357 serve as the essential ubiquitin conjugation sites for the ubiquitin-mediated, proteasome-dependent degradation of Hmg2p. Subtle, mevalonate pathway-regulated alterations in the structure of Hmg2p may change the positions of these lysine residues, thereby allowing their interaction with the ubiquitin conjugation machinery. Perhaps fluctuations in the isoprenoid content of the ER membrane, determined by mevalonate pathway production, affects the precise juxtaposition of the two critical lysine residues resulting in degradation when they are in the correct position. These models are not exclusive and the actual mechanism of Hmg2p regulated degradation may incorporate mechanisms identical or similar to these, as well as others not yet conceived.

By any model, it is clear that Lys6 and Lys357 serve in the specific recognition of Hmg2p for regulated degradation. However, these lysine residues are not needed for *HRD*-gene dependent degradation of many variants, such as 6myc-Hmg2p. In addition,

other regions in Hmg2p required for normal degradation, such as residues 421-497, were similarly not required for *HRD*-gene dependent degradation of the unregulated variants Δ 40-Hmg2p and 6myc-Hmg2p. Thus, Hmg2p has dispersed structural information necessary for its unique targeting as a substrate for regulated degradation, but not generally required for *HRD*-gene dependent degradation. Identifying the molecules that mediate Lys6/Lys357-dependent degradation will help clarify how the Hmg2p distributed degron allows specific recognition of Hmg2p.

Generally, Lys6 and Lys357 function in the Hmg2p distributed degron was quite permissive to changes in local sequence. One striking, and quite unique, exception was the *no*-lysine restriction at position 9. The stabilizing effect of a lysine residue at this position was absolutely specific for that position and residue. A simple interpretation of this restriction is that a lysine residue at position 9 displaces Lys6 or Lys357 in an interaction that normally involves the two critical lysine residues. This interaction could be with a distinct part of the Hmg2p molecule or with a separate molecule that interacts with these critical lysine residues. The new Lys9 interaction could prevent a structural change in the distributed degron required for Hmg2p to become degradation competent. Conservative substitution of position 9 with arginine had no effect, indicating that the action of a position 9 lysine was quite specific. This result favors models of explicit interaction or recognition.

Implications of a distributed degron.

The existence of a distributed degron has important implications in understanding protein degradation at the ER surface, and possibly in general. Many models of protein degradation specificity presume discrete autonomous regions of the protein, degrons, that interact with proteins responsible for degradation. Such discrete, modular degrons can be revealed by primary sequence analysis. Their absence in a particular protein sequence might be considered evidence of independence from the associated degradation pathway. However, our studies have demonstrated that highly selective, regulated degradation can also depend on information that is neither readily apparent at the level of primary sequence nor detectable by homology. The prevalence of distributed degrons in the spectrum of protein degradation is not yet known, but the existence of Hmg2p indicates that it is unwarranted to assume that regulated degradation must always proceed by recognition of discrete, small regions of primary sequence. In this regard, it is worth noting that mammalian HMG-R and yeast Hmg2p have the same enzymatic function, undergo regulated degradation that is in many ways similar, yet have little or no primary sequence homology in the transmembrane regions. Perhaps similar structural features that are transparent to primary sequence comparison mediate their similar degradative behaviors. In fact, precedence already exists for highly divergent sequences that define similar tertiary structures required for identical catalytic mechanisms (Delbaere et al., 1975; Dickerson et al., 1976; Flaherty et al., 1990), indicating that such a phenomenon is not an outlandish consideration in the realm of degradation substrate recognition.

The molecular rules that govern selective protein degradation are still unclear. When they are fully delineated, an entirely new approach to therapeutics will be possible based on using these rules for the design of pharmaceuticals that program or prohibit degradation of clinically important proteins. The Hmg2p distributed degron may be instructive in this regard because it indicates that a generally used, constitutive degradation pathway may be entered in a regulated manner. It is conceivable that molecular processes similar to those used to stringently control Hmg2p entry into the ER quality control pathway could be harnessed to specifically manipulate the degradation of desired clinical targets.

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Chapter IV: Analysis of Hmg2p ubiquitination

"I won't hear of it," laughed Lord Henry, sinking into a chair. "From a label there is no escape! I refuse the title."

> The Picture of Dorian Gray --Oscar Wilde

Side by side, on the meeting-house steps, stood a male and a female figure. The man was a tall, lean, haggard personification of fanaticism, bearing on his breast this label, -- A WANTON GOSPELLER.

> Endicott and the Red Cross --Nathaniel Hawthorne

A. Introduction

Many proteins targeted for destruction by the proteasome require the covalent attachment of the small protein ubiquitin prior to their degradation (Finley and Chau, 1991; Jentsch, 1992). Ubiquitin is a 76 residue protein that is covalently linked to the target substrate via an isopeptide bond between the α -carboxyl group of the C-terminal glycine residue in ubiquitin and the ε -amino group of an internal lysine residue in the target protein. The conjugation of ubiquitin to the target protein is, in general, a threestep mechanism, which is depicted in figure 4-1. Initially, ubiquitin is activated by the ubiquitin-activating enzyme E1, which hydrolyzes ATP and forms a high-energy thiolester intermediate between the active site cysteine in the E1 and the C-terminal glycine residue of ubiquitin. The ubiquitin is then transferred from the E1 to an E2, or ubiquitin-conjugating enzyme (UBC), to which it is linked by a similar high-energy thiol-ester intermediate. The E2 then catalyzes the isopeptide bond formation between ubiquitin and the target protein, often in conjunction with an additional factor called an E3. The E3 proteins generally provide the substrate specificity for the E2 (Hochstrasser, 1996; Hershko et al., 1997), but may also assist in the direct transfer of ubiquitin from the E2 to the substrate (Bartel et al., 1990; Madura et al., 1993; Scheffner et al., 1993; Scheffner et al., 1998; Lorick et al., 1999; Ohta et al., 1999a; Seol et al., 1999; Skowyra et al., 1999). After the first ubiquitin moiety has been linked to the protein target, additional ubiquitin molecules are often covalently added by a similar isopeptide linkage to Lys48 of the attached ubiquitin molecule forming a multiubiquitin chain (Chau *et al.*, 1989). The multi-ubiquitination may be carried out by the



Figure 4-1: Ubiquitin conjugation.

The cycle of ubiquitin conjugation to a protein substrate targeted for degradation can be divided into four steps. First, ubiquitin is activated by the ATP-dependent formation of a thiol ester with the ubiquitin activating enzyme E1 (1). Next, the E1 transfers the ubiquitin to a specific ubiquitin conjugating enzyme (UBC) E2 which also forms a thiol ester linkage with ubiquitin (2). The E2 then directly transfers ubiquitin to the substrate or, more commonly, transfers ubiquitin to the substrate with the help of a ubiquitin ligase E3 (3), perhaps with an additional ubiquitin ligase aiding in the formation of a multi-ubiquitin chain. Finally, the multi-ubiquitinated substrate is recognized as a degradation target by the proteasome and is degraded into small peptides in an ATP-dependent fashion (4). During this process, ubiquitin is removed from the substrate by ubiquitin proteases and recycled for reuse.

Class	Protein	Function	Reference
E1	Uba1p	Ubiquitin activating enzyme	McGrath et al., 1991
E2	Ubc1p	Required for growth after sporulation, for endocytosis of membrane proteins; mediates essential functions with <i>UBC4</i> and <i>UBC5</i> ; Degradation of ER-associated proteins.	Seufert <i>et al.</i> , 1990a Medintz <i>et al.</i> , 1998 Bays <i>et al.</i> , 2000 Friedlander <i>et al.</i> , 2000
	Ubc2p/Rad6p	DNA repair, sporulation, induced mutagenesis, repression of retro-transposition, N-end rule pathway.	Jentsch <i>et al.</i> , 1987 Friedberg, 1988 Lawrence <i>et al.</i> , 1994
	Ubc3p/Cdc34p	G1-S cell cycle progression, DNA replication, spindle pole body separation.	Goebl <i>et al.</i> , 1994 King <i>et al.</i> , 1996a
	Ubc4p, Ubc5p	Degradation of abnormal proteins, MATa2; involved in sporulation, resistance to stress conditions, endocytosis of membrane proteins.	Seufert <i>et al.</i> , 1990a Seufert <i>et al.</i> , 1990b Chen <i>et al.</i> , 1993 Arnason <i>et al.</i> , 1994 Kölling <i>et al.</i> , 1994
	Ubc6p/Doa2p	Degradation of MAT α 2, ER- associated proteins.	Chen <i>et al.</i> , 1994 Biederer <i>et al.</i> , 1993 Galan <i>et al.</i> , 1998
	Ubc7p	Degradation of ER-associated proteins, MATα2, abnormal proteins; cadmium resistance.	Chen <i>et al.</i> , 1993 Sadis <i>et al.</i> , 1995 Hiller <i>et al.</i> , 1995 Hampton <i>et al.</i> , 1997 Galan <i>et al.</i> , 1998
	Ubc8p	Degradation of fructose-1,6-bisphosphatase	Schule <i>et al.</i> , 2000
	Ubc9p	G2-M cell cycle progression; conjugation of ubiquitin-like protein Smt3p.	Blondel <i>et al.</i> , 1996 Johnson <i>et al.</i> , 1997
	Ubc10p/Pas2p	Peroxisome biogenesis	Wiebel et al., 1992
	Ubc11p	Function unknown.	
	Ubc12p	Conjugation of ubiquitin-like protein Rub1p.	Liakopoulos et al., 1998
	Ubc13p	Post-replicative DNA repair; Lys63 linked poly- ubiquitin.	Hofmann et al., 1999
E3	Ubr1p/Ptr1p	Degradation of N-end rule substrates; peptide import; contains RING-H2 finger domain essential for its E3 activity.	Bartel <i>et al.</i> , 1990 Madura <i>et al.</i> , 1993 Byrd <i>et al.</i> , 1998 Xie <i>et al.</i> , 1999
	Ubr2p	Function unknown.	Xie et ut., 1999
	Npi1p/Rsp5p	Endocytosis of Gap1p and Fur4p; subcellular distribution of Mod5p; contains a HECT domain.	Hein <i>et al.</i> , 1995 Galan <i>et al.</i> , 1996 Zoladek <i>et al.</i> , 1997
	Ufd4p	Degradation of ubiquitin fusion proteins.	Johnson <i>et al.</i> , 1995
	Tom1p	G2-M transition of cell cycle.	Utsugi et al., 1999
	Hrd1p/Der3p	Degradation of ER proteins; contains RING-H2 finger domain essential for its activity.	Hampton <i>et al.</i> , 1996a Bordallo <i>et al.</i> , 1998 Bordallo <i>et al.</i> , 1999 Bays <i>et al.</i> , 2000
	Apc11p	Subunit of Anaphase Promoting Complex (APC); contains RING-H2 finger domain.	Zachariae <i>et al.</i> , 1999 Ohta <i>et al.</i> , 1999a Peters <i>et al.</i> , 1999
	Hrt1p, Rbx1p, Roc1p	G1-S transition of cell cycle; contains RING-H2 finger domain and has ubiquitin ligase activity.	Ohta <i>et al.</i> , 1999a Seol <i>et al.</i> , 1999 Skowyra <i>et al.</i> , 1999

Table 4-1: Yeast Ubiquitin Conjugating Enzymes

E2 itself, or by another enzyme termed an E4 (Koegl et al., 1999). The multiubiquitinated substrate is then recognized and degraded by the 26S proteasome. The mechanism of protein ubiquitination is a hierarchy of catalytic activity (Jentsch, 1992; Hochstrasser, 1996; and Table 4-1). In yeast, a single E1 is responsible for the initial activation of ubiquitin in all known substrate targeting pathways (Hochstrasser, 1996). There are 13 distinct E2 enzymes that all share a 16 kDa conserved domain with an active site cysteine residue (Hochstrasser, 1996), which is required for the ubiquitin thiol-ester intermediate formation. Some E2 enzymes contain additional domains that target them to specific regions of the cell or promote their interaction with specific types of substrates (Biederer *et al.*, 1997). From functional genetic studies, it appears that the different E2 enzymes have very different functions in the cell (Haas and Siepmann, 1997; Hochstrasser, 1996), although some do share overlapping functions (Seufert and Jentsch, 1990; Seufert et al., 1990; Chen et al., 1993; Bays et al., 2000). The number of E3 proteins in yeast is currently unknown. The E3 family may be the broadest class of proteins in the ubiquitin conjugation hierarchy, as they are generally required to confer substrate specificity for the E2 (Haas and Siepmann, 1997; Hochstrasser, 1996). Recent studies indicate that some E3 proteins share a conserved domain, named a RING-H2 finger (Kamura et al., 1999; Lorick et al., 1999; Ohta et al., 1999a; Seol et al., 1999; Skowyra et al., 1999; Xie and Varshavsky, 1999; Bays et al., 2000). This domain may be involved in binding the E2 enzyme (Kamura et al., 1999; Lorick et al., 1999; Ohta et al., 1999a; Seol et al., 1999; Skowyra et al., 1999; Xie and Varshavsky, 1999; Bays et al., 2000), thus bringing it in contact with its target substrate.

Covalent attachment of ubiquitin is required for the subsequent destruction of numerous proteasome-dependent degradation substrates, including many that are subject to ER-associated, proteasome-dependent degradation. However, the recognition process for ubiquitin attachment to such ER-associated substrates and the subsequent ubiquitination is not completely understood. The ubiquitination of these substrates is highly dependent upon the ER-associated E2 enzyme Ubc7p (Chen et al., 1993; Biederer et al., 1996; Hiller et al., 1996; Hampton and Bhakta, 1997; Galan et al., 1998). In some cases, another ER-associated E2, Ubc6p, also appears to be involved, although in every case to a lesser extent than Ubc7p (Chen et al., 1993; Biederer et al., 1996; Hiller et al., 1996; Hampton and Bhakta, 1997; Galan et al., 1998). Ubc7p does not contain a membrane anchoring domain like Ubc6p, but instead requires the presence of the ER-associated membrane protein Cue1p (Biederer et al., 1997). In both in vivo and *in vitro* analyses, Cue1p has been shown to bind to Ubc7p and, through this interaction, allow Ubc7p to become very tightly associated with the ER membrane (Biederer et al., 1997). One E3 required for the substrate specificity of Ubc7p appears to be Hrd1p, which contains a RING-H2 finger domain that is required for ubiquitin ligase activity and is conserved among other known E3 proteins (Hampton et al., 1996a; Bays et al., 2000). Consistent with its homology and *in vitro* ubiquitin ligase activity, Hrd1p is required for the *in vivo* ubiquitination and degradation of a number of described ER-associated, proteasome-dependent degradation substrates (Hampton et al., 1996a; Bordallo et al., 1998; Bordallo et al., 1999; Bays et al., 2000).

Previous studies from our laboratory demonstrated that the yeast HMG-CoA reductase (HMG-R) isozyme Hmg2p is multi-ubiquitinated and degraded in an ER-

associated, proteasome-dependent fashion (Hampton *et al.*, 1996a; Hampton and Bhakta, 1997). Furthermore, Hmg2p is ubiquitinated in a manner that is regulated by signals generated from the mevalonate pathway and consistent with the regulation of its degradation (Hampton and Bhakta, 1997). Hmg2p ubiquitination is required for its degradation and depends upon the ER-associated E2 Ubc7p (Hampton and Bhakta, 1997). The absence of a functional *UBC7* gene results in stabilization of Hmg2p. Additionally, the Ubc7p-dependent ubiquitination of Hmg2p requires the ER-associated E3 Hrd1p (Hampton *et al.*, 1996a; Bays *et al.*, 2000). Although it is known that Hmg2p is ubiquitinated by a mechanism that is dependent upon specific and identified proteins, it is not known how these proteins act to covalently attach ubiquitin to Hmg2p. In particular, it is unclear where ubiquitin is attached to Hmg2p or what regions of Hmg2p are specifically required for this process to occur.

Because lack of ubiquitination leads to Hmg2p stability, it was quite likely that many, or all, of the in-*cis* stabilizing mutations in Hmg2p discussed in the previous chapter affected Hmg2p ubiquitination. Therefore, to discover the regions required for this process, we analyzed the effect of each stabilizing mutation on regulated Hmg2p ubiquitination. Through these analyses, we discovered that the entire, correct structure of the Hmg2p transmembrane domain was required for its regulated ubiquitination, consistent with its role in degradation. In addition, Hmg2p ubiquitination required the presence of Lys6 and Lys357 in a manner that suggested their role as the sites for the covalent attachment of ubiquitin to Hmg2p. Lastly, we revealed that attachment of Lys48-linked multi-ubiquitin chains was required for Hmg2p degradation, consistent with its proteasome-dependent degradation.

B. Results

Lys6 and Lys357 were required for Hmg2p ubiquitination.

Covalent attachment of ubiquitin is a critical and regulated step in Hmg2p degradation (Hampton and Bhakta, 1997). Because ubiquitin is usually attached to internal lysine residues within the target protein (Finley and Chau, 1991; Jentsch, 1992), the regulated ubiquitination of the stable K6R- and K357R-Hmg2p mutants described in the previous chapter was examined. To do so, each Hmg2p variant (containing a myc-epitope tag) was expressed in a strain that also expressed HA-epitope tagged ubiquitin (HA-Ub). Hmg2p ubiquitination was assayed by immunoprecipitation of Hmg2p, followed by immunoblotting with anti-HA antibody to detect covalently attached HA-Ub, or with anti-myc antibody to detect total precipitated Hmg2p. Regulation of ubiquitination was also tested by the addition of lovastatin ("Lov"), which decreases ubiquitination, or zaragozic acid ("ZA"), which increases ubiquitination (Hampton and Bhakta, 1997; and Fig. 4-2 "WT").

Individually, K6R-Hmg2p and K357R-Hmg2p showed drastically decreased ubiquitination (Fig. 4-2, "K6R" and "K357R"), even when signals for degradation were elevated by addition of zaragozic acid (ZA). The maximal difference between ubiquitination of normal Hmg2p and K6R-Hmg2p or K357R-Hmg2p observed in the presence of ZA was estimated to be approximately 15-20 fold ("WT" versus "K6R" or "K357R"). It appeared that K357R-Hmg2p showed absolutely no increase in ubiquitination after ZA treatment, while K6R-Hmg2p did show some very slight increase ("K357R" ZA lane versus "K6R" ZA lane). However, K357R-Hmg2p

appeared to have a slightly higher level of basal ubiquitination than K6R-Hmg2p ("K357R" no drug lane versus "K6R" no drug lane). The double mutant, K6R, K357R-Hmg2p, had a similar level of K6R-Hmg2p basal ubiquitination ("K6R, K357R" no drug lane compared with "K6R" no drug lane), but there was no slight stimulation of ubiquitination as seen for K6R-Hmg2p ("K6R, K357R" ZA lane versus "K6R" ZA lane). This lack of stimulated ubiquitination was similar to K357R-Hmg2p ("K6R, K357R" ZA lane compared with "K357R" ZA lane), indicating that the residual ubiquitination in K6R-Hmg2p seen upon ZA treatment was due to the presence of Lys357. Furthermore, the higher basal level of ubiquitination in K357R-Hmg2p was due to the presence of Lys6, as the double mutant had the lower basal ubiquitination levels seen for K6R-Hmg2p.

The minimal ubiquitination levels of the Lys to Arg Hmg2p mutants were reflections of their stability.

In chapter 3, the stability of both K6R-Hmg2p and K357R-Hmg2p was documented (Chapter 3, Fig 3-7&8 and Fig. 3-12&13, respectively). Closer, quantitative examination by flow cytometric analysis of the degradation for both single mutants and the double mutant revealed some intriguing correlates between their ubiquitination patterns and stability. K6R-Hmg2p-GFP was quite stable and there was no further increase in the steady-state level in cells deficient for ER degradation by presence of the $hrd1\Delta$ allele (Chapter 3, Fig. 3-8 and Fig. 4-3a). The steady-state fluorescence of K6R-Hmg2p-GFP was slightly affected by addition of either cycloheximide or zaragozic acid (Fig. 4-3b), both of which dramatically decrease



Figure 4-2: Lys6/Lys357 were required for Hmg2p ubiquitination.

Ubiquitination assays of strains expressing the indicated Hmg2p variant were performed in the presence of no drug ("-"), lovastatin ("Lov", 25μ g/ml), or zaragozic acid ("ZA", 10μ g/ml). Upper panels are the result of anti-HA (" α -HA") immunoblotting for covalently linked HA-tagged Ub. Lower panels are the result of parallel immunoblotting an aliquot (1/8 total volume) of the same immunoprecipitates with the 9E10 anti-myc antibody (" α -myc") to assess total immunoprecipitated Hmg2p. Ubiquitinated Hmg2p is represented as a collection of 3-6 bands with higher molecular weights than normal Hmg2p. Note that the total amount of Hmg2p precipitated in the "WT" ZA lane is less due to stimulated degradation, therefore the degree of ubiquitination in that lane is an underestimate. normal Hmg2p-GFP steady-state fluorescence (Chapter 3). In contrast, K357R-Hmg2p-GFP was modestly degraded as there was a slight increase in the steady-state level in cells containing the *hrd1* Δ allele (Chapter 3, Fig. 3-13 and Fig. 4-3a). The steady-state fluorescence of K357R-Hmg2p-GFP was moderately affected by addition of cycloheximide but not by addition of zaragozic acid (Fig. 4-3b). The introduction of both mutations in the *hmg2::GFP* sequence, to yield K6R, K357R-Hmg2p-GFP, resulted in a completely stable mutant that was unaffected by the presence of the *hrd1* Δ allele (Fig. 4a) or addition of either cycloheximide or zaragozic acid (Fig. 4-3b).

Thus, the ubiquitination patterns of these mutants correlated well with their degradation behaviors. K6R-Hmg2p showed complete loss of basal ubiquitination, but did show a slight increase in ubiquitination after addition of zaragozic acid to the cells. This coincided with a small degree of regulated degradation of K6R-Hmg2p-GFP. K357R-Hmg2p had a slightly higher level of basal ubiquitination than K6R-Hmg2p, but no regulated ubiquitination was observed. This correlated with a modest degree of unregulated degradation of K357R-Hmg2p-GFP. The double mutant, K6R,K357R-Hmg2p, showed complete loss of basal ubiquitination and complete loss of stimulated ubiquitination consistent with its complete stability. The significance of these combined results is discussed below.

All other stabilizing mutants of Hmg2p resulted in decreased Hmg2p ubiquitination.

Stabilizing mutations of Hmg2p that altered the context or position of Lys6 also resulted in decreased Hmg2p ubiquitination. For example, the V9K-Hmg2p showed



Figure 4-3: Comparative analysis of Hmg2p-GFP stability as a result of the K6R and/or K357R mutations.

a) Steady-state levels of each indicated Hmg2p-GFP mutant in *HRD1* or *hrd1* Δ strains. Cells were grown to mid-log-phase, then analyzed by flow cytometry. b) Effect of either cycloheximide (50µg/ml) or zaragozic acid (10µg/ml) addition on the steady-state levels of each Hmg2p-GFP mutant. Drugs were added to cells grown into early log-phase and the cells were incubated for an additional 4 hours at 30°C. Cellular fluorescence was analyzed by flow cytometry. c) Comparison of the steady-state levels of either single K6R- or K357R- Hmg2p-GFP mutant with the double mutant, K6R,K357R-Hmg2p-GFP. Fluorescence histograms are from the same samples generated in (a).

decreased ubiquitination (Fig. 4-4, "V9K"), as did the Hmg2p mutant with a single alanine inserted between residues 18 and 19 ("+1A"). However, both these mutants showed a moderate increase in ubiquitination after the addition of zaragozic acid (compare ZA lanes). If the K6R replacement was introduced into any of these stable mutants, the residual ubiquitination was decreased to that of the K6R mutant (data not shown). Thus, Hmg2p mutants that altered the context or position of Lys6 were still subject to specific, albeit poor, recognition of this critical lysine if it was present.

Stabilizing mutations in the rest of the Hmg2p transmembrane domain also decreased Hmg2p ubiquitination, and their phenotypes could be separated into two distinct classes. The first class is defined as having a similar ubiquitination phenotype as V9K-Hmg2p. The members of this class showed decreased Hmg2p ubiquitination, but some stimulated ubiquitination was evident after addition of zaragozic acid (Fig. 4-5a). The second class is defined as having reduced basal ubiquitination that was completely unresponsive to the addition of zaragozic acid (Fig. 4-5b). From these ubiquitination analyses, all mutations that stabilized Hmg2p also resulted in reduced ubiquitination. No stabilizing mutations that allowed normal ubiquitination were ever observed. Although all the stabilizing mutations reduced Hmg2p ubiquitination, it was unclear if the normal regions of Hmg2p encompassed by the mutations were specifically involved in ubiquitination or if they were required for formation of the correct structure of the Hmg2p transmembrane domain, whereby their alteration no longer allowed efficient recognition of the critical regions directly involved in ubiquitination. However, because all stabilizing mutations resulted in some form of reduced Hmg2p



Figure 4-4: Mutations that altered context or position of Lys6 reduced Hmg2p ubiquitination.

Ubiquitination assays of strains expressing the indicated Hmg2p variant were performed in the presence of no drug ("-"), lovastatin ("Lov", 25μ g/ml), or zaragozic acid ("ZA", 10μ g/ml). Upper panels are the result of anti-HA (" α -HA") immunoblotting for covalently linked HA-tagged Ub. Lower panels are the result of parallel immunoblotting an aliquot (1/8 total volume) of the same immunoprecipitates with the 9E10 anti-myc antibody (" α -myc") to assess total immunoprecipitated Hmg2p. Note that the total amount of Hmg2p precipitated in the "WT" ZA lane is less due to stimulated degradation, therefore the degree of ubiquitination in that lane is an underestimate.



Figure 4-5: Small scale exchange or replacement mutants of Hmg2p also reduced Hmg2p ubiquitination.

Ubiquitination assays of strains expressing the indicated Hmg2p variant were performed in the presence of no drug ("-") or zaragozic acid ("ZA", 10μ g/ml). Upper panels are the result of anti-HA (" α -HA") immunoblotting for covalently linked HA-tagged Ub. Lower panels are the result of parallel immunoblotting an aliquot (1/8 total volume) of the same immunoprecipitates with the 9E10 anti-myc antibody (" α -myc") to assess total immunoprecipitated Hmg2p. **a**) Stable Hmg2p mutants that show some regulation of ubiquitination. **b**) Stable Hmg2p mutants that show no regulation of ubiquitination. ubiquitination, it was clear that the correct structure of the Hmg2p transmembrane domain was required for the normal attachment of ubiquitin to Hmg2p.

Hmg2p degradation required the covalent attachment of multi-ubiquitin chains.

For many ubiquitin-dependent degradation substrates, ubiquitin is covalently attached to the substrate as a multi-ubiquitin chain (Chau *et al.*, 1989; Hochstrasser *et al.*, 1991; Johnson *et al.*, 1992; Finley *et al.*, 1994; Schork *et al.*, 1995; Hiller *et al.*, 1996). The ubiquitin molecules within this chain are connected to each other through an isopeptide linkage on Lys48 of ubiquitin (Fig. 4-6a). Disruption of multi-ubiquitin chain formation, by substitution of an arginine for Lys48 in ubiquitin, results in stabilization of such substrates (Chau *et al.*, 1989; Hochstrasser *et al.*, 1991; Finley *et al.*, 1994; Schork *et al.*, 1995; Hiller *et al.*, 1989; Hochstrasser *et al.*, 1991; Finley *et al.*, 1994; Schork *et al.*, 1995; Hiller *et al.*, 1989; Hochstrasser *et al.*, 1991; Finley *et al.*, 1994; Schork *et al.*, 1995; Hiller *et al.*, 1996), indicating that the specific, covalent attachment of a Lys48-linked multi-ubiquitin chain to the substrate is required for degradation.

Ubiquitination of Hmg2p is a prerequisite for its degradation (Hampton and Bhakta, 1997; and above results). Furthermore, Hmg2p ubiquitination often appeared as a collection of discrete, higher molecular weight ubiquitin-Hmg2p conjugates (Fig. 4-7a), which are representative of the covalent attachment of multiple ubiquitin molecules to Hmg2p. The total number of observable bands in the ubiquitin conjugation ladder and calculation of the molecular weight for each band indicated that 3-8 observable ubiquitin molecules were attached to Hmg2p. However, whether the ubiquitin molecules were linked singly to multiple lysine residues or linked as multiubiquitin chains to a single or a few lysine residues was not clear. Therefore,



Figure 4-6: Proteasome-dependent degradation utilizes Lys48-linked multiubiquitin chains.

a) Proteasome-dependent degradation of many substrates requires the addition of a multi-ubiquitin chain to the substrate. Each ubiquitin in the chain is linked through an iso-peptide bond via Lys48. b) K48R ubiquitin cannot support the formation of the Lys48-linked multi-ubiquitin chain. The single K48R ubiquitin attached to the degradation substrate cannot be recognized for degradation by the proteasome leading to stabilization of the degradation substrate.

the dependence of Hmg2p degradation on multi-ubiquitin chain formation was determined by observing the effect of K48R ubiquitin over-expression on Hmg2p degradation. To do so, a yeast strain that expressed 1myc-Hmg2p was transformed with a 2 micron plasmid that allowed expression of K48R ubiquitin from the strong, inducible *CUP1* promoter (Hochstrasser *et al.*, 1991), from which transcription is induced by high concentrations (100μ M) of Cu²⁺ (Ecker *et al.*, 1987). When K48R ubiquitin was over-expressed from the *CUP1* promoter by addition of 100μ M CuSO₄ to log-phase cells, Hmg2p degradation was significantly decreased (Fig. 4-7b, "K48R Ub" versus "wt Ub"). Thus, Hmg2p degradation required the attachment of Lys48-linked multi-ubiquitin chains to Hmg2p, suggesting that only a few lysine residues at best were covalently linked with ubiquitin. This correlated well with the observation that only two lysine residues, Lys6 and Lys357, were absolutely required for Hmg2p degradation. By extension, it is likely that these two lysine residues serve as the sites for the covalent attachment of ubiquitin to the Hmg2p transmembrane domain.



Figure 4-7: Hmg2p degradation required the formation of multi-ubiquitin chains.

a) Ubiquitination assay of a strain expressing 1myc-Hmg2p was performed in the presence of no drug ("-") or zaragozic acid ("ZA", $10\mu g/ml$). Upper panels are the result of anti-HA (" α -HA") immunoblotting for covalently linked HA-tagged Ub. Lower panels are the result of parallel immunoblotting an aliquot (1/8 total volume) of the same immunoprecipitates with the 9E10 anti-myc antibody (" α -myc") to assess total immunoprecipitated Hmg2p. Ubiquitinated Hmg2p is represented as a collection of 5-8 bands with higher molecular weights than normal Hmg2p. b) Hmg2p degradation required Lys48-linked multi-ubiquitin chains. Otherwise identical strains co-expressing 1myc-Hmg2p and either normal ubiquitin ("wt Ub") or K48R ubiquitin ("K48R Ub") from the *CUP1* promoter were subject to a cycloheximide-chase assay. Cells were grown exponentially in the presence of CuSO₄ (final concentration 100 μ M) for 2 hours prior to the addition of cycloheximide. Lysates for each indicated time point were made and immunoblotted to determine the level of 1myc-Hmg2p.

C. Discussion

Ubiquitination of Hmg2p requires the ER-associated E2 Ubc7p and occurs in a manner that is regulated by the mevalonate pathway and consistent with Hmg2p regulated degradation (Hampton and Bhakta, 1997). Utilizing the collection of stable Hmg2p mutants constructed in the previous chapter, we have analyzed the effect of the stabilizing mutations on Hmg2p degradation and discovered that the entire, correct structure of the Hmg2p transmembrane domain was required for normal, regulated ubiquitination. We also revealed that ubiquitination of Hmg2p occurred by attachment of Lys48-linked multi-ubiquitin chains. Prevention of multi-ubiquitin chain formation, by introduction of the K48R mutation in ubiquitin, resulted in stabilization of Hmg2p.

The correct structure of the distributed degron was required for normal, regulated ubiquitination.

All mutations that stabilized Hmg2p resulted in reduced ubiquitination of Hmg2p. The effects of the mutations on Hmg2p ubiquitination could be separated into two phenotypic classes: mutations that reduced ubiquitination, but still allowed a small amount of zaragozic acid-stimulated ubiquitination, and mutations that resulted in reduced ubiquitination that was unresponsive to the stimulating effect of zaragozic acid. It was difficult to ascertain whether each critical region of Hmg2p was directly involved in the process of Hmg2p ubiquitination by direct participation in E2/E3 binding and/or ubiquitin attachment, or if each region was indirectly involved in Hmg2p ubiquitination by only contributing to the correct structure of the transmembrane domain. However, it was clear that the normal regions encompassed by some of these mutations were required for the maintenance of a correct structure (Chapter 3). Thus, because all stabilizing mutations resulted in reduced ubiquitination and at least some of these were required for the correct structure of the Hmg2p transmembrane domain, it appeared that the correct structure of the transmembrane domain was essential for both normal, regulated degradation and normal, regulated ubiquitination. This is not surprising as ubiquitination appears to be the precursor for Hmg2p degradation (Hampton and Bhakta, 1997), and elimination of ubiquitination in-*trans* or in-*cis* results in stabilization. Unfortunately, the exact degree of participation for each critical region in Hmg2p ubiquitination will in all likelihood have to wait until more detailed structural information is available for the Hmg2p transmembrane domain.

Lys48-linked multi-ubiquitin chains were required for Hmg2p degradation.

The degradation of Hmg2p required its initial ubiquitination (Hampton and Bhakta, 1997). However, Hmg2p degradation did not proceed through just the simple mono-ubiquitination of these critical lysines. Rather, Hmg2p degradation required the conjugative attachment of multi-ubiquitin chains that were linked through an isopeptide bond defined by the internal Lys48 of the ubiquitin molecules. Loss of Lys48 in ubiquitin, by replacement with arginine, prevents multi-ubiquitin formation (Chau *et al.*, 1989; Hochstrasser *et al.*, 1991), and resulted in the strong stabilization of Hmg2p. Thus, Hmg2p degradation occurred by recognition of a multi-ubiquitinated form of Hmg2p, which is consistent with the known, Lys48-linked, multi-ubiquitin chainbinding activities of the 26S proteasome (Hough *et al.*, 1987; Chau *et al.*, 1989; Hochstrasser *et al.*, 1991; Beal *et al.*, 1996; Thrower *et al.*, 2000).

Recently, it was demonstrated that the minimal length of the Lys48-linked, multi-ubiquitin chain required for recognition and binding of the 26S proteasome was 4 ubiquitin molecules (Thrower *et al.*, 2000). In fact, it appeared that the 26S proteasome recognized a Lys48-linked multi-ubiquitin chain comprised of 4 ubiquitin molecules as a single unit (Thrower *et al.*, 2000). Furthermore, optimal recognition of a degradation substrate by the 26S proteasome occurred with a multi-ubiquitin chain comprised of two units, 8 total ubiquitin molecules, as no significant increase in affinity of the 26S proteasome for larger multi-ubiquitin chains was observed (Thrower *et al.*, 2000). Interestingly, we observed that the maximal number of detectable ubiquitin molecules attached to Hmg2p was 8 (Fig. 4-7a). Because Hmg2p required Lys48-linked multiubiquitin chain attachment for degradation, the number of ubiquitin molecules attached to Hmg2p suggested that either two units of Lys48-linked, multi-ubiquitin tetramers were attached to two separate lysine residues within the Hmg2p transmembrane domain, or two units were attached to one single lysine residue.

Lys6 and Lys357 may serve as the sites for the covalent attachment of ubiquitin.

Lysine residues are the usual residues to which ubiquitin is attached within a degradation substrate (Finley and Chau, 1991; Jentsch, 1992). Although we have yet to determine the actual sites of ubiquitin attachment within Hmg2p, the fact that two critical lysine residues, Lys6 and Lys357, were required for degradation (Chapter 3) suggested that these lysine residues may serve in this capacity. Based upon the
degradation phenotypes and ubiquitination patterns of the K6R- and K357R-Hmg2p mutants, we propose a putative model for the attachment of ubiquitin to these residues, which is pictured in figure 4-8. In normal Hmg2p, an initial ubiquitin attachment event may occur on Lys357. This ubiquitination event would be regulated by the mevalonate pathway and would likely occur through the exposure of Lys357 to the ubiquitination machinery via alteration of the transmembrane domain structure under the appropriate mevalonate pathway conditions. The ubiquitination of Lys357 would then lead to the covalent attachment of a multi-ubiquitin chain to Lys6. The multi-ubiquitin chain on Lys6 may be comprised of two units of a Lys48-linked, multi-ubiquitin tetramer or only one unit. If the latter is the case, then it is likely that the other unit of a Lys48-linked, multi-ubiquitin tetramer is attached to Lys357. The recognition of Lys6 for the covalent conjugation of ubiquitin would be allowed by structural changes induced by the initial ubiquitination of Lys357. However, the ubiquitination of Lys6 would not be directly regulated by the mevalonate pathway, but would be absolutely required for the degradation of normal Hmg2p.

Both the ubiquitination and degradation phenotypes of the K6R and K357R mutants support this model. The K6R-Hmg2p mutant was completely stable (Chapter 3, and Fig. 4-3), but was still subject to a very small amount of mevalonate pathway-regulated ubiquitination. The low level of regulated ubiquitination likely occurred on Lys357, as the double K6R, K357R-Hmg2p mutant was no longer subject to any observable zaragozic-acid stimulated ubiquitination. Because Lys6 was substituted with arginine in the K6R-Hmg2p mutant, no Lys6-dependent ubiquitination could occur and this coincided with an almost complete lack of degradation. In contrast, the



Figure 4-8: Mechanism of Hmg2p ubiquitination.

Cartoon representation of the putative model for Hmg2p ubiquitination. When mevalonate pathway production is abundant, the action of an FPP-derived signal, combined with the action from an oxysterol-derived signal, results in alteration of the Hmg2p transmembrane domain such that Lys357 is exposed to the ER ubiquitination machinery. Lys357 is mono-ubiquitinated, which results in further alteration of the Hmg2p transmembrane domain structure thereby exposing Lys6 for multiubiquitination and subsequent degradation of the multi-ubiquitinated Hmg2p. Two possible models exist for Hmg2p multi-ubiquitination: either Lys6 is the only lysine multi-ubiquitinated (Model 1), or both Lys6 and Lys357 are multi-ubiquitinated (Model 2). K357R-Hmg2p mutant had a slightly higher level of basal ubiquitination and a modest degree of degradation (Chapter 3; and Fig. 4-3), but both occurred in a manner that was unregulated by the mevalonate pathway. The small amount of unregulated ubiquitination and degradation was Lys6 dependent, as the double K6R, K357R-Hmg2p mutant was no longer subject to the slight basal ubiquitination and degradation associated with K357R-Hmg2p (Chapter 3, and Fig. 4-3). Thus, some ubiquitination likely occurred on Lys6 in an unregulated, albeit highly inefficient, manner in the absence of Lys357. From our model, the mevalonate pathway-regulated, covalent attachment of ubiquitin to Lys6, which is required for subsequent Hmg2p degradation.

In fact, other recent observations may support our model of regulated Hmg2p ubiquitination. Two ubiquitin conjugating enzymes have been shown to act in Hmg2p ubiquitination: Ubc7p and Ubc1p (Hampton and Bhakta, 1997; Bays *et al.*, 2000). Loss of Ubc7p function in the cell results in stabilization of Hmg2p (Hampton and Bhakta, 1997; Bays *et al.*, 2000). Furthermore, loss of Ubc1p function in the cell also results in significant stabilization of Hmg2p (Bays *et al.*, 2000). In the absence of Ubc7p, over-expression of the ER-associated ubiquitin ligase Hrd1p, which is required for Hmg2p degradation (Hampton *et al.*, 1996a; Bays *et al.*, 2000), allows Hmg2p ubiquitination and degradation through enhanced recruitment of Ubc1p by Hrd1p (Bays *et al.*, 2000). The Hmg2p ubiquitination pattern in *ubc7* Δ cells with increased Hrd1p expression demonstrates a build-up of what appears to be mono-ubiquitinated Hmg2p with decreased Hmg2p multi-ubiquitination (Bays *et al.*, 2000). Additional loss of Ubc1p function in these cells eliminates Hmg2p ubiquitination altogether (Bays *et al.*, 2000).

From these results, it is conceivable that in normal cells Ubc1p acts in the initial monoubiquitination of Lys357 in Hmg2p, which leads to the subsequent multi-ubiquitination of Lys6, and possibly Lys357, by the action of Ubc7p. The fact that multiubiquitination is not completely eliminated in the *ubc7* Δ cells indicates that Ubc1p may be able to inefficiently substitute for Ubc7p in Hmg2p multi-ubiquitination (Bays *et al.*, 2000). In contrast, loss of Ubc1p function results in loss of the majority of Hmg2p ubiquitination (Bays *et al.*, 2000), possibly by the decreased initial mono-ubiquitination of Lys357, which by our model is required to initiate the structural change essential for further multi-ubiquitination. However, as Hmg2p is not completely stable in *ubc1* Δ cells (Bays *et al.*, 2000), it is likely that Ubc7p can substitute for Ubc1p function in mono-ubiquitination of Lys357 at a low efficiency and then effect the multiubiquitination of Lys6 and Lys357 by its normal function.

Implications of the mechanism of Hmg2p ubiquitination.

An understanding of the mechanism of Hmg2p ubiquitination is important from not just the aspect of sterol synthesis regulation, but also from the standpoint of ER degradation, and quite possibly general ubiquitin-dependent degradation. It is a frequent observation that covalent attachment of ubiquitin to degradation substrates depends upon specific lysines within the target protein, either on a single lysine residue (Chau *et al.*, 1989; Bachmair and Varshavsky, 1989; Sokolik and Cohen, 1991, 1992; Nishizawa *et al.*, 1993; Trovato *et al.*, 1999), or multiple, but limited, lysine residues (Chau *et al.*, 1989; Hou *et al.*, 1994; Scherer *et al.*, 1995; Rodriguez *et al.*, 1996; Shirane *et al.*, 1999). In some substrates however, it appears that specific lysine residues are not required for ubiquitination (Hochstrasser et al., 1991; Treier et al., 1994; Crook et al., 1996; King et al., 1996; Yamano et al., 1998). Thus, it may be that the presence of any single lysine residue within these substrates can effectively serve as a site for ubiquitin conjugation. Alternatively, it may be that the right combination of lysine residue substitutions, which would specifically pinpoint the limited group of lysine residues involved in the target substrate's ubiquitination, have not been constructed and analyzed. However, in some cases, elimination of all lysine residues within the target protein has been demonstrated to still allow substrate ubiquitination and degradation (Yu et al., 997; Breitschopf et al., 998). It may be that in these instances, the extreme N-terminal amino acid residue acts as the target site for covalent attachment of ubiquitin. The primary α -amino group on the N-terminal residue of a protein can often be similarly chemically modified as the primary ε -amino group in a lysine side chain in vivo (Strous et al., 1974; Jörnvall, 1975; Driessen et al., 1985; Rainwater and Kolattukudy, 1982; L'Hernault and Rosenbaum, 1985), and in vitro (Lomant and Fairbanks, 1976; Partis *et al.*, 1983). If unmodified *in vivo*, the primary α amino group on the N-terminal residue may be similarly reactive in the process of ubiquitination. Indeed, the N-terminal residue of the transcriptional activator MyoD does appear to be utilized as the primary site for substrate ubiquitination (Breitschopf et al., 1998). It may also be possible that the ubiquitin-dependent degradation of such substrates occurs by ubiquitination of an effector protein that subsequently targets these substrates to the proteasome. In all of these cases that have been highlighted, it is quite possible that in the future only a small group of residues within each target substrate

will be identified as ubiquitin conjugation sites, whether they are internal lysine residues or the amino group of the extreme N-terminal residue remains to be seen.

Most of the attempts to identify specific lysine residues utilized for the covalent attachment of ubiquitin within the target substrate have been performed with regulated degradation substrates. Few studies have been performed to identify specific ubiquitin conjugation sites within the sequences of quality control substrates. This is not surprising in that these proteins are mutant and/or misfolded and most likely present multiple, independently acting lysine residues to the ubiquitin-dependent, quality control degradation apparatus. These lysine residues would ordinarily not come into contact with the ubiquitination machinery in the normal and stable version of the protein. However, knowledge of such lysine residues would have potential value in prevention of the aberrant degradation of quality control substrates like PiZ α -1 antitrypsin or CFTR Δ F508 (Ciccarelli *et al.*, 1993; Jensen *et al.*, 1995; Ward *et al.*, 1995), or in allowing enhanced entry of HMG-R into the ER quality control degradation pathway in order to slow cellular cholesterol production.

Understanding the mechanism of Hmg2p ubiquitination may be informative in this regard. Hmg2p is a normally folded protein that enters the ER quality control degradation pathway in a regulated manner. We have clearly demonstrated that Hmg2p required specific lysine residues to effect ubiquitination and degradation. This is an essential characteristic of Hmg2p necessary for the regulation of its ubiquitination and degradation. That is, for regulation to effectively function, Hmg2p must restrict access to all lysine residues when conditions of mevalonate pathway flux signal for Hmg2p stability, otherwise constitutive ubiquitination and degradation would occur. To accomplish this, the structure of the Hmg2p transmembrane domain likely renders all lysine residues inaccessible to the ubiquitination machinery except for Lys6 and Lys357, which would only be accessible when mevalonate pathway production is abundant or appropriately manipulated with drugs. Thus, Hmg2p appears to have evolved an intact structure for the precise, conditional presentation of only specifically placed lysine residues to the ubiquitination machinery, thereby allowing Hmg2p to elude recognition for degradation under the appropriate mevalonate pathway conditions in a compartment where the degradation targeting machinery may be constantly scanning for quality control substrates (see Chapter 5). Consistent with this are the numerous structural mutants of Hmg2p subject to rapid, unregulated, ubiquitindependent degradation that no longer requires either critical Lys6 or Lys357 (see Chapter 3). It is quite probable that these unregulated mutants are constitutively degraded because additional lysine residues are now constitutively accessible for ubiquitination due to the structural alterations imposed by the mutations. Such Hmg2p mutants may be useful in designing strategies to effect decreased cholesterol production in humans.

Our studies with Hmg2p may indicate a divergence between the ubiquitination of quality control substrates and the ubiquitination of regulated degradation substrates. For instance, it may be a desirable cellular strategy for quality control substrates to utilize multiple and independent lysine residues for ubiquitination to ensure their constitutive destruction; whereas, it may be desirable for regulated degradation substrates to utilize only particular lysine residues, with the capacity to alter access of the ubiquitination machinery to such lysine residues according to the specific and current physiological conditions. This is reflected by the observation that normal Hmg2p is a regulated degradation substrate degraded by way of a conditional ubiquitination that depends upon specific lysine residues, but some structural mutants of Hmg2p, such as 6myc-Hmg2p (Hampton et al., 1996a; and Chapter 3), are quality control substrates that are constitutively degraded in a ubiquitin-dependent manner that no longer solely depends upon these specific lysine residues. In this regard, other regulated degradation substrates have also been found to require a small number of critical sites for their ubiquitination, such as IkBa (Scherer et al., 1995; Rodriguez et al., 1996), the tumor suppressor protein p53 (Crook et al., 1996), the TCR ζ chain (Hou et al., 1994), the cyclin-dependent kinase (CDK) inhibitory protein p27(Kip1) (Shirane et al., 1999), the transcriptional activator MyoD (Breitschopf et al., 1998), cytochrome c (Sokolik and Cohen, 1991, 1992), and the HTLV-1 protein p12(I) (Trovato et al., 1999). It is important to note that the strategy of employing restricted access to specific lysine residues to effect regulated degradation need not be brought about solely through in-cis structural mechanisms, but also may be accomplished through in-trans mechanisms (Li and Coffino; 1992; Murakami et al., 1992; Qu et al., 1996), or a combination of both.

Actual identification of the ubiquitination sites in Hmg2p will help clarify any putative model based on the existing data. The existence of Hmg2p suggests that ubiquitination by the ER-associated ubiquitin need not proceed by the covalent attachment of ubiquitin to any random lysine residue within a region of the target protein. This should be taken into consideration when formulating the general mechanistic features of ubiquitin attachment and ubiquitin-dependent degradation at the ER.

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Chapter V: Mechanism of recognition and ubiquitination by the ER E3 complex

Holmes took each face of the house in turn, and examined it with great interest. He then led the way inside, and went over the whole building from basement to attic. Most of the rooms were unfurnished, but none the less Holmes inspected them all minutely. Finally, on the top corridor, which ran outside three untenanted bedrooms, he again was seized with a spasm of merriment.

> The Adventure of the Norwood Builder --Sir Author Conan Doyle

A. Introduction

Proteasome-dependent destruction of proteins is a vital component of cellular physiology. Numerous essential and important cellular processes utilize proteasomedependent degradation as a means to control the levels of specific proteins. Such processes include cell-cycle progression (Glotzer *et al.*, 1991; King *et al.*, 1996a; Hershko, 1997), metabolic pathway regulation (Edwards *et al.*, 1983; Nakanishi *et al.*, 1988; Murakami *et al.*, 1992; Li and Coffino, 1992; Hampton and Rine, 1994; Hayashi and Murakami, 1995; Schork *et al.*, 1995; Coleman and Pegg, 1997; Gross-Mesilaty *et al.*, 1997), signal transduction (Scherer *et al.*, 1995), and quality control (Cheng *et al.*, 1990; Finger *et al.*, 1993; Sommer and Jentsch, 1993; Ciccarelli *et al.*, 1993; Jensen *et al.*, 1995; Ward *et al.*, 1995; Qu *et al.*, 1996). In fact, proteasome-dependent degradation is so critical for cellular function that loss of proteasome function leads to cell death (Heinemeyer *et al.*, 1991; Ghislain *et al.*, 1993; Hilt and Wolf, 1995).

The proteasome is a complex general protease located in the cytosol and nucleus of eukaryotes (Peters *et al.*, 1994; Enenkel *et al.*, 1998; Wilkinson *et al.*, 1998; Russell *et al.*, 1999). To prevent the random destruction of all proteins in these compartments by the general proteolytic activity of the proteasome, unique targeting and regulatory mechanisms are associated with proteasome-dependent degradation (Hershko and Ciechanover, 1992; Hochstrasser, 1996; Hershko *et al.*, 1997; Baumeister *et al.*, 1998). Proteins destined for proteasome-dependent degradation are usually targeted initially by the covalent attachment of the small protein ubiquitin by action of a collection of defined enzymes (Finley and Chau, 1991; Jentsch, 1992). In addition, the proteasome

contains a 19S regulatory particle that specifically recognizes such ubiquitin-tagged substrates and regulates their unfolding and entry into the catalytic portion of the proteasome (Chu-Ping *et al.*, 1994; DeMartino *et al.*, 1994; Deveraux *et al.*, 1994; Tsurumi *et al.*, 1996; van Nocker *et al.*, 1996; Kominami *et al.*, 1997; Baumeister *et al.*, 1998; Fu *et al.*, 1998), where they are cleaved into small peptides (Heinemeyer *et al.*, 1993; Enenkel *et al.*, 1994; Arendt and Hochstrasser, 1997). For the proteasome to function in such a diverse array of degradation processes, the targeting mechanism must be general enough to accommodate the different substrates, but specific enough to prevent the degradation of proteins whose stability is desired. Thus, the essential function of the proteasome not only requires its complete catalytic activity, but also the complete and accurate activities of the targeting and regulatory mechanisms. Loss of function for these mechanisms results in similar deleterious effects as loss of proteasomal catalytic activity (McGrath *et al.*, 1991; Ghislain *et al.*, 1993; Finley *et al.*, 1994; Schnall *et al.*, 1994; Dohmen *et al.*, 1995).

Proteasome-dependent degradation of critical cellular substrates is so heavily dependent upon these targeting and regulatory processes that numerous focused studies have been conducted to elucidate their mechanistic details. In the case of the ubiquitin-targeting mechanism, much has been revealed. The covalent attachment of ubiquitin to a desired degradation substrate is brought about by a collection of enzymes that form a hierarchy of activity (Finley and Chau; 1991; Jentsch, 1992; Hochstrasser, 1996). In yeast, ubiquitin is activated by the formation of a high-energy, thiol-ester bond with the commonly used E1 protein , or ubiquitin activating enzyme, Uba1p (McGrath *et al.*, 1991). The activated ubiquitin is then transferred to one of numerous E2 proteins, or

ubiquitin conjugating enzymes (UBCs) (Finley and Chau; 1991; Jentsch, 1992; Hochstrasser, 1996). The transfer of ubiquitin to a target protein is effected by the action of the ubiquitin-charged E2 and by additional proteins known as E3 proteins, or ubiquitin ligases (Finley and Chau; 1991; Jentsch, 1992; Hochstrasser, 1996). The E3 proteins appear to provide the specificity required for the E2 to recognize its specific target and may comprise the largest class of proteins in the ubiquitin conjugation hierarchy (Hochstrasser, 1996). Although the number of E1 and E2 proteins is known in yeast, the actual number of E3 proteins has not yet been determined, as their function in ubiquitin-conjugation has been difficult to elucidate. Additional genetic and biochemical studies on the mechanisms of substrate targeting should provide further insight into the number of E3 proteins within yeast.

Despite an incomplete tally of E3 proteins, both *in vitro* and *in vivo* studies have revealed some common characteristics among the known E3 proteins. Of the few yeast E3 proteins characterized, all of them appear to be absolutely essential for the degradation of their associated degradation substrates (Bartel *et al.*, 1990; Hampton *et al.*, 1996a; Ohta *et al.*, 1999a; Soel *et al.*, 1999; Skowyra *et al.*, 1999). Furthermore, many of these characterized yeast E3 proteins contain a highly conserved RING-H2 finger motif (Hampton *et al.*, 1996a; Kamura *et al.*, 1999; Lorick *et al.*, 1999; Ohta *et al.*, 1999a; Seol *et al.*, 1999; Skowyra *et al.*, 1999; Bays *et al.*, 2000). When included as a heterologous sequence, these domains appear specifically to bind E2 proteins and direct the transfer of ubiquitin to the associated fusion protein *in vitro* (Lorick *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999; Bays *et al.*, 2000). Directed mutations in this domain abolish the *in vitro* ubiquitin ligase activity (Bays *et al.*, 2000), and prevent the *in vivo* degradation of their corresponding substrates (Bordallo and Wolf, 1998; Xie and Varshavsky, 1999; Bays *et al.*, 2000). Although the RING-H2 finger motifs of these E3 proteins appear able to bind a variety of E2 proteins *in vitro* (Lorick *et al.*, 1999; Bays *et al.*, 2000), the binding of the E3 proteins is limited to very specific E2 proteins *in vivo* (Madura *et al.*, 1993; Seol *et al.*, 1999; Skowyra *et al.*, 1999; Bays *et al.*, 2000). Thus, the E3 proteins appear to contain the *in vivo* specificity to distinguish between the variety of divergent proteasome-dependent degradation substrates.

While the E2 binding and ubiquitin ligase activities of E3 proteins are beginning to be revealed, little is known about how these proteins actually recognize and target their specific degradation substrates. From two limited examples, the mode of substrate recognition has been revealed, but in each instance appears to be effected through very different mechanisms. In the case of the SCF ubiquitin ligase complex, the E3 protein Hrt1p/Rbx1p/Roc1p employs a scaffold complex containing an effector protein that specifically binds to the target substrate (Feldman *et al.*, 1997; Skowyra *et al.*, 1999a; Seol *et al.*, 1999; Skowyra *et al.*, 1999), thereby bringing the substrate into proximity to the ubiquitin ligase. In contrast, the N-end rule ubiquitin ligase complex appears to contain multiple substrate binding activities within the E3 protein itself (Reiss *et al.*, 1988; Gonda *et al.*, 1989; Bartel *et al.*, 1990; Baker and Varshavsky, 1991).

The conjugation of ubiquitin to an individual substrate is effected by a specific ubiquitin ligase complex (Hochstrasser, 1996), which specifically recognizes intrinsic molecular information within the target substrate. However, the cell also employs proteasome-dependent degradation as a quality control mechanism, destroying mutant, damaged and misfolded proteins in the cell to prevent their accumulation. How do ubiquitin ligase complexes recognize this immense and diverse array of substrates? Degradation for purposes of physiological regulation often utilizes specific modular sequences within the target substrate (Bachmair et al., 1989; Hochstrasser and Varshavsky, 1990; Glotzer et al., 1991; Nishizawa et al., 1992; Whiteside et al., 1995; King et al., 1996; Hershko, 1997; Varshavsky, 1996; Johnson et al., 1998), thereby allowing recognition by a specific ubiquitin ligase complex (Bartel et al., 1990; King et al., 1996; Peters, 1999; Xie and Varshavsky, 1999). Such small, linear sequences are called "degrons" (Varshavsky, 1991), and, in most cases, a degron will function autonomously when fused to a stable protein as a heterologous sequence (Hochstrasser and Varshavsky, 1990; Glotzer et al., 1991; King et al., 1996). In contrast, degradation used for purposes of quality control requires recognition of a diverse collection of proteins that may share no primary sequence homology (Finger et al., 1993; Biederer et al., 1996; Hampton et al., 1996; Bordallo et al., 1998; Plemper et al., 1998). More importantly, the ubiquitin ligase complexes must be able to distinguish correctly folded proteins from the same proteins that are mutant, damaged, or misfolded. As in regulated degradation, quality control recognition requires a high degree of accuracy, but it appears to be based on common structural hallmarks indicative of damage or misfolding rather than specific, degradation-targeting sequences.

To understand more completely the selection and targeting mechanisms of ubiquitin ligase complexes, we have been studying the process of ubiquitin-dependent, ER-associated degradation. Degradation associated with the ER provides a unique opportunity to study the mechanistic details of substrate selection because it is employed for both regulated degradation of normal proteins and quality control degradation of mutant or misfolded proteins (Inoue et al., 1991; Meigs and Simoni, 1992; Ciccarelli et al., 1993; Finger et al., 1993; Hampton and Rine, 1994; Ward et al., 1995; Biederer et al., 1996; Hampton et al., 1996). In order to learn more about the process of substrate selection and targeting of ER-associated degradation substrates, we have been exploring the ER-associated degradation of the yeast HMG-CoA Reductase (HMG-R) isozyme Hmg2p (Hampton and Rine, 1994; Hampton et al., 1996; Hampton and Bhakta, 1997). HMG-R catalyzes the rate-limiting, irreversible conversion of HMG-CoA to mevalonate in the mevalonate pathway (Goldstein and Brown, 1990), from which sterols and essential isoprenoids are synthesized. In mammals and yeast, HMG-R is subject to proteasome-dependent, ER-associated degradation that is feedback regulated by signals generated downstream in the mevalonate pathway (Edwards et al., 1983; Nakanishi et al., 1988; Chun et al., 1990; Hampton et al., 1994; Hampton et al., 1996). Genetic analysis of the degradation of the yeast HMG-R isozyme Hmg2p revealed that Hmg2p degradation strongly depended on the E2 protein Ubc7p (Hampton and Bhakta, 1997), the proteasome subunit Hrd2p (Hampton et al., 1996), and two ER membrane proteins Hrd1p and Hrd3p (Hampton et al., 1996).

Further characterization of the proteins involved in Hmg2p degradation revealed that some of them form an ER localized ubiquitin ligase complex. Analysis of Hrd1p function demonstrated that it contained a RING-H2 finger motif (Hampton *et al.*, 1996a; Bordallo *et al.*, 1998), which was capable of ubiquitin ligase activity *in vitro* and *in vivo* (Bays *et al.*, 2000). Additional studies revealed a functional genetic and biochemical interaction between Hrd1p and Hrd3p (Plemper *et al.*, 1999b; Gardner *et* *al.*, 2000), which appeared to form a complex that protected Hrd1p from selfprogrammed ubiquitination and subsequent proteasomal degradation (Plemper *et al.*, 1999b; Gardner *et al.*, 2000). Also, it appeared that Hrd1p interacted with the ERassociated and membrane-bound E2 protein Ubc7p *in vivo* and, to a lesser extent, with the E2 protein Ubc1p (Bays *et al.*, 2000). Thus, the ER-associated, ubiquitin-mediated, proteasome-dependent degradation of Hmg2p required a ubiquitin ligase complex comprised of Hrd1p, Hrd3p, Ubc7p or sometimes Ubc1p. Although the identity and architecture of the ER-associated, ubiquitin-targeting complex is coming into focus, it is still completely unknown how this complex recognizes and targets individual degradation substrates.

To address this deficiency, we have initiated an analysis of the interactions between the proteins of the ER ubiquitin ligase complex and one of its target substrates, Hmg2p. Hmg2p is a useful substrate for such studies because its degradation is not constitutive; rather, Hmg2p degradation is regulated by the cellular abundance of mevalonate pathway products (Hampton and Rine, 1994; Hampton and Bhakta, 1997; Gardner and Hampton, 1999b). Accordingly, Hmg2p is increasingly recognized for degradation under conditions of increased mevalonate pathway production, but eludes such recognition under conditions of decreased pathway production, providing a convenient assay to test the mechanism of interaction. To analyze such direct interactions, we employed an *in vivo*, chemical cross-linking assay utilizing epitopetagged and functional versions of each protein. From this analysis, we have demonstrated that some components of the ER ubiquitin ligase complex interacted with the degradation substrate in the absence of other components, but only a completely intact ER ubiquitin ligase complex allowed interaction of all components. Furthermore, it appeared that the complete complex targeted degradation substrates through a quality control scanning mechanism that brought the complex into close proximity of both stable and degradation-competent proteins. However, only the degradation-competent proteins were ubiquitinated and degraded, most likely by exposure of key lysine residues to the ubiquitination machinery.

B. Results

In our previous genetic analysis of ER degradation in yeast, we discovered the identity of genes required for the degradation of the yeast HMG-R isozyme Hmg2p (Hampton et al., 1996a), which were termed HRD genes. Two of these genes, HRD1 and *HRD3*, encode proteins that contain putative membrane spanning domains (Hampton et al., 1996a), and the respective proteins have subsequently been localized to the ER membrane (Bordallo et al., 1998; Saito et al., 1999; Plemper et al., 1999b). In addition to the *HRD*-gene encoded proteins, we also revealed that Hmg2p degradation required the presence of the ubiquitin conjugating enzyme (E2), Ubc7p (Hampton and Bhakta, 1997). Additional studies, which detailed the different interactions between these proteins and the *in vitro* ubiquitin ligase activity of Hrd1p, indicated that these three proteins form an E2/E3 complex with ubiquitin ligase activity in vitro and in vivo (Bays et al., 2000; Gardner et al., 2000). The ER-membrane protein Cue1p may also be a component of this complex as it is required for the ERmembrane association of Ubc7p (Biederer et al., 1997). Because these proteins are required for the ubiquitination and degradation of Hmg2p (Bays et al., 2000; N. Bays and R. Hampton, unpublished observations), it is quite likely that these proteins directly interact with Hmg2p to target it for destruction by the covalent addition of ubiquitin under the appropriate mevalonate pathway conditions.

To test this hypothesis, we initiated chemical cross-linking studies between Hmg2p and the individual proteins of the ER ubiquitin ligase complex. Co-immunoprecipitation was not chosen as a viable assay due to the membrane localization of the

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proteins, which would require harsh solubilizing conditions to release them from their integral membrane association that would most likely disrupt any protein-protein interactions. Instead, an *in vivo* chemical cross-linking assay was developed to reveal interactions between Hmg2p and the ER ubiquitin ligase complex under the cellular conditions that such interactions would most likely be formed. Chemical cross-linking results in the reversible, covalent attachment of proteins that are within close proximity to one another, thereby allowing the use of harsh detergent conditions to solubilize the membrane proteins.

We designed an *in vivo* chemical cross-linking assay for use with whole, exponentially growing cells (Fig. 5-1), which was an adaptation of the procedure used with cell lysates (Marcusson et al., 1994). The assay was performed by growing the appropriate strains into log phase and then removing all free amino acids from the media. The cells were placed in osmotically balanced media and the membranepermeable, chemical cross-linker DSP (dithiobis(succinimidyl-proprionate)) was added to the cells. We chose DSP as the cross-linking agent because it was membrane permeant, contained a thiol bond that was cleavable under reducing conditions, was easily quenched by addition of primary amines, and contained a spacer arm of 12 Å that limited its ability to cross-link only closely associated proteins. Zymolase was also added to the cells to aid in lysis and the cells were incubated for 30 min in the presence of both the lytic enzyme and the cross-linking agent DSP. Cells were subsequently osmotically lysed in a detergent solution containing a quenching agent to inhibit further chemical cross-linking and to solubilize and denature the cross-linked proteins. The Hmg2p molecules were immunoprecipitated utilizing antisera specific for the Hmg2p



Figure 5-1: *In vivo* cross-linking assay to determine interactions between Hmg2p and the *HRD*-gene encoded degradation machinery.

Live, whole cells are treated with increasing concentrations of the membrane permeable, thiol-containing, cross-linking agent DSP, which reacts with the primary amines within lysine residues. After incubation with DSP, cells are osmotically lysed and the proteins resuspended under non-reducing conditions. Protein Z molecules are immunoprecipitated with an antisera specific for only Protein Z. The immunoprecipitate is subject to denaturation under reducing conditions, and then analyzed by immunoblotting. Protein Z is tagged with a myc-epitope sequence and Protein X is tagged with an HA-epitope sequence for ease of detection. The presence of these added sequences does not impair the functions of either Protein X or Protein Z.

catalytic domain. The subsequent immunoprecipitate was subject to Western analysis under reducing conditions to separate the cross-linked proteins in order that they migrate according to their molecular weight.

For ease of detection, we constructed functional, epitope-tagged versions of each protein. From previous studies, Hmg2p contained a single myc-epitope inserted between residues 618 and 619 in its poorly conserved linker region (Hampton and Bhakta, 1997). The presence of the myc-epitope tag does not effect the normal, regulated degradation of Hmg2p (Hampton and Bhakta, 1997; and see previous Chapters 3 and 4). In addition, a single myc-epitope tag was inserted in a similar position, between residues 622-623, in the stable HMG-R isozyme Hmg1p in order to assess interactions with a similar, yet stable protein. A triple HA-epitope tag was placed on the extreme C-terminus of Hrd1p, and the addition of this protein sequence did not effect the ability of Hrd1p to program the regulated degradation of Hmg2p (Fig. 5-2a). Previously, a triple HA-epitope tag was inserted between residues 20 and 21 of the Hrd3p sequence (Saito *et al.*, 1999), and the presence of this sequence had no effect on the ability of Hrd3p to function in Hmg2p regulated degradation (Fig. 5-2b). Lastly, a double HA-epitope tag was placed on the C-terminus of Ubc7p, and the presence of this tag allowed normal regulated Hmg2p degradation (Fig. 5-2c). Together, with these functional and epitope-tagged proteins, we characterized the ability of each myc-tagged HMG-R isozyme to be cross-linked to each HA-tagged protein under various degradation-enhancing or degradation-inhibiting conditions of the mevalonate pathway.



Figure 5-2: Epitope-tagged versions of each protein required for Hmg2p degradation were functional.

Each HA-epitope tagged protein was transformed into a strain that expressed 1myc-Hmg2p and contained its respective null allele to allow a complementation test. Each strain carrying the indicated allele of the gene, marked above each experiment, was subject to a cycloheximide-chase assay to determine the degree of 1myc-Hmg2p degradation. Cells were grown to mid-log phase and cycloheximide ($50\mu g/ml$) was added to inhibit protein synthesis. Lysates from the indicated time point after addition of cycloheximide were prepared and analyzed by immunoblotting to determine the degradation of the pool of protein made prior to addition of cycloheximide. Myctagged proteins were detected using the 9E10 antibody (α -myc) and HA-tagged proteins were detected using the 12CA5 antibody (α -HA}). In all cases, the HA-tagged protein was able to complement its respective null allele. **a**) 3HA-Hrd1p complements a *hrd1* Δ allele. **b**) 3HA-Hrd3p complements a *hrd3* Δ allele. **c**) 2HA-Ubc7p complements a *ubc7* Δ allele.

Ubc7p cross-linked to degraded Hmg2p, but did not cross-link to stable Hmg1p.

We first tested the ability of Hmg2p to cross-link to Ubc7p, the ubiquitin conjugating enzyme required for its degradation (Hampton and Bhakta, 1997). When Hmg2p was immunoprecipitated from cells incubated with increasing concentrations of the cross-linker DSP, Ubc7p co-immunoprecipitated with Hmg2p in a cross-linker concentration-dependent fashion (Fig. 5-3a, "Hmg2p"). A small amount of Ubc7p was co-immunoprecipitated with Hmg2p in the absence of added cross-linker, but this same amount was also observed in the absence of immunoprecipitated Hmg2p (data not shown). In contrast, the other ER-associated ubiquitin conjugating enzyme Ubc6p, which does not significantly participate in the ubiquitination and degradation of Hmg2p (Hampton and Bhakta, 1997), did not increasingly co-immunoprecipitate with Hmg2p in the presence of cross-linker (data not shown). Furthermore, when the stable HMG-R isozyme Hmg1p was immunoprecipitated from cells treated with DSP, very little Ubc7p co-immunoprecipitated in a DSP concentration-dependent manner (Fig. 5-3a, "Hmg1p"). Thus, it appeared that the cross-linking assay revealed an interaction between Hmg2p and Ubc7p that was physiologically relevant. Only the ubiquitin conjugating enzyme required for Hmg2p degradation cross-linked to Hmg2p and this cross-linker dependent interaction was observed with only the HMG-R isozyme targeted for ubiquitination and degradation, not the stable HMG-R isozyme.

Ubc7p did not cross-link to Hmg1p, but that fact does not necessarily preclude an interaction between Ubc7p and Hmg1p. It is possible that Ubc7p did not cross-link to Hmg1p because Hmg1p was simply recalcitrant to the cross-linking protocol. In order for two proteins to be cross-linked by DSP, free and accessible lysine residues



Figure 5-3: Ubc7p cross-linked to the degraded Hmg2p, but not the stable Hmg1p.

Interaction of 2HA-Ubc7p with 1myc-Hmg2p was assessed by the *in vivo* cross-linking assay described in figure 5-1. Cells were grown to mid-log phase and removed to amine free media. DSP was added in increasing concentrations (same volume of DMSO) to separate aliquots of cells, which were subsequently incubated for 30 minutes at 30°C. Cells were osmotically lysed and denatured under non-reducing conditions. 1myc-HMG-R was immunoprecipitated with antisera raised against its catalytic domain. Immunoprecipitates were denatured under reducing conditions and analyzed by immunoblotting with either the 9E10 antibody to detect 1myc-HMG-R or the 12CA5 antibody to detect 2HA-Ubc7p. a) Ubc7p cross-linked to Hmg2p, but did not cross-link to Hmg1p. Cross-linking assay with strains expressing either 1myc-Hmg2p or 1myc-Hmg1p and co-expressing 2HA-Ubc7p. The reduced immunoreactivity of 1myc-HMG-R in the 400μ g/ml DSP lane is due to modification of the lysine residue in the myc epitope sequence, not reduced immunoprecipitation of HMG-R (data not shown). b) Ubc7p cross-linked to a degraded version of Hmg1p. Cross-linking assay with strains expressing either 1myc-Hmg2p, 1myc-Hmg1p, or hydrophilic 1myc-Hmg1p and coexpressing 2HA-Ubc7p. c) Ubc7p did not cross-link to K6R, K357R-Hmg2p Hmg1p. Cross-linking assay with strains expressing either 1myc-Hmg2p, 1myc-K6R-Hmg2p, 1myc-K357R-Hmg2p, or 1myc-K6R, K357R-Hmg2p and co-expressing 2HA-Ubc7p.

within a target protein must be within a short distance of lysine residues within the bait protein. Perhaps Ubc7p interacted with Hmg1p but did not form cross-links because there were no accessible lysine residues within Hmg1p that were in close proximity to Ubc7p. As lysine residues are generally the sites for attachment of ubiquitin (Finley and Chau, 1991; Jentsch, 1992), it may be a structural strategy of the stable Hmg1p to prevent access of Ubc7p to its lysine residues.

To test if Ubc7p could be cross-linked to Hmg1p, we performed the crosslinking assay on a mutant and degraded version of Hmg1p. From our previous mutagenic analyses that dissected the in-*cis* determinants for Hmg2p degradation (see Chapter 3), we constructed a number of Hmg1p mutants that were no longer stable, but were subject to Ubc7p-dependent degradation (data not shown). One of these degraded Hmg1p mutants, termed hydrophilic-Hmg1p, was the result of a limited number of substitutions that converted the hydrophobic residues in first 26 residues to a hydrophilic ones, but did not change the number of lysine residues within Hmg1p, similar to hydrophilic-Hmg2p previously described (see Chapter 3, Fig. 3-12b). When hydrophilic-Hmg1p was immunoprecipitated from cells treated with DSP, Ubc7p was now co-immunoprecipitated with the mutant Hmg1p in a cross-linker concentrationdependent manner (Fig. 5-3b, "hydrophilic" versus "wt"). This suggested that the lack of Ubc7p cross-linking to Hmg1p was a consequence of its stability rather than an intrinsic inability of Hmg1p to cross-link to Ubc7p, as the degraded form of Hmg1p readily cross-linked to Ubc7p. Thus, it may be likely that Hmg1p interacts with Ubc7p but does not cross-link because Hmg1p, as a stable protein, restricts its surface lysine residues from access to Ubc7p.

If a stable protein cannot be cross-linked to Ubc7p because its lysine residues are inaccessible, then it may be that Ubc7p cross-linking to a degraded protein is contingent upon key lysine residues that serve as the ubiquitination sites within the target substrate. Previously in Chapters 3 and 4, we demonstrated that Hmg2p degradation required the presence of two critical lysine residues, 6 and 357, which may serve as the major sites for the attachment of ubiquitin to Hmg2p. By this simple model, elimination of these two critical lysine residues should abrogate Ubc7p crosslinking to Hmg2p. In fact, when the same Ubc7p cross-linking assay was performed with K6R, K357R-Hmg2p in place of normal Hmg2p, Ubc7p cross-linking was almost completely eliminated in the double mutant (Fig. 5-3c, "K6R, K357R" versus "wt"). In contrast, when the assay was performed with K6R-Hmg2p or K357R-Hmg2p, Ubc7p was capable of being cross-linked to each single mutant ("K6R" and "K357R"), although the cross-linking was moderately reduced. The results with each single mutant were not unexpected, as each single Hmg2p mutant demonstrated some small degree of ubiquitination on the remaining lysine residue (Chapter 4). Because the remaining critical lysine residue in each single mutant may be inefficiently ubiquitinated by Ubc7p, it would still be accessible to Ubc7p and able to serve in the formation of a covalent cross-link. Elimination of Ubc7p cross-linking by substitution of both lysine residues in Hmg2p supported the simple model that Ubc7p cross-linking was dependent upon the accessible lysine residues required for the covalent attachment of ubiquitin, although this is not the exclusive interpretation.

The fact that Ubc7p cross-linked to a target protein was indicative of an interaction between Ubc7p and that protein, and could be used as measure of Ubc7p

access to potential ubiquitination sites within a target protein. However, it is important to recognize that a lack of Ubc7p cross-linking does not necessarily imply the lack of an interaction between Ubc7p and the target protein. In the very least, it does indicate that the target protein does not have free, accessible lysine residues in proximity to Ubc7p.

Ubc7p cross-linking to Hmg2p was regulated by the mevalonate pathway.

We previously demonstrated that the ubiquitination of Hmg2p was regulated by the mevalonate pathway (Hampton and Bhakta, 1997). If drugs that slow synthesis of downstream products of the mevalonate pathway are added to cells, such as the HMG-R inhibitor lovastatin, Hmg2p ubiquitination is greatly reduced. In contrast, addition of the squalene synthase inhibitor zaragozic acid to cells results in a dramatic increase in Hmg2p ubiquitination. This is due to an increase in the cellular levels of farnesyl diphosphate, which is the substrate for squalene synthase (Hampton and Bhakta, 1997; and see Chapter 2). Because Hmg2p ubiquitination is regulated by the mevalonate pathway, we tested whether the cross-linking of Ubc7p to Hmg2p was also mevalonatepathway regulated. When cells were pre-incubated with the HMG-R inhibitor lovastatin, Ubc7p cross-linking to Hmg2p was greatly reduced (Fig. 5-4a, "Lov"). However, this could be due to the fact that lovastatin directly binds to Hmg2p and disrupts the cross-linking through a mechanism that is independent of mevalonate pathway production. Therefore, we tested the effect of the HMG-CoA synthase inhibitor L659,699 on Ubc7p cross-linking to Hmg2p. L659,699 has the same inhibitory effect on mevalonate pathway production and Hmg2p ubiquitination as lovastatin (Hampton and Bhakta, 1997), but does not bind to Hmg2p. Similar to

lovastatin addition, addition of L659,699 to cells resulted in greatly decreased Ubc7p cross-linking to Hmg2p (Fig. 5-4a, "L659"), indicating that Ubc7p cross-linking to Hmg2p was physiologically relevant. In contrast to lovastatin or L659,699, addition of zaragozic acid, which results in increased Hmg2p ubiquitination, resulted in a modest increase in Ubc7p cross-linking to Hmg2p (Fig. 5-4a, "ZA"). This increase was not as profound as the decrease observed with lovastatin or L659,699, but that could be due to the limited amount of time the cells were incubated with zaragozic acid. Long incubation periods (greater than 10 minutes) with zaragozic acid results in decreased Hmg2p steady-state levels due to enhanced degradation (Hampton and Bhakta, 1997), resulting in reduced amounts of immunoprecipitated Hmg2p. Therefore, our ability to determine the effect of zaragozic acid on Ubc7p cross-linking to Hmg2p was limited by the restrictions of the assay. Despite this limitation, the effects of the mevalonate pathway inhibitors on Ubc7p cross-linking to Hmg2p were consistent with their effects on ubiquitination.

It was possible that the cross-link reducing effects of the mevalonate pathway inhibitors lovastatin and L659,699 were a coincidence and these inhibitors actually disrupted Ubc7p cross-linking to all proteins rather than just specifically to Hmg2p. To test this possibility, we examined Ubc7p cross-linking to 6myc-Hmg2p, a mutant of Hmg2p subject to Ubc7p-dependent degradation that is not regulated by the mevalonate pathway (Hampton *et al.*, 1996a; N. Bays and R. Hampton, unpublished results). When 6myc-Hmg2p was immunoprecipitated from cells that were treated with DSP, Ubc7p cross-linked to 6myc-Hmg2p in a cross-linker concentration-dependent fashion (Fig. 5-4b, "no drug"), similar to Hmg2p. Pretreatment of the cells with either lovastatin or



Figure 5-4: Ubc7p cross-linking to Hmg2p was regulated by the mevalonate pathway.

Regulated Ubc7p cross-linking to Hmg2p was observed by pre-incubating the cells in either no drug, 25μ g/mlZA ("ZA") for 10 minutes, 50μ g/ml lovastatin for 2 hours ("Lov"), or 10μ g/ml L659,599 ("L659") for 2 hours prior to addition of DSP. Cross-linking assay was performed identical to that in figure 5-3. **a**) Cross-linking of Ubc7p to 1myc-Hmg2p was regulated by the mevalonate pathway. Addition of ZA increased Ubc7p cross-linking, while addition of either lovastatin or L659,699 decreased Ubc7p cross-linking. **b**) Cross-linking of Ubc7p to 6myc-Hmg2p was not regulated by the mevalonate pathway, consistent with the unregulated Ubc7p-dependent degradation of 6myc-Hmg2p. Addition of lovastatin or L659,699 had no effect on Ubc7p cross-linking to 6myc-Hmg2p.

L659,699 had no effect on Ubc7p cross-linking to 6myc-Hmg2p (Fig. 5-4b, "Lov" and "L659"). Thus, only Ubc7p cross-linking to Hmg2p was affected by the addition of mevalonate pathway inhibitors, indicating that the interaction between Ubc7p and Hmg2p revealed by cross-linking was physiologically relevant.

Ubc7p cross-linking to Hmg2p required the presence of Hrd1p, Hrd3p and Cue1p.

Hmg2p degradation occurs through the combined action of a collection of proteins (Hampton *et al.*, 1996a). From those identified proteins required for Hmg2p degradation, a subset have been shown to genetically and biochemically interact. That is, Hrd1p and Hrd3p form a complex (Gardner *et al.*, 2000), and the absence of Hrd3p results in the auto-ubiquitination and degradation of Hrd1p (Plemper *et al.*, 1999b; Gardner *et al.*, 2000). Furthermore, Hrd1p and Ubc7p interact and this interaction is required for the transfer of ubiquitin from Ubc7p to the substrate by the action of the ubiquitin ligase activity of Hrd1p (Bays *et al.*, 2000). Additionally, the ER membrane protein Cue1p is required for Ubc7p association with the ER membrane (Biederer *et al.*, 1997), implicating Cue1p as a potential member of the functional complex. Based upon these observations, Hrd1p, Hrd3p, Ubc7p and most likely Cue1p form a functional ER ubiquitin ligase complex.

If such a complex exists, it is likely that the absence of Hrd1p, Hrd3p or Cue1p would alter the interaction of Ubc7p with its target substrates. To test this idea, null alleles of the genes encoding these proteins were introduced into the strain that co-expressed the epitope-tagged versions of Hmg2p and Ubc7p. As expected, the presence of each of these null alleles resulted in complete stabilization of Hmg2p (Fig. 5-5a).

The effect of the null alleles was then assessed utilizing the cross-linking assay described above. Consistent with their effect on Hmg2p degradation, the presence of each null allele resulted in a drastic reduction in Ubc7p cross-linking to Hmg2p (Fig. 5-5b, " $hrd1\Delta$ ", " $hrd3\Delta$ ", and " $cue1\Delta$ " versus "wt"). In contrast, the presence of the hrd2-l allele, which encodes a disabled subunit of the 26S proteasome, had no effect on Ubc7p cross-linking to Hmg2p (Fig. 5-5b, "hrd2-l"). These results indicated that each protein of the ER ubiquitin ligase complex was required for the Ubc7p-Hmg2p interaction, as only mutant alleles of these genes and not mutant alleles of genes encoding proteins involved in downstream processes of Hmg2p degradation, affected this interaction.

Previously, it was demonstrated that Cue1p was required for Ubc7p association with the ER membrane and the absence of Cue1p resulted in greatly reduced levels of Ubc7p (Biederer *et al.*, 1997). Thus, one explanation for the reduced levels of Ubc7p cross-linking to Hmg2p was that the presence of these null alleles resulted in a reduced steady-state level of Ubc7p. We tested this by measuring the effect of the *hrd1* Δ , *hrd3* Δ , and *cue1* Δ alleles on the steady-state level and stability of Ubc7p. In strains with normal alleles of these genes, Ubc7p appeared to be quite stable, showing essentially no loss of protein over the 4 hour degradation period (Fig. 5-6a, "wt"). In both the *hrd1* Δ and *hrd3* Δ strains, Ubc7p showed a slight degree of degradation, but the Ubc7p steady-state level was unaltered (Fig. 5-6a, "*hrd1* Δ " and "*hrd3* Δ "). However, in the presence of the *cue1* Δ allele, Ubc7p degradation was dramatically increased and its corresponding steady-state level decreased (Fig. 5-6a, "*cue1* Δ "). Interestingly, Ubc7p had significantly greater steady-state levels in the *hrd2-1* strain (Fig. 5-6a, "*hrd2-1*"), indicating that the majority of synthesized Ubc7p in normal cells was degraded in a proteasome-dependent fashion. In fact, Cue1p bound a stoichiometric equivalent amount of Ubc7p in normal cells, thereby protecting only the pool of bound Ubc7p from proteasome degradation (R. Gardner and R. Hampton, unpublished observations). This was similar to Hrd3p control over Hrd1p degradation (Gardner et al., 2000).

Consistent with the degradation of Ubc7p, only the presence of the *cue1* Δ allele prevented Ubc7p from associating with the ER membrane (Fig. 5-6b, "*cue1* Δ "). All other *hrd* Δ alleles allowed a similar amount of Ubc7p ER-membrane association. From these observations, it appeared that only the *cue1* Δ allele affected Ubc7p cross-linking to Hmg2p through the mis-localization of Ubc7p, which resulted in enhanced proteasome-dependent degradation of Ubc7p. The *hrd1* Δ and *hrd3* Δ alleles did not alter Ubc7p localization and most likely altered Ubc7p cross-linking to Hmg2p through disruption of the functional E2/E3 complex.



Figure 5-5: *HRD*-gene encoded proteins were required for Ubc7p-Hmg2p cross-linking.

a) The appropriate null alleles of each gene required for Hmg2p degradation were introduced into the strain co-expressing 1myc-Hmg2p and 2HA-Ubc7p. Correct introduction of each null allele was determined by observation of 1myc-Hmg2p stabilization in a cycloheximide-chase assay, performed as in figure 5-2. Lysates from each indicated time point after addition of cycloheximide were prepared and immunoblotted to determine the level of 1myc-Hmg2p. The presence of each respective allele resulted in stabilization of 1myc-Hmg2p as expected. b) The presence of $cue1\Delta$, $hrd1\Delta$, and $hrd3\Delta$, but not hrd2-1, resulted in a drastic reduction of Ubc7p cross-linking to Hmg2p. Cross-linking assay was performed as previously described.



Figure 5-6: Presence of the *hrd1* Δ or *hrd3* Δ alleles did not alter Ubc7p degradation or membrane localization.

a) Cells expressing 2HA-Ubc7p and the indicated *hrd* allele were grown to log phase. Lysates from each indicated time point after addition of cycloheximide were prepared and immunoblotted to determine the level of 2HA-Ubc7p. 2HA-Ubc7p levels and degradation were unaffected in *hrd1* Δ or *hrd3* Δ cells, but 2HA-Ubc7p was rapidly degraded in *cue1* Δ cells. b) Membrane fractionation of the cells from (a) was performed by osmotically lysing cells and preparing a crude microsomal fraction. The ability of 2HA-Ubc7p to remain membrane bound was assayed under the varying conditions of either buffer, 2.5M NaCl, 2.5M urea, 0.8M potassium acetate (KOAc), pH11.6, or 1%Triton X100. Supernatant fractions are lanes denoted "S". Pellet fractions are lanes denoted "P".
Hrd1p cross-linked to both the stable Hmg1p and the degraded Hmg2p.

From the Ubc7p cross-linking results, it appeared that the entire functional ER ubiquitin ligase complex was required for an interaction between Ubc7p and Hmg2p. We next characterized the ability of the ubiquitin ligase Hrd1p to be cross-linked with Hmg2p. To do so, functional epitope-tagged versions of Hrd1p and either Hmg2p or Hmg1p were co-expressed in cells that were subject to the identical cross-linking assay as was performed with Ubc7p. When Hmg2p was immunoprecipitated from cells treated with increasing amounts of DSP, Hrd1p co-immunoprecipitated in a cross-linker concentration-dependent manner (Fig. 5-7, "Hmg2p"). It must be noted that a small amount of Hrd1p was co-immunoprecipitated in the absence of cross-linker. However, this amount was equivalent to the amount observed when the assay was performed with cross-linker but in the absence of Hmg2p-specific antibodies (Fig. 5-7, compare 0µg/ml DSP with 200µg/ml DSP + pre-sera), indicating that these were background levels of precipitated Hrd1p. In contrast to Ubc7p cross-linking, when Hmg1p was immunoprecipitated from cells treated with increasing amounts of DSP, Hrd1p was coimmunoprecipitated in a cross-linker concentration-dependent fashion (Fig. 5-7, "Hmg1p"). Thus, it appeared that Hrd1p was capable of being cross-linked to both degraded proteins and stable proteins.

The fact that Hrd1p cross-linked to both Hmg2p and Hmg1p indicated that the function of Hrd1p may be to scan all proteins in the ER to assess their degradation competency. If this were the case, then it might be expected that the observed Hrd1p cross-linking to Hmg2p would be unregulated by the mevalonate pathway. Indeed, when the same cross-linking assay was performed in the presence of either lovastatin or



Figure 5-7: Hrd1p cross-linked to both the degraded Hmg2p and the stable Hmg1p.

Strains expressing either 1myc-Hmg2p or 1myc-Hmg1p and co-expressing 3HA-Hrd1p were subject to the cross-linking assay as described in figure 5-3. 1myc-Hmg2p and 1myc-Hmg1p were detected using the 9E10 antibody and 3HA-Hrd1p was detected using the 12CA5 antibody. The lower immunoreactivity of 1myc-HMG-R in the 400 μ g/ml DSP lane is due to modification of the lysine residue in the myc epitope sequence, not reduced immunoprecipitation of HMG-R (data not shown). Pre-sera substituted for α -Hmg2p anti-sera in the indicated sample is indicated as "Pre".



Figure 5-8: Hrd1p cross-linking with Hmg2p was not regulated by the mevalonate pathway.

Lack of regulated Hrd1p cross-linking to Hmg2p was observed by pre-incubating the cells in either no drug, 50µg/ml lovastatin for 2 hours ("Lov"), or 10µg/ml L659,599 ("L659") for 2 hours prior to addition of DSP. Cross-linking assay was performed identical to that in figure 5-3. 1myc-Hmg2p was detected using the 9E10 antibody and 3HA-Hrd1p was detected using the 12CA5 antibody.

L659,699, Hrd1p cross-linking to Hmg2p was unaffected by these mevalonate pathway manipulations and was similar to that observed in the untreated cells (Fig. 5-8, "Lov" and "L659" versus "no drug"). Thus, not only did Hrd1p cross-link to both the degraded Hmg2p and the stable Hmg1p, but Hrd1p cross-linking to Hmg2p was not regulated by the mevalonate pathway. These results together supported a scanning mechanism for Hrd1p function.

Hrd1p cross-linking to either Hmg2p or Hmg1p was affected by the absence of Hrd3p, but not the absence of Ubc7p or Cue1p.

The prior results with Ubc7p indicated that the entire functional ubiquitin ligase complex was required for Ubc7p to cross-link with Hmg2p. We assessed if this were also the case for Hrd1p by introducing the appropriate null alleles of *hrd3*, *ubc7*, and *cue1* into the strains used to determine Hrd1p cross-linking. Introduction of these alleles resulted in stabilization of Hmg2p as expected (Fig. 5-9a). However, unlike the results seen with Ubc7p cross-linking, Hrd1p cross-linking to Hmg2p was drastically reduced only by the absence of *hrd3* (Fig. 5-9b, "*hrd3Δ*"), not by the absence of *ubc7* nor *cue1* (Fig. 5-9b, "*ubc7Δ*" and "*cue1Δ*"). The identical result was observed with Hrd1p cross-linking to Hmg1p (Fig. 5-9c). Accordingly, it appeared that the entire functional complex was not required for Hrd1p interaction with either Hmg2p or Hmg1p, only the presence of Hrd3p was a prerequisite.

It was previously demonstrated that the absence of Hrd3p results in the proteasome-dependent degradation of Hrd1p (Plemper *et al.*, 1999b; Gardner *et al.*, 2000). Therefore, the degradation of Hrd1p was determined in the strains carrying each

of the null alleles. Similar to the previously reported results, the presence of the $hrd3\Delta$ allele allowed the rapid degradation of Hrd1p, which resulted in a greatly reduced steady-state level of Hrd1p (Fig. 5-10, " $hrd3\Delta$ "). In contrast, the presence of either the $ubc7\Delta$ or the $cue1\Delta$ alleles had no effect on Hrd1p stability (Fig. 5-10, " $ubc7\Delta$ " and " $cue1\Delta$ "). Interestingly, these null alleles did result in the appearance of an increased molecular weight form of Hrd1p (Fig. 5-10, arrow), although the identity of the modification that created this slower migrating band is currently unknown.

The fact that Hrd3p was required for Hrd1p stability suggested that the reduced Hrd1p cross-linking to Hmg2p in the $hrd3\Delta$ strain was simply a consequence of the drastically reduced levels of the degraded Hrd1p. To test this possibility, we determined the cross-linking of Hrd1p to Hmg2p in strains that carried the $hrd3\Delta$ allele and either over-expressed Hrd1p or normally expressed the C399S-Hrd1p mutant described previously (Bordallo et al., 1998). Over-expression of Hrd1p allows the normal, regulated degradation of Hmg2p and the normal, constitutive degradation of CPY* in the absence of Hrd3p (Plemper et al., 1999b; Gardner et al., 2000), indicating that sufficient levels of Hrd1p can program the degradation of these substrates in the absence of Hrd3p. The C399S-Hrd1p mutant contains a point mutation in the RING-H2 finger domain and cannot effect the normal Hrd1p-dependent degradation of either Hmg2p or CPY* due to a loss in ubiquitin ligase function (Bordallo et al., 1998; Bays et al., 2000). However, because loss of the ubiquitin ligase function does not necessarily eliminate the substrate binding activity of ubiquitin ligases (Xie and Varshavsky, 1999), and because C399S-Hrd1p is stable in the absence of Hrd3p (Plemper *et al.*, 1999b;



Figure 5-9: Only the presence of Hrd3p was required for Hrd1p cross-linking to Hmg2p or Hmg1p.

a) The appropriate null alleles of each gene required for Hmg2p degradation were introduced into the strain co-expressing 1myc-HMG-R and 3HA-Hrd1p. Correct introduction of each null allele was determined by observation of 1myc-Hmg2p stabilization in a cycloheximide-chase assay, performed as in figure 5-2. Lysates from each indicated time point after addition of cycloheximide were prepared and immunoblotted to determine the level of 1myc-Hmg2p. The presence of each respective allele resulted in stabilization of 1myc-Hmg2p as expected. b) The presence of *hrd3A*, but not *cue1A* or *ubc7A*, resulted in a drastic reduction of Hrd3p cross-linking to Hmg1p. Cross-linking assays were performed as previously described in figure 5-7.

Hrd3p (Plemper *et al.*, 1999b; Gardner *et al.*, 2000), examination of cross-linking between C399S-Hrd1p and Hmg2p would also be instructive. When the cross-linking assay was performed on strains that either over-expressed the HA-epitope tagged version of Hrd1p or normally expressed HA-epitope tagged version of the C399S Hrd1p mutant, Hrd1p cross-linking to Hmg2p was now observed in both strains containing the modified *HRD1* alleles in the absence of Hrd3p (Fig. 5-11, "wt" compared with "C399S, *hrd3Δ*" and "P_{*TDH3*}, *hrd3Δ*"), although C399S-Hrd1p did show somewhat reduced levels of cross-linking ("C399S, *hrd3Δ*" versus "wt"). Thus, Hrd1p required the presence of Hrd3p for stability, which subsequently was required for Hrd1p to cross-link with Hmg2p under normal genetic conditions. However, Hrd1p cross-linking to Hmg2p did not absolutely require Hrd3p, as it could be observed under conditions in which Hrd3p was absent. This suggested that Hrd3p was not necessarily a requirement of the ER ubiquitin ligase complex, but was employed under normal conditions to maintain the stability of Hrd1p and possibly increase the efficiency of substrate binding.

In the previous section detailing Ubc7p interaction with Hmg2p, we observed that Ubc7p cross-linking to Hmg2p depended upon the presence of both Hrd3p and Hrd1p. However, because loss of Hrd3p was equivalent to loss of Hrd1p due to the drastically decreased stability of Hrd1p in the $hrd3\Delta$ strain, it was likely that Ubc7p did not cross-link to Hmg2p in the $hrd3\Delta$ strain due to the reduction of Hrd1p steady-state levels below a critical concentration. If this were the case, then the fact that Hrd1p cross-linked to Hmg2p in the absence of Hrd3p under certain genetic conditions might mean that Ubc7p would cross-link to Hmg2p in the absence of Hrd3p under the same



Figure 5-10: Absence of Hrd3p resulted in the rapid degradation of Hrd1p.

Strains used for the previous cross-linking assay in figure 5-9, which expressed 3HA-Hrd1p and contained the indicated null alleles, were subject to a cycloheximide-chase assay to determine Hrd1p stability. Lysates from the indicated time points after cycloheximide addition were prepared and immunoblotted to assess the levels of Hrd1p. As previously reported (Plemper *et al*, 1999b), Hrd1p was rapidly degraded in a $hrd3\Delta$ genetic background. However, the presence of either the *cue1* Δ or *ubc7* Δ alleles resulted in the appearance of a higher molecular weight form of Hrd1p (annotated with an arrow).



Figure 5-11: The presence of Hrd3p was not required for Hrd1p cross-linking to Hmg2p when levels of Hrd1p were elevated by over-expression or mutation.

Cells expressing 1myc-Hmg2p and co-expressing either 3HA-Hrd1p from its native promoter in the presence of the wild-type *HRD3* allele ("wt"), 3HA-Hrd1p from its native promoter in the presence of the *hrd3* Δ allele ("*hrd3* Δ "), 3HA-C399S-Hrd1p from its native promoter in the presence of the *hrd3* Δ allele ("C399S, *hrd3* Δ "), or 3HA-Hrd1p from the strong, constitutive *TDH3* promoter in the presence of the *hrd3* Δ allele ("P_{TDH3}, *hrd3* Δ ") were subject to the same cross-linking assay as performed in figure 5-9.

genetic conditions. Therefore, we tested the ability of Ubc7p to cross-link to Hmg2p in $hrd3\Delta$ cells when either when Hrd1p was over-expressed from the TDH3 promoter or when C399S-Hrd1p was expressed from the native *HRD1* promoter. When the crosslinking assay was performed on $hrd3\Delta$ cells co-expressing 2HA-Ubc7p, 1myc-Hmg2p and increased levels of Hrd1p, Ubc7p cross-linked to Hmg2p in a cross-linker, concentration-dependent manner similar to Ubc7p in a wild-type HRD1/HRD3 strain (Fig. 5-12, " P_{TDH3} , hrd3 Δ " compared with "wt"). In contrast, Ubc7p demonstrated limited cross-linking to Hmg2p in $hrd3\Delta$ cells expressing C399S-Hrd1p as the sole source of Hrd1p ("C399S, $hrd3\Delta$ "), and the level of cross-linking was not much greater than in $hrd3\Delta$ cells (" $hrd3\Delta$ "). In fact, Ubc7p cross-linking to Hmg2p was similarly limited in cells expressing C399S-Hrd1p and carrying the wild-type *HRD3* allele ("C399S, HRD3"). This result was not surprising as it has been shown that Ubc7p has limited interaction with Hrd1p when the RING-H2 finger motif is altered (Bays et al., 2000). Thus, although Hrd1p cross-linking to Hmg2p did not depend upon an intact RING-H2 finger, Ubc7p cross-linking to Hmg2p did require a fully functional RING-H2 finger motif of Hrd1p. However, the fact that Ubc7p cross-linked to Hmg2p in the absence of Hrd3p when Hrd1p was over-expressed indicated that the Ubc7p interaction with Hmg2p was absolutely dependent upon Hrd1p, but conditionally dependent upon Hrd3p.

Hrd3p also cross-linked to both the degraded Hmg2p and the stable Hmg1p.

Although it appeared that Hrd3p was not required for degradation of Hmg2p or Hrd1p cross-linking to Hmg2p under altered genetic conditions, it was quite possible



Figure 5-12: The presence of Hrd3p was not required for Ubc7p cross-linking to Hmg2p when levels of Hrd1p were elevated by over-expression, but not by mutation.

Cells expressing 1myc-Hmg2p, 2HA-Ubc7p and co-expressing either Hrd1p from its native promoter in the presence of the wild-type *HRD3* allele ("wt"), Hrd1p from its native promoter in the presence of the *hrd3* Δ allele ("*hrd3* Δ "), Hrd1p from the strong, constitutive *TDH3* promoter in the presence of the *hrd3* Δ allele ("*P*_{*TDH3*}, hrd3 Δ "), C399S-Hrd1p from its native promoter in the presence of the *hrd3* Δ allele ("*C399S*, *hrd3* Δ "), or C399S-Hrd1p from its native promoter in the presence of the *hrd3* Δ allele ("*C399S*, *hrd3* Δ "), or C399S-Hrd1p from its native promoter in the presence of the wild-type *HRD3* allele ("*C399S*, *HRD3*") were subject to the same cross-linking assay as performed in figure 5-3.

that Hrd3p played a more significant role in the ER ubiquitin ligase complex under normal genetic conditions than just the stabilization of Hrd1p. For instance, it was possible that over-expression of Hrd1p in the absence of Hrd3p resulted in recruitment of a cellular protein that functioned similar to Hrd3p and compensated for the absence of Hrd3p's necessary activity. In fact, we have already observed a similar scenario for over-expression of Hrd1p in the absence of Ubc7p, which resulted in the recruitment of Ubc1p, a ubiquitin conjugating enzyme that functioned similarly to the missing Ubc7p for Hmg2p degradation (Bays et al., 2000). Therefore, the results above do not necessarily indicate that Hrd3p is dispensable for recognition of Hmg2p or interaction with Hmg2p under normal genetic conditions. Furthermore, we have recently discovered that a mutant of Hrd3p that allows normal stabilization of Hrd1p, but does not allow ER degradation to proceed (Gardner et al., 2000). This indicated that Hrd3p does serve a significant function in ER degradation. For this reason, we performed a similar cross-linking assay as those used above to determine if Hrd3p could be crosslinked to Hmg2p or Hmg1p. Strains were constructed that expressed an HA-epitope tagged version of Hrd3p together with either the myc-epitope tagged Hmg2p or Hmg1p. When Hmg2p was immunoprecipitated from cells treated with increasing concentrations of DSP, Hrd3p was co-immunoprecipitated in a cross-linker, concentration-dependent manner (Fig. 5-13,"Hmg2p"). In a similar assay, Hrd3p was also cross-linked to Hmg1p in a cross-linker concentration-dependent fashion (Fig. 5-13,"Hmg1p"). Thus, Hrd3p cross-linked to both degraded proteins and stable proteins, similar to Hrd1p but unlike Ubc7p.

Hrd3p cross-linked to both Hmg2p and Hmg1p and this indicated that Hrd3p,

like Hrd1p, may function as part of a scanning mechanism in the ER to assess the degradation competency of all proteins. Again, if this were the case, then it is likely that Hrd3p cross-linking to Hmg2p would be unregulated by the mevalonate pathway. To test this, the same cross-linking assay was performed in the presence of either no drug, lovastatin or L659,699. Similar to Hrd1p, Hrd3p cross-linking to Hmg2p was unaffected by incubation with mevalonate pathway drugs and was similar under all conditions tested (Fig. 5-14). Thus, Hrd3p cross-linked to Hmg2p in a manner that was not regulated by the mevalonate pathway. Combined with the observation that Hrd3p also cross-linked to the stable Hmg1p, it appeared that Hrd3p, together with Hrd1p, acted as part of an ER ubiquitin ligase complex scanning for degradation substrates.

Hrd3p cross-linking to either Hmg2p or Hmg1p was unaffected by the absence of Hrd1p, Ubc7p, or Cue1p.

The studies with Ubc7p and Hrd1p indicated that the presence of particular proteins in the ER ubiquitin ligase complex was required to allow cross-linking to Hmg2p, or Hmg1p. We assessed if this were also the case for Hrd3p by introducing the appropriate null alleles of *hrd1*, *ubc7*, and *cue1* into the strains used to determine Hrd3p cross-linking in the above assays. Introduction of these alleles into the strains used for the Hrd3p cross-linking assay resulted in stabilization of Hmg2p as expected (Fig. 5-15a). However, unlike the results seen with Ubc7p cross-linking and Hrd1p cross-linking, Hrd3p cross-linking to Hmg2p was unaffected by the presence of either the *hrd1* Δ , *ubc7* Δ or *cue1* Δ alleles (Fig. 5-15b). The identical result was observed with Hrd3p cross-linking to Hmg1p (Fig. 5-15c). Thus, it appeared that Hrd3p did not

require any of these proteins to cross-link to Hmg2p or Hmg1p.

Because Ubc7p and Hrd1p steady-state levels and stability were determined by the presence of specific proteins, Cue1p and Hrd3p respectively, we determined the stability of Hrd3p in each of the strains carrying null alleles for *cue1*, *hrd1*, and *ubc7*. Hrd3p was stable in the presence of either *cue1* Δ , *hrd1* Δ , or *ubc7* Δ (Fig. 5-16), and this was consistent with the ability of Hrd3p to interact with target substrates in the presence or absence of each functional protein of the putative ER ubiquitin ligase complex.

A structural transition of the Hmg2p transmembrane domain is a precursor for Hmg2p recognition and subsequent degradation.

To this point, we have presented data that implicates a scanning mechanism for the ER E3 complex, in which the complex interacts with all accessible proteins, whether they are stable proteins or degradation substrates, and assesses their degradation competency. Hmg2p, a regulated degradation substrate, presents itself as a degradation substrate under the appropriate mevalonate pathway conditions, but acts as a stable protein when the signals for degradation are not sufficiently high. Because the ER E3 complex functions largely as a quality control apparatus destroying a variety of divergent mutant and/or misfolded proteins, it is likely that the complex recognizes intrinsic structural features within the degradation substrates that universally mark them as quality control targets. But how does Hmg2p enter this degradation pathway? Does Hmg2p present itself as a misfolded protein under conditions of high mevalonate pathway production? Is Hmg2p more tightly folded under mevalonate pathway conditions that promote stability? To address these questions, an examination of



Figure 5-13: Hrd3p cross-linked to both the degraded Hmg2p and the stable Hmg1p.

Strains expressing either 1myc-Hmg2p or 1myc-Hmg1p and co-expressing 3HA-Hrd3p were subject to the cross-linking assay as described in figure 5-3. 1myc-Hmg2p and 1myc-Hmg1p were detected using the 9E10 antibody and 3HA-Hrd3p was detected using the 12CA5 antibody. The lower immunoreactivity of 1myc-HMG-R in the 400 μ g/ml DSP lane is due to modification of the lysine residue in the myc epitope sequence, not reduced immunoprecipitation of HMG-R (data not shown).



Figure 5-14: Hrd3p cross-linking to Hmg2p was not regulated by the mevalonate pathway.

Lack of regulated Hrd3p cross-linking to Hmg2p was observed by pre-incubating the cells in either no drug, 50μ g/ml lovastatin for 2 hours ("Lov"), or 10μ g/ml L659,599 ("L659") for 2 hours prior to addition of DSP. Cross-linking assay was performed identical to that in figure 5-3. 1myc-Hmg2p was detected using the 9E10 antibody and 3HA-Hrd3p was detected using the 12CA5 antibody.



Figure 5-15: Hrd3p interaction with Hmg2p did not require the presence of the other *HRD* pathway proteins.

a) The appropriate null alleles of each gene required for Hmg2p degradation were introduced into the strain co-expressing 1myc-HMG-R and 3HA-Hrd3p. Correct introduction of each null allele was determined by observation of 1myc-Hmg2p stabilization in a cycloheximide-chase assay, performed as in figure 5-2. Lysates from each indicated time point after addition of cycloheximide were prepared and immunoblotted to determine the level of 1myc-Hmg2p. b) Neither the presence of $cue1\Delta$, $hrd1\Delta$, nor $ubc7\Delta$ altered Hrd3p cross-linking to Hmg2p. c) Neither the presence of $cue1\Delta$, $hrd1\Delta$, nor $ubc7\Delta$ altered Hrd3p cross-linking to Hmg1p. Cross-linking assays were performed as previously described in figure 5-13.



Figure 5-16: Hrd3p stability and steady-state levels were unaffected by the presence of either the *cue1* Δ , *hrd1* Δ , or *ubc7* Δ alleles.

Strains used for the previous cross-linking assay in figure 5-15, which expressed 3HA-Hrd3p and contained the indicated null alleles, were subject to a cycloheximide-chase assay to determine Hrd3p stability. Lysates from the indicated time points after cycloheximide addition were prepared and immunoblotted to assess the levels of Hrd3p. Hmg2p structure was required. Therefore, we explored ways in which we could manipulate Hmg2p structure biochemically and observe alterations in Hmg2p degradation behavior according to the predicted effect of the manipulation. In addition, we designed experiments that allowed us to probe for any changes in the structure of Hmg2p under varying mevalonate pathway conditions.

We first determined if Hmg2p degradation could be altered through biochemical manipulation of Hmg2p structure. To do so, we examined the effect of incubating cells with compounds that enhance protein folding on Hmg2p degradation. Chemical compounds that enhance protein folding, such as glycerol, are known as "chemical chaperones" (Welch and Brown, 1996). Direct alteration of protein folding and stability has been observed by addition of these compounds to cells. For example, addition of glycerol to cells protects them from the lethal effects of thermal denaturation of cellular proteins (Back *et al*, 1979; Lin *et al.*, 1981; Gekko and Koga, 1983; Henle *et al.*, 1983; Edington *et al.*, 1989). In a number of instances, the addition of glycerol to cells enhanced both the folding and stability of specific mutant or misfolded proteins (Brown *et al.*, 1996a, b, c; Sato *et al.*, 1996; Brown *et al.*, 1997). Because of the readily observable effects of glycerol on protein folding and stability, we assessed whether incubation of cells with glycerol had any stabilizing effect on Hmg2p degradation.

For a complete and quantitative analysis we used strains that expressed either the regulated degradation substrates 1myc-Hmg2p and Hmg2p-GFP or the constitutively degraded misfolded substrates 6myc-Hmg2p and 6myc-Hmg2p-GFP. When cells that expressed Hmg2p-GFP were incubated in glycerol, a concentration dependent increase in the cellular steady-state Hmg2p-GFP fluorescence was observed with the greatest effect, a 10-fold increase, resulting from incubation with 10% glycerol (Fig. 5-17a, Hmg2p-GFP, "+glycerol" versus "no drug"). The glycerol stabilizing effect was similar to the stabilizing effect observed when cells were incubated with lovastatin ("+glycerol" compared with "+Lovastatin"), indicating that stabilization of Hmg2p by incubation with glycerol was complete. Incubation of cells in glycerol also blunted the Hmg2p degradation-enhancing effect of zaragozic acid (Fig. 5-17b; "+glycerol" versus "no glycerol"), which allows increased degradation of Hmg2p by build-up of the mevalonate pathway product FPP (Hampton and Bhakta, 1997; see Chapter 2). Because glycerol reduced the effect of zaragozic acid, it appeared that Hmg2p was recognized for degradation by a mevalonate-pathway regulated change in the Hmg2p structure from a state that was tightly folded to a less folded state.

Other explanations for the glycerol effect were possible. For instance, it was possible that glycerol addition affected general ER degradation, and so we assessed the effect of glycerol addition on the steady-state level of 6myc-Hmg2p, a highly misfolded, mutant version of Hmg2p (Hampton *et al.*, 1996a). Only a modest increase in the steady-state fluorescence of the constitutively degraded, misfolded 6myc-Hmg2p-GFP was observed upon incubation of the cells with glycerol (Fig. 5-17a, "6myc-Hmg2p-GFP"). Alternatively, the glycerol effect observed with Hmg2p-GFP could also be the result of a global effect on increasing GFP fluorescence, so we assessed the effect of glycerol addition on the steady-state level of Hmg1p-GFP, a normally stable ER protein (Hampton *et al.*, 1996c). Again, addition of glycerol to cells resulted in only a modest increase the steady-state fluorescence of the stable protein Hmg1p-GFP ("Hmg1p-GFP").

These results implied that glycerol's effect was through stabilization of protein degradation, possibly by enhancing protein folding. To verify that glycerol directly affected degradation, we determine the effect of glycerol on the degradation of 1myc-Hmg2p and 6myc-Hmg2p by a cycloheximide-chase degradation assay. Incubation of cells in glycerol resulted in stabilization of 1myc-Hmg2p to a similar extent as incubation of cells with lovastatin (Fig. 5-17c, 1myc-Hmg2p: "glycerol" and "Lovastatin" versus "no drug"). In a similar experiment, the degradation of 6myc-Hmg2p was unaltered by incubation of cells with glycerol (Fig. 5-17c, 6myc-Hmg2p: "glycerol" versus "no drug"). This confirmed that the increases in Hmg2p steady-state levels due to incubation of cells with glycerol were the result of stabilization of Hmg2p degradation.

Because glycerol enhances the folding of proteins, it is reasonable to speculate that the glycerol-induced stabilization of Hmg2p was the result of altered recognition for degradation due to a tightly folded Hmg2p structure. Although the ER E3 complex interacts with both stable proteins and degradation substrates, it is capable of distinguishing between the two types of proteins by altering the degree of Ubc7p engagement. By the cross-linking assay described above, Ubc7p was the only component of the ER E3 complex that failed to cross-link with stable proteins. Furthermore, Ubc7p was the only complex protein that demonstrated an altered interaction with Hmg2p under stabilizing and degradation-enhancing conditions. From this, we postulated that Hmg2p cross-linked to Ubc7p by altering its structure to present critical lysine residues for ubiquitination by Ubc7p under appropriate mevalonate pathway conditions. If glycerol were affecting this structural transition, then we should see an alteration in Ubc7p cross-linking to Hmg2p. In fact, when the cross-linking assay was performed with cells incubated with glycerol, Ubc7p cross-linking to Hmg2p was reduced (Fig. 5-17d, "glycerol" versus "no drug"), and the reduction was similar in magnitude to that observed by incubation of cells with lovastatin ("glycerol" compared with "lovastatin"). Thus, glycerol blocked Ubc7p cross-linking to Hmg2p, most likely by maintaining the tightly folded structure of stable Hmg2p and preventing the transition to the less folded structure of degradation-competent Hmg2p.

The experiments with glycerol strongly implied that the Hmg2p structure was different between the stable state and the degradation-competent state, with the stable state having a more tightly folded structure. To determine if Hmg2p did have two different folding states, we examined if the structure of Hmg2p had altered sensitivity to exogenously added proteases under different mevalonate pathway conditions. We probed the protease sensitivity of Hmg2p by isolating microsomes from yeast cells that were treated with either no drug, lovastatin, which stabilizes Hmg2p by blocking production of the downstream signal required for degradation (see Chapter 2), zaragozic acid, which enhances Hmg2p degradation by allowing build-up of the signal for Hmg2p degradation (see Chapter 2), or glycerol. A similar assay was successfully employed to assess the structural differences between the wild-type form of CFTR and the rapidly degraded CFTR mutant, CFTR Δ F508, in mammalian cells (Zheng *et al.*, 1998).

When microsomes were isolated from cells treated with either no drug, lovastatin or zaragozic acid, a noticeable difference was observed in the proteolytic sensitivity of the zaragozic acid-treated cells compared to the no drug cells or the lovastatin-treated cells (Fig. 5-18, 8% gel: "Z" lane versus "nd" lane or "L" lane). The

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complete absence of the lower molecular weight fragments in the zaragozic acid-treated cells (15% gel, marked with brackets), with no corresponding appearance of higher molecular weight bands, suggested that they were further degraded to smaller fragments due to greater proteolytic sensitivity of Hmg2p from these cells. The fact that Hmg2p from cells treated with no drug had a proteolytic sensitivity more closely resembling that of Hmg2p from cells treated with lovastatin is not surprising, in that Hmg2p is fairly stable under the normal growth conditions and a large portion of its pool would exist in stable structure. The change in Hmg2p proteolytic sensitivity seen with the zaragozic acid-treated cells was blocked by pre-incubation of these cells in lovastatin prior to addition of zaragozic acid ("ZL" lane), indicating that the effect was regulated by upstream blockade of the mevalonate pathway. More importantly, the change in Hmg2p proteolytic sensitivity seen with the zaragozic acid-treated cells was also blocked by co-incubation of the cells with glycerol ("ZG" lane), demonstrating that the structural transition of Hmg2p from cells treated with zaragozic acid was a folding transition that was prevented from occurring by addition of the chemical chaperone.

Together, these results demonstrated that Hmg2p underwent a mevalonate pathway-regulated structural transition from a stable state to a degradation-competent state. The stable state was less sensitive to exogenously added proteases than the degradation-competent state. Furthermore, because the chemical chaperone glycerol stabilized Hmg2p and prevented the greater proteolytic sensitivity, it appeared that the degradation-competent state was more loosely folded and resembled that of a misfolded quality control substrate.



a) Incubation of cells with glycerol increased the steady-state levels of Hmg2p-GFP. Cells expressing the indicated HMG-R-GFP variant were grown in minimal media to mid log-phase in the presence or absence of glycerol (final concentration 10%). Lovastatin (final concentration 25μ g/ml) was added to the indicated sample and all samples were incubated at 30°C for an additional 4 hours. Steady-state GFP fluorescence was analyzed by flow cytometry. b) Glycerol blunted the degradation enhancing effect of zaragozic acid. Cells expressing Hmg2p-GFP were grown in minimal media to mid log-phase. Zaragozic acid (final concentration 10μ g/ml) and/or glycerol (final concentration 10%) were added to the indicated samples, which were incubated at 30°C for an additional 4 hours. Steady-state GFP fluorescence was analyzed by flow cytometry. c) Glycerol stabilized 1myc-Hmg2p, but not 6myc-Hmg2p. Cells expressing either 1myc-Hmg2p or 6myc-Hmg2p were grown in minimal media to mid log-phase. Glycerol ("glycerol", final concentration 15%) or lovastatin ("lovastatin", final concentration $25\mu g/ml$) was added to the indicated samples and the cells were incubated at 30°C for 4 hours. Cells were then subjected to a cycloheximidechase assay. Lysates were prepared at the indicated time points after cycloheximide addition and immunoblotted with the 9E10 antibody to assess the levels of myc-HMG-R. d) Glycerol blocked Ubc7p cross-linking to Hmg2p. Cells co-expressing 1myc-Hmg2p and 2HA-Ubc7p were grown in minimal media to mid log-phase. Cells were treated with either no drug ("no drug"), 15% glycerol ("glycerol"), or 25mg/ml lovastatin ("lovastatin") for 2 hours at 30°C. A similar cross-linking assay was performed as in figure 5-3.



Figure 5-18: The Hmg2p transmembrane domain underwent a structural transition from a stable state to a degradation-competent state.

Limited proteolytic analysis of intact microsomes containing 1myc-Hmg2p revealed an altered sensitivity to trypsin when cells were incubated with zaragozic acid. Cells expressing 1myc-Hmg2p were grown to mid log-phase in minimal media. The cells were treated with either no drug ("nd"), lovastatin ("L", 100μ g/ml final concentration), zaragozic acid ("Z", 10μ g/ml final concentration), or glycerol ("G", 15% final concentration) for 2 hours at 30°C. Intact, isolated microsomes were prepared from these cells. Separate aliquots of isolated microsomes were treated with 0.5μ g/ml trypsin for 30 min at 0°C in the absence of detergent. The degree of protease digestion was observed by immunoblotting with a polyclonal antisera specific for Hmg2p sequences located in both the lumen and cytosol. Full length Hmg2p is indicated with an arrow. Lower molecular weight bands absent in zaragozic acid-treated cells are indicated with the two different types of brackets.

Discussion

The ER-associated proteins Hrd1p, Hrd3p, Ubc7p and Cue1p are required for the ER-associated, proteasome-dependent degradation of both ER lumenal proteins, such as CPY* (Biederer et al., 1997; Hiller et al., 1996; Bordallo et al., 1998; Plemper et al., 1999), and ER membrane proteins, such as Hmg2p (Hampton et al., 1996; Hampton and Bhakta, 1997; this work). The protein Ubc7p is a ubiquitin conjugating enzyme (E2) that functions in the conjugative attachment of ubiquitin to target substrates (Chen et al., 1993; Biederer et al., 1996; Hiller et al., 1996; Hampton and Bhakta, 1997). Cue1p binds Ubc7p and promotes its ER-membrane association (Biederer *et al.*, 1997). Hrd1p possesses a RING-H2 finger domain that is highly homologous with other known ubiquitin ligase proteins (Hampton et al., 1996; Lorick et al., 1999; Bays et al., 2000), and interacts with Ubc7p to promote E2-dependent ubiquitination of substrates through the ubiquitin ligase activity of its conserved RING-H2 finger domain (Bays et al., 2000). Hrd3p shares homology with other identified proteins (Hampton et al., 1996), but its only known function is interaction with and stabilization of Hrd1p (Plemper et al., 1999; Gardner et al., 2000). Together, these proteins form a functional ubiquitin ligase complex required for the ER-associated targeting and ubiquitination of both ER lumenal and ER membrane proteasomedependent degradation substrates (Bays et al., 2000; Gardner et al., 2000). However, the actual targeting mechanism used by this complex is not known.

We have employed an *in vivo* chemical cross-linking assay to determine how these proteins interact with a target substrate. From our analyses, we have determined that the complex employs a quality control scanning mechanism that inspects all accessible ER proteins to determine if they are degradation competent. Hrd1p and Hrd3p function in such a scanning mechanism in the absence of Ubc7p or Cue1p, and thus appear to form the core of the complex. Ubc7p did not interact with target substrates or ubiquitinate target substrates in the absence of Hrd1p or Hrd3p (Bays *et al.*, 2000; this work), further indicating that these two proteins functioned together as the core ubiquitin ligase complex.

The scanning mechanism of the ER ubiquitin ligase complex.

The minimal components of the ER ubiquitin ligase complex are Hrd1p, Hrd3p, Ubc7p and Cue1p. A model of the organization of the complex and its action in Hmg2p degradation is depicted in figure 5-19. Hrd1p, Cue1p and Ubc7p are all primarily cytosolic (Biederer *et al.*, 1997; Bays *et al.*, 2000), although Hrd1p and Cue1p both contain ER-membrane spanning domains that anchor them to the ER and present small regions of sequence to the ER lumen (Hampton *et al.*, 1996; Biederer *et al.*, 1997). The RING-H2 finger domain of Hrd1p, which is required for its ubiquitin ligase activity (Bays *et al.*, 2000), is present in the cytosol and in the same compartment as the ubiquitin conjugating enzyme Ubc7p (Gardner *et al.*, 2000). In contrast, Hrd3p is predicted to be almost entirely lumenal (Hampton *et al.*, 1996; Saito *et al.*, 1999; Plemper *et al.*, 1999; Bays *et al.*, 2000). Together, the complex has sequences on both sides of the ER membrane.

Figure 5-19: Model for Hmg2p ubiquitination by the ER E2/E3 complex.

Top panel: Functional ER E2/E3 complex binds Hmg2p. However, under conditions of low mevalonate pathway production, critical lysine residues Lys6 and Lys357 are not accessible. Thus, Hmg2p is released without the covalent attachment of ubiquitin to these lysine residues. **Bottom panel:** Functional ER E2/E3 complex binds Hmg2p. In this case, conditions of high mevalonate pathway production are present within the cell and the critical lysine residues Lys6 and Lys357 are now accessible. Ubc7p, with the aid of Hrd1p, catalyzes the attachment of ubiquitin chains to these lysine residues. Ubiquitinated Hmg2p is released with the covalent attachment of multi-ubiquitin chains to its critical lysine residues. This form of Hmg2p is recognized by the proteasome and subsequently degraded.

What might the mechanism be for the ER ubiquitin ligase complex? From the chemical cross-linking results, it appeared that the ubiquitin ligase complex interacted with both stable and targeted degradation substrates. This suggested that the complex actively scanned all accessible proteins to assess their degradation competency. Thus, we propose in our current model that the complex inspects all proteins with which it comes into contact, whether they are stable proteins or proteins destined for degradation. This interaction would position Ubc7p in a correct orientation with the substrate to allow subsequent ubiquitination of those proteins that are to be degraded.

The fact that only Ubc7p cross-linked with degradation substrates and not stable proteins leads to two possible models for the mechanism of Ubc7p orientation with Hrd1p/Hrd3p-bound substrate. First, it is possible that Hrd1p/Hrd3p core complex undergoes an allosteric alteration only upon the binding of degradation substrates. The structural alteration subsequently brings Ubc7p into a correct orientation with the substrate, thereby allowing a detectable interaction and substrate ubiquitination. Binding of stable proteins would not induce such a change and therefore would not result in a detectable interaction with Ubc7p. Alternatively, it is possible that the Ubc7p position with the complex is fixed and the complex does not undergo any structural alteration. Instead, the complex distinguishes degradation substrates from stable proteins by accessibility of lysine residues, which are used as the sites for the covalent attachment of ubiquitin (Finley and Chau, 1991; Jentsch, 1992). Those proteins that are to be degraded would have lysine residues accessible to Ubc7p in order for the substrates to be covalently tagged with ubiquitin. As DSP is a chemical cross-linker that reacts with the primary amines in the side-chains of lysine residues, this would

result in an interaction that could be readily detectable by DSP cross-linking. Conversely, stable proteins may restrict surface access of their lysine residues from Ubc7p to prevent the aberrant ubiquitination of these residues. Thus, it may be that Ubc7p interacts with stable proteins, but cannot ubiquitinate them due to restricted access of the lysine residues. The interaction of Ubc7p with stable proteins would also not be detectable by DSP cross-linking.

In this regard, the regulated cross-linking of Ubc7p to Hmg2p may be instructive. That is, degradation of Hmg2p requires two critical lysine residues, Lys6 and Lys357, for Ubc7p-dependent ubiquitination under the appropriate mevalonate pathway conditions (Chapter 3 & 4). It is quite conceivable that these lysine residues serve as the major ubiquitin attachment sites in Hmg2p and would, by necessity, be within close contact with Ubc7p under conditions that allow Hmg2p degradation. Cross-linking of Ubc7p to Hmg2p is similarly regulated by the mevalonate pathway, supporting the idea that the lysine residues required for cross-linking are accessible under some mevalonate pathway conditions but not others. Interestingly, both Lys6 and Lys357 were required for Ubc7p cross-linking to Hmg2p in addition to Hmg2p ubiquitination. Thus, Ubc7p cross-linking to Hmg2p appeared to utilize the same lysine residues as those essential for Hmg2p ubiquitination. By extension, stable proteins most likely do not present accessible lysine residues to Ubc7p and would not be able to form DSP-dependent cross-links with Ubc7p. Accordingly, Ubc7p, as part of an ubiquitin ligase complex, may interact with stable proteins, but this interaction would not be revealed through the DSP cross-linking assay. When we sufficiently altered the structure of the stable Hmg1p so that it was subsequently a Ubc7p-dependent

degradation substrate, Ubc7p cross-linked to this degradation-competent target substrate, indicating that stable proteins can cross-link to Ubc7p if they are altered to become degradation substrates without adding any lysine residues. Alternatively, it may be that the interaction of only recognized degradation substrates results in an alteration of the Hrd1p/Hrd3p complex structure such that Ubc7p is brought into correct position for substrate ubiquitination. Further studioes on the ER E3 complex will delinieate these possibilities.

To interact with such a diverse array of proteins, the complex must have a general binding mechanism. We cannot rule out that the complex recognizes specific sequences in each protein that program its degradation, such as degrons (Varshavsky, 1991), but no consensus sequence has been revealed through our identification attempts by mutagenesis or computer analysis (Chapter 3; R. Gardner and R. Hampton, unpublished observations). In fact, our recent analysis of the Hmg2p sequence suggests that the complex recognizes structural cues within degraded proteins rather than specific sequences (Chapter 3). To allow recognition of common structural features between a variety of proteins, perhaps a protein in the complex has a similar binding site as a chaperone. It is quite possible that the substrate scanning site is in the lumen of the ER, where both lumenal proteins and ER membrane proteins would exist. In this regard, it is interesting to note that the degradation of CPY*, a lumenal substrate targeted by this complex, appears to employ the activity of the ER chaperone Kar2p (Plemper et al., 1997). However, Kar2p may serve in the formation of the ubiquitin ligase complex and not as an integral complex protein. Hrd3p is the only protein of the established ER ubiquitin ligase complex that is almost entirely lumenal (Hampton et al., 1996; Plemper

et al., 1999; Bays *et al.*, 2000), and may be a likely candidate for such a chaperone-like binding activity. From our cross-linking data, it appeared that Hrd1p could interact with degradation substrates in the absence of Hrd3p, implying that a portion of substrate recognition is encoded by the ubiquitin ligase protein, as would be expected. However, Hrd3p could interact with all tested substrates in the absence of the other complex components, and the presence of Hrd3p increased the efficiency of Hrd1p-substrate interaction. This hinted that Hrd3p may serve a primary role in substrate recognition. In fact, the function of substrate recognition may be encoded by the Hrd3p N-terminal region (residues 1-390), as the loss of this region still allowed normal Hrd1p stabilization, but did not allow ER degradation to proceed (Gardner *et al.*, 2000). Continued analysis of Hrd3p function will help identify any further role it might have in ER degradation.

A structural transition may enhance Hmg2p susceptibility for degradation.

Hmg2p is not degraded constitutively, as are other ER degradation substrates (Finger *et al.*, 1993; Biederer *et al.*, 1996; Plemper *et al.*, 1998). Rather, Hmg2p is subject to degradation depending upon the physiological state of mevalonate pathway production in the cell (Hampton and Rine, 1994; Chapter 2). How is Hmg2p recognized as a degradation substrate under certain mevalonate pathway conditions, but left as a stable protein under other conditions? From our proteolytic sensitivity glycerol addition experiments, it appears that the Hmg2p transmembrane domain undergoes a structural transition from a stable, tightly folded state, or "closed" state, to a less folded state, or "open" state, when signals from downstream in the mevalonate pathway are abundant. The acquisition of the "open" state would result in an Hmg2p molecule that looks misfolded, thus acquiring the ability to be recognized for degradation by the ER E3 complex. Accordingly, the structure of the "open" state may likely contain similar structural elements that the ER ubiquitin ligase complex uses to recognize constitutive ER-degradation substrates.

The mutagenesis of the Hmg2p transmembrane domain conducted in Chapter 3 also strongly supports that Hmg2p degradation proceeds by structural alteration of this domain. Numerous structural elements were discovered within this domain that were required for normal Hmg2p regulated degradation. Mutations that abolished these structural elements often had drastic consequences for Hmg2p degradation including stability, poorer recognition of degradative signals, or enhanced recognition of degradative signals. Thus, it is quite possible that physiological control of Hmg2p degradation is brought about by induced changes in the structure of the Hmg2p transmembrane domain, perhaps of a similar magnitude to those we introduced by mutation.

Implications for the quality control scanning ER ubiquitin ligase complex: conservation among RING-H2 finger ubiquitin ligase complexes?

The structure of the ER ubiquitin ligase complex and its mechanism for ubiquitination closely resembles that of other ubiquitin ligase complexes, most notably the well-defined SCF complex (Kamura *et al.*, 1999; Ohta *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999). A schematic representation comparing the ER complex and the SCF complex is shown in figure 5-20. Both complexes parallel each other by



Figure 5-20: Comparison of cellular E2/E3 complexes.

Functional similarities are observed between all three known RING-H2 finger containing E3 complexes. All utilize a specific E2 (Cdc34p, Ubc7p or Ubc2p) and a specific RING-H2 finger E3 (Rbx1p, Hrd1p or Ubr1p). Parallel components to the SCF substrate binding protein Cdc4p or Grr1p are the substrate-binding portion of Ubr1p in the N-end rule complex and possibly Hrd3p in ER complex. Parallel components to the SCF scaffold protein Cdc53p are the Ubc2p-binding region of Ubr1p in the N-end rule complex and possibly Cue1p in the ER complex. The component comprising the linker protein Skp1p in the SCF complex may similarly exist as part of Hrd1p in the ER complex.

participation of a specific E2 and involvement of a RING-H2 finger-containing protein, both of which are required for ubiquitin ligase activity (Kamura et al., 1999; Ohta et al., 1999a; Seol et al., 1999; Skowyra et al., 1999; Bays et al., 2000). Although the substrate binding component of the SCF complex has been identified as the F-box proteins Cdc4p or Grr1p (Feldman et al., 1997; Skowyra et al., 1997; Kishi et al., 1998; Galan and Peter; 1999), the parallel component of the ER complex has not been conclusively determined. Whether Hrd3p serves in this capacity or is only required for Hrd1p stability is currently under investigation. However, it is intriguing that absence of Cdc4p prevented all other subunits of the SCF complex from binding to the substrate Sic1p (Feldman *et al.*, 1997), and the absence of Hrd3p similarly prevented all other subunits of the ER complex from binding to Hmg2p. The SCF complex requires the protein Skp1p to bridge a connection between the substrate binding proteins Grr1p or Cdc4p and the E2 complex of Cdc53p and Cdc34p. A similar component has not been identified for the ER complex. Hrd3p interacts with the membrane binding domain of Hrd1p (Gardner et al., 2000), which is an additional domain of the RING-H2 finger component of the ER complex that is not observed in other RING-H2 finger proteins. Perhaps this additional domain serves a similar function as Skp1p. In fact, multiple domains contained within a single protein of a ubiquitin ligase complex may have precedent. The ubiquitin ligase complex required for degradation of N-end rule substrates appears to have a similar architecture as the other ubiquitin ligase complexes, but contains fewer proteins (Madura et al., 1993). The N-end rule complex utilizes the E2 Ubc2p (Dohmen et al., 1991; Madura et al., 1993), and the RING-H2 finger protein Ubr1p (Madura et al., 1993; Xie and Varshavsky, 1999). Ubr1p is a very large protein

that also contains multiple substrate binding domains (Reiss *et al.*, 1988; Gonda *et al.*, 1989; Bartel *et al.*, 1990; Baker and Varshavsky, 1991) and an E2 binding domain (Madura *et al.*, 1993; Watkins *et al.*, 1993; Xie and Varshavsky, 1999). The presence of such domains would obviate the need for separate substrate binding proteins, such as Cdc4p or Grr1p of the SCF complex, or separate E2 binding proteins, such as Cdc53p of the SCF complex or Cue1p of the ER complex. Thus, the N-end rule complex may be constructed in a similar fashion as the SCF complex or the ER complex (Fig. 5-20), indicating an evolutionary conservation among such ubiquitin ligases.

A very intriguing similarity between both the SCF complex and the ER complex is that individual components of each complex are subject to auto-ubiquitination and subsequent proteasome-dependent degradation. The SCF complex E2, Cdc34p, is subject to auto-ubiquitination and degradation (Seol et al., 1999; Skowyra et al., 1999). Similarly, the ER complex E2, Ubc7p, also undergoes auto-ubiquitination and proteasomal dependent degradation (Biederer et al., 1997; R. Gardner and R. Hampton, unpublished observations). In addition, the F-box proteins of the SCF complex Grr1p and Cdc4p, which are required for substrate binding (Feldman et al., 1997; Skowyra et al., 1997), are both intrinsically unstable and degraded in a ubiquitin-dependent manner in the cell (Galan and Peter, 1999). In the ER complex, the RING-H2 finger-containing component, Hrd1p, is degraded in a ubiquitin-proteasome dependent fashion in the absence of Hrd3p (Plemper et al., 1999; Bays et al., 2000; Gardner et al., 2000). Furthermore, only the amount of Hrd1p that is equivalent to the cellular amount of Hrd3p is spared from degradation, as a degradation impaired mutant of Hrd1p is present at much higher levels in the cell when expressed from the same promoter in the

presence of Hrd3p (Gardner *et al.*, 2000). Similarly the RING-H2 finger protein of the SCF complex, ROC1, requires the interaction with CUL1/CDC53 to prevent its proteasome-dependent degradation (Ohta *et al.*, 1999b). Thus, some components of both the SCF complex and the ER complex are auto-ubiquitinated and degraded in a proteasome-dependent manner, indicating that this may be a common theme among E2/E3 complexes.

Continued studies with ubiquitin ligase complexes should provide additional insight into their targeting and ubiquitination mechanisms. It is expected that further similarities between the ER complex and other such complexes will be revealed. However, the ER complex may provide particularly useful insights into the process of general proteasome-dependent protein degradation in that it is designed to recognize a variety of divergent degradation substrates, whereas other known ubiquitin ligase complexes function only in the recognition of very specific and select substrates.

Acknowledgements

For the results encompassed by this chapter, I was the primary researcher and Dr. Randolph Hampton directed and supervised the research. Alex Shearer conducted the original pilot experiments on the effect of glycerol addition on Hmg2p degradation. The steady-state GFP data in figure 5-17 are the direct result of his experimentation.

Chapter VI: Conclusions

That the self advances and confirms the myriad things is called delusion. That the myriad things advance and confirm the self is called enlightenment.

--Dogen Zenji
ER-associated protein degradation: A current view

The body of work contained in this dissertation was initiated when our general understanding of ER-associated degradation was in a nascent state. Beyond identification of the proteasome as the major cellular protease involved in ERassociated degradation (Ward *et al.*, 1995; Biederer *et al.*, 1996; Hampton *et al.*, 1996a; Hiller *et al.*, 1996; Hughes *et al.*, 1997; Qu *et al.*, 1996; Fisher *et al.*, 1997; Yu *et al.*, 1997; Plemper *et al.*, 1998), little was known about the selection and targeting of proteins in the ER destined for destruction, including a paucity of knowledge about the proteins in the ER required to effect ER-associated degradation.

During the course of this research, ER molecular chaperones have been implicated in some cases as part of the selection mechanism (McCracken and Brodsky, 1996; Plemper *et al.*, 1997; de Virgilio *et al.*, 1998; Brodsky *et al.*, 1999), but the degree of their involvement and the mechanism by which they function in ERassociated degradation is still unclear. It is possible that these chaperones act as the initial mode of recognition, binding to a misfolded or mutant protein in an attempt to correctly fold the protein. Prolonged interaction with the chaperone may result in its delivery to the retro-translocation/ubiquitin ligase machinery of the ER. In one case, it has been demonstrated that interaction of the degradation substrate with a chaperone leads to the ubiquitination of the chaperone itself, thereby leading to destruction of the substrate (Qu *et al.*, 1996).

The main targeting mechanism that initiates cellular proteasome-dependent degradation is the covalent attachment of ubiquitin to the selected substrate (Finley and Chau, 1991; Jentsch, 1992). Over the period of this work, post-translational

ubiquitination as a prerequisite for proteasome-dependent, ER-associated degradation has been demonstrated for numerous ER substrates (Ward *et al.*, 1995; Hiller *et al.*, 1996; Biederer *et al*; 1997; Hampton and Bhakta, 1997; Yang *et al.*, 1998; Wilhovsky *et al.*, 2000). Ubiquitination at the co-translational level has also been demonstrated for select substrates (Liao *et al.*, 1998; Zhou *et al.*, 1998). Whether ubiquitination at the cotranslational level and the post-translational level occur by different mechanisms is currently unknown, as the proteins required for such processes have yet to be elucidated.

Ubiquitination of a target substrate is usually carried out by a specific collection of proteins that comprise a functional ubiquitin ligase complex (Hochstrasser, 1996). In yeast cells, an ER-associated ubiquitin ligase complex has been discovered for the first time by our group (Hampton *et al.*, 1996a; Bays *et al.*, 2000; Gardner *et al.*, 2000), and this complex shares many similarities with other known ubiquitin ligase complexes (Madura *et al.*, 1993; Ohta *et al.*, 1999a; Ohta *et al.*, 199b; Seol *et al.*, 1999; Skowyra *et al*, 1999; Xie and Varshavsky *et al.*, 1999). However, this complex is not required for the degradation of many ER-associated substrates (Hill and Cooper, 2000; Wilhovsky *et al.*, 2000), indicating that other ER ubiquitin ligase complexes are likely to exist.

Despite these impressive and detailed analyses, the actual mechanism of degradation substrate targeting in the ER remained elusive. The results presented in Chapter 5 represent the first detailed examination of the targeting process and provide valuable insights into how the ER ubiquitin ligase selects degradation substrates and distinguishes them from otherwise stable proteins. This work should serve as a sound base for further exploration of the targeting and selection process.

Eukaryotic conservation of HMG-R degradation.

A great effort has been expended upon elucidating the mechanism of the ERassociated, mevalonate pathway-regulated degradation of HMG-R for its value in understanding both the details of ER-associated degradation and this clinically and physiologically important mode of cellular cholesterol regulation. The early, and still continuing, studies of HMG-R degradation were conducted using mammalian cell systems to identify the molecular signals that control the rate of HMG-R degradation (Edwards *et al.*, 1983; Nakanishi *et al.*, 1988; Chun *et al.*, 1990; Meigs and Simoni, 1992; Meigs *et al.*, 1996; Meigs and Simoni, 1997). However, although mammalian cell systems intimately approximate human systems, the limitations of mammalian cell systems has thus far stymied the identification of the molecular components that effect the regulated destruction of HMG-R, except for elucidation of the proteasome as the protease involved in HMG-R degradation (Inoue *et al.*, 1991; McGee *et al.*, 1996).

Recent studies of HMG-R degradation in yeast have demonstrated significant similarities to the analogous process in mammalian cells. Such similarities include: degradation without exit from the ER (Chun *et al.*, 1990; Meigs and Simoni, 1992; Hampton and Rine, 1994), mevalonate pathway feedback regulation of HMG-R degradation by both an isoprenoid-derived signal and an oxysterol-derived signal (Chin *et al.*, 1985; Panini *et al.*, 1992; Roitelman *et al.*, 1992; Correll and Edwards, 1994; Hampton and Rine, 1994; Meigs *et al.*, 1996; Hampton and Bhakta, 1997; Chapter 2), the necessity and sufficiency of the HMG-R N-terminal transmembrane domain for regulated degradation (Gil *et al.*, 1985; Nakanishi *et al.*, 1988; Hampton and Rine, 1994; Hampton *et al.*, 1996b), and involvement of the proteasome in HMG-R degradation (Inoue *et al.*, 1991; Hampton and Rine, 1994; Hampton *et al.*, 1996a; McGee *et al.*, 1996). In contrast to mammalian cells, the studies in yeast have identified many of the protein components that program the regulated degradation of HMG-R (Hampton *et al.*, 1996a; Bays *et al.*, 2000).

This conservation of HMG-R degradation between mammalian cells and yeast holds the promise of discovering the underlying molecular mechanism for HMG-R degradation in the more genetically tractable organism yeast. The acquired knowledge from yeast may then serve as a basis to focus the experimentation in mammalian cells. In fact, our studies have already provided a solid foothold on the identity and mechanism of the ER-associated, ubiquitin ligase complex that mediates HMG-R degradation in yeast (Hampton *et al.*, 1996a; Hampton and Bhakta, 1997; Bays *et al.*, 2000; Gardner *et al.*, 2000). As a number of these components have homologues in mammalian cells (Donoviel *et al.*, 1998; Katsanis and Fisher, 1998; Lin *et al.*, 1999), it is likely that mammalian HMG-R will utilize a highly similar machinery for its own targeting and selection.

Continuing studies in yeast on the function and mechanism of the ER ubiquitin ligase and the retro-translocation process required for removal of degradation substrates from the ER will further cement the foundation for an understanding of the specific mode of HMG-R degradation, and ER-associated degradation in general. These future studies will no doubt usher in a new era of depth and knowledge in the process of mammalian HMG-R degradation and may lead to new clinical therapies to control cholesterol production and heart disease. The insights provided by the studies in yeast ER-associated degradation may also aid in an understanding of diseases resulting from aberrant, ER-associated degradation in humans, such as cystic fibrosis, panlobular emphysema and some neurodegenerative disorders (Carlson *et al.*, 1989; Kerem *et al.*, 1989; Ciccarelli *et al.*, 1993; Ward et al., 1995; Qu *et al.*, 1996; Johnston et al., 1998), thereby providing better clinical therapies for treatment of such disorders.

A current model for HMG-R degradation in yeast.

The bulk of the work during my dissertation research has focused on elucidating the molecular mechanism for the degradation of the yeast HMG-R isozyme Hmg2p. I have detailed in Chapter 2 a genetic and pharmacological dissection of the mevalonate pathway derived molecules that act as positive signals for Hmg2p degradation. In Chapter 3, I demonstrated that Hmg2p utilizes the entire structure of its N-terminal transmembrane domain to sense these signals and alter its structure accordingly. This alteration in structure allows two critical lysine residues, Lys6 and Lys357, to function in Hmg2p ubiquitination, which I examined in Chapter 4. My work culminated with Chapter 5, were I explored the mechanism by which the ER ubiquitin ligase complex recognizes proteins destined for destruction. From these results, it appeared that the ER ubiquitin ligase actively scans all accessible ER proteins and ubiquitinates only those proteins that expose lysine residues to the ubiquitin conjugating enzyme component of the complex. Thus, the ER ubiquitin ligase functions as a quality control inspector, assessing the degradation status of its subset of accessible ER proteins and initiating degradation of those proteins that expose lysine residues to its ubiquitination apparatus. Hmg2p appears to utilize a structural alteration to expose the critical Lys6 and Lys357 to the ubiquitin conjugating enzyme Ubc7p under conditions of abundant mevalonate

pathway production, but can prevent such access to those lysines under conditions of insufficient pathway production.

Cartoons of the Hmg2p degradation mechanism is represented in figure 6-1 and figure 6-2. Figure 6-1 represents the cycle of Hmg2p interaction with the ER ubiquitin ligase complex under conditions of insufficient mevalonate pathway production. Hmg2p is accessible to the ER ubiquitin ligase complex, but does not present any lysine residues for ubiquitination. Hmg2p dissociates from the complex without becoming covalently attached with ubiquitin, and therefore remains stable. Figure 6-2 represents the cycle of Hmg2p interaction with the ER ubiquitin ligase complex and subsequent degradation under conditions of abundant mevalonate pathway production. A signal generated downstream in the mevalonate pathway from the molecule FPP acts to alter the Hmg2p transmembrane domain structure. This could occur by either direct association of the FPP-derived molecule with Hmg2p, association of the FPP-derived molecule with an effector molecule that directly binds Hmg2p, or changes in the ER membrane composition of the FPP-derived molecule that result in alteration of the ER membrane structure. The ability of the FPP-derived molecule to alter Hmg2p structure is enhanced by the presence of an oxysterol-derived signal that may also elicit a change in the Hmg2p transmembrane domain structure through similar mechanisms as the FPPderived signal. The structurally altered Hmg2p interacts with the ER ubiquitin ligase machinery as it did when mevalonate pathway production was insufficient. However, the structural alterations within the Hmg2p transmembrane domain now allow presentation of key lysine residues to the ubiquitin conjugating portion of the ubiquitin ligase complex. This results in the attachment of multi-ubiquitin chains to the lysine



Figure 6-1: The life cycle of Hmg2p under conditions of insufficient mevalonate pathway production.

Hmg2p interacts with the ER ubiquitin ligase complex, but rapidly dissociates without being ubiquitinated because it prevents access to key lysine residues.



Figure 6-2: The life cycle of Hmg2p under conditions of abundant mevalonate pathway production.

Signals generated from the mevalonate pathway interact with Hmg2p and alter its structure, exposing the critical lysine residues. Hmg2p interacts with the ER ubiquitin ligase complex and is now ubiquitinated on those exposed lysine residues. The ubiquitinated Hmg2p is recognized by the proteasome for destruction. Removal of Hmg2p from the ER membrane may proceed with help from the Sec61p translocon complex, or may occur solely through the ATPase action of the proteasome.

residues, resulting in the subsequent recognition and destruction of the ubiquitinated Hmg2p molecule by the proteasome.

Future directions and considerations.

Despite the molecular insights gained from the results detailed in this work, much still needs to be done before a complete understanding of the ER-associated degradation of Hmg2p can be claimed. Indeed, further experimentation on all aspects of Hmg2p recognition, targeting and delivery to the proteasome still need to be conducted (Fig. 6-3). Many exciting details of Hmg2p degradation and its regulation will no doubt be discovered by far more in depth analyses.

For instance, it is not clear if the FPP-derived or oxysterol-derived signals directly interact with the Hmg2p transmembrane domain or if they act through more indirect means (Fig. 6-3a). Genetic analyses of the components required for the regulation of Hmg2p degradation may reveal proteins that serve as effectors that bind Hmg2p and alter its structure according to mevalonate pathway production. Binding studies between the Hmg2p transmembrane domain and FPP or oxysterols may help delineate whether the effect of such molecules on Hmg2p degradation is direct or indirect.

In addition to exploring the mechanism of regulation, the process of degradation requires further examination in order to determine how Hmg2p is removed from the ER membrane (Fig. 6-3b). It is not clear if the proteasome extracts Hmg2p from the membrane through the collected action of its ATPase subunits, or if Sec61p and a retro-translocation apparatus are used to facilitate this process. It could also be that the



Figure 6-3: Future considerations for ER-associated Hmg2p degradation.

a) Do the signals from the mevalonate pathway directly interact with Hmg2p, or do they exert their effect through association with an effector molecule? **b)** Does the proteasome directly remove ubiquitinated Hmg2p from the membrane, or does its action require the Sec61p pore complex for retro-translocation? Does the proteasome pull Hmg2p from the membrane or shear off pieces of Hmg2p? **c)** Is ubiquitin attachment solely a tag for proteasomal recognition, or does it also have other functions such as structure destabilization?

proteasome acts to shear off the portions of Hmg2p that are exposed to the cytosol, leaving the rest to be destroyed by ER lumenal proteases. A reconstituted *in vitro* assay may go a long way in helping to determine if any or all of these processes are actually used in Hmg2p degradation.

The role of ubiquitin attachment to Hmg2p should be further clarified (Fig. 6-3c). Do Lys6 and Lys357 actually serve as the ubiquitination sites in Hmg2p? Understanding the mode of action of these two critical lysines is required before the complete model of Hmg2p targeting and degradation is formulated. Also, ubiquitination may only serve as the universal molecular tag necessary for the proteasome to recognize and bind a diverse array of degradation substrates. However, the covalent attachment of ubiquitin may have additional roles in ER-associated degradation, such as serving as a ratchet mechanism to aid in the retro-translocation of substrates out of the ER or acting as a modification that destabilizes and unfolds the structure of target proteins. In depth retro-translocation assays with functional or disabled ubiquitination machinery could help identify a ratchet function. Limited protease protection assays on ubiquitinated substrates may identify a destabilizing function for ubiquitin. These further considerations are certainly not all encompassing and each new experiment may provide not only additional insight into the mechanism of ER-associated degradation, but future directions for experimentation that cannot be visualized at this current time.

Proteasomal protein degradation...just the beginning.

From the collected work of numerous labs, it has become quite evident that proteasomal protein degradation is an essential part of cellular functioning and physiology, from cell cycle progression and transcriptional regulation to metabolic pathway regulation and quality control. Research into the different aspects of proteasomal protein degradation has blossomed productively over the past several years and promises to bear abundant, fruitful insight into many of the cellular processes that use the proteasome as a key part of their mechanisms. Indeed, regulation of protein levels by proteasomal protein degradation will one day rival regulation by transcriptional regulation. And the emergence of proteomics as a high throughput assay will only enhance the exploration into cellular degradation by the proteasome, allowing greater and more in-depth analyses than currently have been employed. To have played a small role in the emerging field of proteasome-dependent degradation has certainly been a highlight of my graduate studies. As I move on, I find myself with the comfort that my small contribution may aid future researchers in the quest for understanding these processes. I will continue to look upon the ongoing research with a keen eye, heightened curiosity and pleasurable interest as a result of my dedicated years in this pursuit. It is my hope that those who read this labor of love will find inspiration and discover within themselves a similar sense of excitement and joy that I have found.

Appendix 1-Materials and Methods

A. Materials and reagents.

All enzymes used in these studies were obtained from New England Biolabs (Beverly, MA). Chemical reagents were obtained from Sigma Chemical (St. Louis, MO), except for DSP, which was obtained from Pierce Chemicals (Rockford, IL). ECL chemiluminescence immunodetection reagents were from Amersham Corp. (Arlington Heights, IL). Lovastatin, L659,699, and zaragozic acid were generously provided by Merck & Co. (Rahway, NJ). Terbinafine was commercially obtained as a 1% Lamisil[®] solution from Novartis (East Hanover, NJ). Ro48-8071 was generously provided by O. Morand (Hoffmann-LaRoche Ltd., Basel, Switzerland). The 9E10 anti-myc antibody was used directly from a hybridoma (ATCC CRL 1725) supernatant. The anti-HA antibody was an ascites fluid obtained from Babco (Berkeley, CA). The anti-HSV-Tag antibody was obtained from Novagen (Madison, WI). Affinity-purified goat anti-mouse HRP conjugated antiserum was obtained from Sigma Chemical. Protein A-sepharose CL-4B was obtained from Pharmacia Biotech (Piscataway, NJ). Sequencing was performed using the T7 Sequenase v2.0 sequencing kit from Amersham (Arlington Heights, II) and a model SA sequencing apparatus (Life Technologies, Madison, WI).

B. Site-directed mutagenesis and DNA cloning

The overlap extension method (Ho *et al.*, 1989) was used to create site-specific mutations in *HMG2*. All PCR was performed using Vent DNA polymerase in 100 μ l

reaction volumes (1x Thermopol buffer, 200 μ M dNTPs, 1 μ g template, 1 μ M primers and 0.5 μ l Vent DNA polymerase). PCR was carried out with a dwell at 94°C for 5 minutes; followed by 15 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds; then a final dwell at 72 °C for 7 minutes. The splicing by overlap extension method, or "SOEing" (Horton *et al.*, 1989), was used to synthesize the chimeric and point mutant portions of each plasmid. PCR products (50 μ l samples) representing the two halves for the SOEing PCR were loaded onto 0.8% agarose (1xTAE) gels and sufficiently resolved. Appropriate DNA-containing gel slices were cut from the resolved gel and eluted through a siliconized glass wool filter. The recovered DNA was washed 1x in an ULTRAFREE-MCTM 30,000 molecular weight cut-off filter unit (Millipore, Bedford, MA), and recovered as a 10 μ l volume. 5 μ l of each DNA solution were used for the SOEing PCR.

The regions of *HMG2* generated from the SOEing PCR were each subcloned into pRH377 or pRH423 (Hampton and Bhakta, 1997), both of which contained the sequence of a single myc epitope tag was inserted between codons 618 and 619 in *HMG2* (Hampton and Bhakta, 1997). PCR products with mutations created in codons 1-228 were cloned between the *Aft*II and *Sph*I sites in *ImycHMG2* in pRH423. PCR products with mutations in codons 228-370 were cloned between the *Sph*I and *BgI*II sites in *ImycHMG2* in pRH377. PCR products with mutations between codons 371-523 were cloned between the *BgI*II and *Nru*I sites in *ImycHMG2* in pRH377. All *HMG2* mutants were tested for complementation of mevalonate auxotrophy in yeast lacking both endogenous HMG-R genes. Mutations in codons 1-26 were introduced into the *hmg2::GFP* reporter gene by replacement of the *Sph*I-*Sal*I region of the mutant *hmg2* coding regions with the *SphI-Sal*I GFP-containing region from pRH469 (Hampton *et al.*, 1996b). Mutations in codons 353-370 were introduced into the *hmg2::GFP* reporter gene by replacement of the *Bgl*II-*Sal*I region of the mutant *hmg2* coding regions with the *Bgl*II-*Sal*I GFP-containing region from pRH469.

pRH1100 is an Ade⁺ selectable, ARS-CEN vector containing a triple HAepitope tagged ubiquitin expressed from the *TDH3* promoter. Construction of pRH1100 was as follows: The 900bp *Eco*RI-*Cla*I fragment from YEp112 (Hochstrasser *et al.*, 1991), which contains the HA-Ub gene, was cloned between the *Eco*RI-*Cla*I sites in pBluescript KS II (Stratagene, La Jolla, CA) to yield pRH381. The coding region for a triple HA-epitope tag was PCR amplified and cloned between the *Eco*RI and *Bgl*II sites in pRH381 to yield pRH964. The 4.2kb *PstI-Cla*I region from pRH423 was replaced with the 900bp *PstI-Cla*I region from pRH964 to yield pRH988, resulting in placement of the 3HA-Ub gene under control of the *TDH3* promoter. The 3HA-Ub containing, 2.2kb *Pvu*II-*Sac*I fragment from pRH988 was cloned between the *SmaI-Sac*I sites in pASZ11 (Stotz and Linder, 1990) to yield pRH1100.

The genes encoding squalene synthase (*ERG9*), farnesyl diphosphate synthase (*ERG20*), squalene epoxidase (*ERG1*) and oxidosqualene-lanosterol cyclase (*ERG7*) were PCR amplified from yeast strain RHY623 genomic DNA, isolated by the Winston method (Hoffman and Winston, 1987), using separate primers that contained *Pst*I and *Bam*HI or *Bgl*II sites in the upstream primers and *Nhe*I and *Sal*I sites in the downstream primers. The amplified *ERG9* and *ERG20* genes were cloned between the *Pst*I and *Sal*I sites in pRH423, thereby placed under control of the glyceraldehyde-3-phosphate dehydrogenase gene (*TDH3*) promoter (P_{TDH3}) (a.k.a. GAPDH promoter, Bitter and

Egan, 1984). The squalene synthase containing plasmid was named pRH440 and the farnesyl diphosphate synthase containing plasmid was named pRH830. The amplified *ERG7* gene was digested with *Bgl*II and *Sal*I and the 2.22 kb fragment was then cloned between the *BamH*I and *Sal*I sites in pRH440 to yield P_{TDH3} -*ERG7* (pRH1205). The amplified *ERG1* gene was digested with *BamH*I and *Sal*I and the 1.5 kb fragment was then cloned between the *BamH*I and *Sal*I sites in pRH440 to yield P_{TDH3} -*ERG7* (pRH1205). The amplified *ERG1* gene was digested with *BamH*I and *Sal*I and the 1.5 kb fragment was then cloned between the *BamH*I and *Sal*I sites in pRH440 to yield P_{TDH3} -*ERG1* (pRH1203).

Squalene synthase (*ERG9*) was tagged at the C-terminus with the HSV epitope sequence (Isola *et al.*, 1989). The plasmid containing an HSV-tagged squalene synthase expressed from the *TDH3* promoter was made by annealing primers that encoded the HSV epitope sequence (QPELAPEDPED) and cloning the resulting DNA fragment between the *Nhe*I and *Sal*I sites in pRH440 to yield pRH442 (*ERG9*-HSV). The plasmid to tag *ERG9* at its genomic locus with HSV was made by digesting pRH442 with *Mun*I and the 5.4kb vector fragment was reclosed to yield pRH885. The remaining portion of *erg9* included codons 208-446 and the 3' HSV sequence.

Plasmids that allowed expression of the genomic copy of either *ERG9*, *ERG20*, *ERG7* or *ERG1* from the *MET3* promoter (P_{MET3}) (Cherest *et al.*, 1985; Mountain *et al.*, 1991) were constructed as follows: pRH442 was digested with *Eco*RI and pRH448 was digested with *Kpn*I. Each vector was reclosed with the insert removed. The *erg9* vector was named pRH948 and the *erg20* vector was named pRH950. The *MET3* promoter was cloned into pRH948 and pRH950 by digesting each plasmid with *SspI* and *PstI* and replacing the insert with the *MET3* promoter containing *SspI-PstI* fragment from pHAM8 obtained from Dr. Harry Mountain (Staffordshire, UK). The P_{MET3}-*erg9* plasmid was named pRH973 and the P_{MET3} -erg20 plasmid was named pRH975. A PCR product containing the ERG1 coding region was digested with BamHI and PvuII. The 840 bp fragment was then cloned between the BamHI and HpaI sites in pRH973 and the resulting plasmid was named pRH1204. A PCR product containing the ERG7 coding region was digested with BglII and HpaI. The 840 bp fragment was then cloned between the BamHI and pRH1204. A PCR product containing the ERG7 coding region was digested with BglII and HpaI. The 840 bp fragment was then cloned between the BamHI and HpaI. The 840 bp fragment was then cloned between the BamHI and HpaI.

The plasmid to delete *HRD1* was constructed as follows: A 1.45 kb *Bgl*II-*Xho*I fragment from pUG6 (Güldener *et al.*, 1996), which contained the *kanMX* expression module (Wach *et al.*, 1994), replaced the 1.43 kb *Bgl*II-*Sal*I fragment in pRH507, which contained the *HRD1* gene (Hampton *et al.*, 1996a). The resulting plasmid was named pRH1122.

The plasmid that expressed a single, myc-epitope tagged version of Hmg1p (1myc-Hmg1p) was constructed as follows: Partial complementary primers that encoded a single myc epitope sequence (EQKLISEEDL) were used to amplify a fragment of *HMG1* from pRH144-2 (Hampton and Rine, 1994), which resulted in a single, myc-epitope coding sequence inserted between codons 621-622 of *HMG1*. The PCR fragment was cloned between the *Bsr*GI and *Pfl*MI sites in pRH144-2. The resulting plasmid was named pRH945.

The plasmid that expressed a triple, HA-epitope tagged version of Hrd1p (3HA-Hrd1p) was constructed as follows: The *HRD1* gene was amplified from pRH507. A DNA fragment that encoded three tandem HA-epitope tags was amplified from pGTEP (obtained from B. Futcher). The two PCR fragments were spliced together by PCR, and the resulting DNA fragment was cloned between the *Bgl*II and *Sph*I sites in pRH507, which resulted in pRH642. pRH642 was digested with *BstE*II and the vector fragment was reclosed to yield pRH1196, which results in the only genomic copy of *HRD1* tagged with the triple HA-epitope upon integration at the *HRD1* genomic locus.

The plasmid that expressed a triple, HA-epitope tagged version of Hrd3p (3HA-Hrd3p) was constructed as follows: The 2.7kb *XhoI-SacI* fragment from pYS14 (Saito *et al.*, 1999), which contained the *3HA-HRD3* gene, was used to replace the 3.1kb *XhoI-SacI* fragment from pRH507, yielding pRH1154. A PCR fragment containing the downstream terminator from *HRD3* was amplified from RHY623 genomic DNA and cloned between the *SacI-SacII* sites in pRH1154 to yield pRH1263. Integration of pRH1263 at the *HRD3* genomic locus results in the only genomic copy of *HRD3* tagged with the triple HA-epitope.

The plasmid that expressed a double, HA-epitope tagged version of Ubc7p (2HA-Ubc7p) was constructed as follows: The *UBC7* gene was amplified from RHY623 genomic. A DNA fragment that encoded two tandem HA-epitope tags was amplified from pGTEP. The two PCR fragments were spliced together by PCR, and the resulting DNA fragment was cloned between the *Pst*I and *Sal*I sites in pRH423 (Hampton and Bhakta, 1997), which resulted in pRH685. The 1.3kb *Pvu*II-*Sph*I fragment from pRH685, which contained the *2HA-UBC7* gene, was cloned between the *Ecl*136II-*Sph*I sites in pRH507. The resulting *TRP1*, *2HA-UBC7* containing plasmid was called pRH373.

The plasmid that expressed a triple, HA-epitope tagged version of Ubc6p (3HA-Ubc6p) was made as follows: The *UBC6* gene was amplified from RHY623 genomic DNA and cloned between the *Pst*I and *Nhe*I sites in pRH442. The resulting plasmid was called pRH1149. A DNA fragment encoding a triple, HA-epitope tag was amplified from pGTEP and cloned between the *Nhe*I and *Sal*I sites in pRH1149, which resulted in pRH1151. A 1.6kb fragment, which contained the *3HA-UBC6* gene, was cloned between the *PstI-Kpn*I sites in pRH507. The resulting *TRP1*, *3HA-UBC6* containing plasmid was called pRH1217.

The plasmid to make $hrd1\Delta::LEU2$ was constructed as follows: A 3.1 kb BamHI-EcoRI fragment of the HRD1 gene from was inserted between the BamHI-EcoRI sites in pBluescript KS II (Stratagene, La Jolla, CA), which resulted in pRH1174. The LEU2 gene was PCR amplified from pRS405, digested with SphI, and inserted between the StuI-SphI sites in pRH1174. The resulting $hrd1\Delta::LEU2$ plasmid was named pRH1184.

The plasmid to make $hrd3\Delta::LEU2$ was constructed as follows: A 3.1 kb *XhoI-SpeI* fragment from pRH508, containing the *HRD3* gene (Hampton *et al.*, 1996a), was inserted between the *Bam*HI-*Eco*RI sites in pBluescript KS II, which resulted in pRH1175. The *LEU2* gene was PCR amplified from pRS405, digested with *Xba*I, and inserted between the *Bsa*BI-*Nhe*I sites in pRH1175. The resulting *hrd3*\Delta::*LEU2* plasmid was named pRH1185.

The plasmid to make $ubc7\Delta::LEU2$ was constructed as follows: A 650 bp fragment containing a non-functional ubc7 gene was PCR amplified from pRH685 and inserted between the *PstI-Sal*I sites in pBluescript KS II, which resulted in pRH1176. *pR*H1176 contained an HpaI and a BsrGI site with the *ubc*7 sequence. A *1.5* kb HpaI-BsrGI fragment from pRS405, containing the LEU2 gene, was inserted between the HpaI-BsrGI sites in pRH1176. The resulting $ubc7\Delta$::LEU2 plasmid was named pRH1186.

The plasmids containing C399S-*HRD1* were constructed as follows: the C399S mutation was introduced into the *HRD1* coding region by the "SOEing" PCR method. The resulting PCR fragment was digested with *Nco*I and *Bgl*II and used to replace the *NcoI-Bgl*II fragment in pRH642 to yield pRH1245. The 1.1kb *NcoI-Sph*I fragment from pRH1245 was used to replace the 1.1kb *NcoI-Sph*I fragment from pRH1153 to yield pRH1257. An untagged version of C399S-*HRD1* expressed from the *TDH3* promoter was constructed by replacing the 800bp *NsiI-Bgl*II fragment in pRH1316 with the 800bp *NsiI- Bgl*II fragment from pRH1245, yielding pRH1346. An untagged version of C399S-*HRD1* promoter was constructed by replacing the *HRD1* promoter was constructed by replacing the *HRD1* promoter was constructed by replacing from the *HRD1* promoter was constructed by replacing the *HRD1*

C. Strains and Media

Growth and transformation of *E. coli* or yeast strains were performed as previously described (Hampton and Rine, 1994). The lithium acetate (LiOAc) method was used to transform yeast with plasmid DNA (Ito *et al.*, 1983).

Mevalonate auxotrophic yeast strain RHY468 (*a his3* Δ 200 lys2-801 ade2-101 ura3-52 met2 hmg1::LYS2 hmg2::HIS3) was used as a host strain for all plasmids expressing the enzymatically active HMG-R mutants. Each integrating plasmid containing a mutant HMG2 gene was introduced into the recipient strain by targeted integration at the BamHI site of the hmg2 Δ ::HIS3 genomic locus, followed by selection for mevalonate prototrophy on YPD medium. In all cases, the mutant plasmids were able to restore mevalonate prototrophy.

Plasmids with mutant hmg2::GFP fusions were transformed into strain RHY519 (*a his3* $\Delta 200$ *lys2-801 ade2-101 ura3-52::hmg2cd met2 hmg1::LYS2 hmg2::HIS3*). RHY519 expressed a soluble, enzymatically active Hmg2p catalytic domain as the sole source of HMG-R activity. Thus, all GFP-derived optical reporter fusions were expressed in strains with identical mevalonate pathway activity. GFP fusion plasmids were introduced into RHY519 by targeted integration at the *Stu*I site of the *ura3-52* allele followed by selection for Ura⁺ prototrophy.

Yeast strain RHY871 (*a his3* Δ 200 lys2-801 ade2-101 leu2 Δ ura3-52::LEU2::hmg2-GFP met2 hmg1::LYS2 hmg2::HIS3::1MYC-HMG2) was the parent strain for transformation of plasmids containing *TDH3* expressed mevalonate pathway genes. RHY871 co-expressed 1myc-Hmg2p (Hampton and Bhakta, 1997) and the autofluorescent Hmg2p-GFP (Hampton *et al.*, 1996b). Each integrating plasmid was introduced by targeted integration at the *Stu*I site of the *ura3-52* genomic locus. Yeast transformants were selected for Ura⁺ prototrophy.

Yeast strain RHY1326 (*a his3* Δ 200 lys2-801 ade2-101 leu2 Δ ura3-52::LEU2::hmg2-GFP MET2 hmg1::LYS2 hmg2::HIS3::1MYC-HMG2) was made by transforming RHY871 with a functional MET2 gene fragment from pGMET (Baroni *et al.*, 1990), followed by selection for Met⁺ prototrophy. RHY1326 and RHY1462 (*a his3D200 lys2-801 ade2-101 leu2* Δ ::6myc-hmg2-GFP::LEU2 ura3-52::6MYC-HMG2 MET2 hmg1::LYS2 hmg2 Δ ::HIS3) were the parent strains for transformation of all plasmids containing P_{MET3} expressed mevalonate pathway genes. Plasmid pRH973 (P_{MET3}-erg9) was introduced by targeted integration at the *Hpa*I site of *ERG9*. Plasmid pRH975 (P_{MET3}-erg20) was introduced by targeted integration at the *Hind*III site of *ERG20*. Plasmid pRH1204 (P_{MET3}-erg1) was introduced by targeted integration at the *Age*I site of *ERG1*. Plasmid pRH1206 (P_{MET3}-erg7) was introduced by targeted integrated integration at the *BamH*I site of *ERG7*. Yeast transformants were selected for Ura⁺ prototrophy.

Yeast strains RHY1914 (α his3 $\Delta 200$ lys2-801 ade2-101 ura3-52::1mycHMG2::URA3 met2 hmg1::LYS2 trp1::HISG leu2 Δ) and RHY1915 (α his3 $\Delta 200$ lys2-801 ade2-101 ura3-52::1mycHMG1::URA3 met2 hmg1::LYS2 trp1::HISG leu2 Δ) were the parent strains for the cross-linking assays. HA-epitope tagged versions of the HRD genes were integrated at their respective loci and transformants were selected for Trp⁺ prototrophy. The hrd Δ alleles were integrated at their respective loci and yeast transformants were selected for Leu⁺ prototrophy.

As in previous studies, all Hmg2p proteins, both normal and mutant, were expressed from the strong, constitutive *TDH3* promoter in order to separate degradation from other possible modes of regulation (Hampton and Rine, 1994; Hampton *et al.*, 1996a). In addition, all *HMG2* coding regions had a single copy of the myc-epitope coding sequence inserted between codons 618 and 619 located in the poorly conserved *HMG2* linker region (Hampton and Bhakta, 1997). Presence of this single myc epitope had no effect on the essential HMG-R activity or regulated, *HRD*-dependent degradation of Hmg2p (Hampton and Bhakta, 1997).

D. Degradation Assays

Degradation of Hmg2p and its variants was assessed by cycloheximide-chase assays as previously described (Hampton and Rine, 1994), except that the lysis buffer was 1%SDS, 8M urea, 10 mM MOPS, pH 6.8, and 10 mM EDTA (abbreviated SUME). Briefly, yeast strains were grown in 25 ml supplemented minimal media to an approximate optical density (OD_{600}) of 0.3. Cells were pelleted by centrifugation and resuspended in 6 ml of fresh supplemented minimal media. Cycloheximide was added to each culture at a final concentration of 50 μ g/ml and vortexed briefly. Each culture was separated into three 2 ml samples with lovastatin added to one of the samples to a final concentration 25 μ g/ml. One of the no drug samples was then immediately lysed for the zero time point. The remaining pair, with and without drug, was incubated at 30°C for 4 hours and then lysed. Lysis of cells for immunoblotting was performed as described (Hampton and Rine, 1994). Briefly, cells were pelleted by centrifugation and resuspended in 100 μ l SUME + protease inhibitors (10 mM PMSF, 100 μ g/ml TPCK, 100 μ g/ml leupeptin and 100 μ g/ml pepstatin); 100 μ l acid-washed glass beads were added and the mixture vortexed at full speed for 3 min; 100μ l of 2x USB was added to the mixture and incubated at 55°C for 10 min; the liquid lysate was removed from the glass beads and clarified by centrifugation for 5 min.

The log-phase steady-state assays were performed using the same lysis and immunoblotting procedure described above. The steady-state assays were performed as follows: Yeast strains were grown in 25 ml of supplemented minimal medium to an approximate OD_{600} of 0.1. Each culture was separated into three 8 ml samples with

drugs added to the indicated concentrations. The samples were incubated at 30°C for 3 hours and then lysed for immunoblotting.

Methionine-chase assays were performed as follows: Cells were grown to early log phase with an optical density (OD_{600}) of 0.01. Methionine was added to a final concentration of 2mM and the cells grown at 30°C for 15 hours. Cells were then either used for the cycloheximide-chase assay as described above or for the FACS analysis described below.

Cellular lysates were immunoblotted as described (Hampton and Rine, 1994). Briefly, 15 μ l samples were resolved on 8% SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with the 9E10 anti-myc antibody. In some circumstances, relative levels of immunoreactivity were compared between samples by assessing the exposure times required to give similar autoradiographic intensities. Although we only used this method to approximate differences in levels, it is a method that has been successfully applied in previous semi-quantitative studies in yeast (Zhang *et al.*, 1993). Furthermore, we have directly confirmed the use of exposure time as a relative gauge of immunoreactivity in this study by performing controls with different volumes of identical lysates loaded on the same gels (data not shown).

E. Ubiquitination Assays

Strains expressing individual Hmg2p variants were transformed with pRH1100, which expressed a triple HA-epitope tagged ubiquitin from the constitutive *TDH3* promoter. Transformants were selected for Ade⁺ prototrophy. Ubiquitination assays were performed with these strains similarly to that previously described (Hampton and Bhakta, 1997), except no CuSO₄ was added. Briefly: Cells were grown in 20 ml cultures to an OD₆₀₀ of 0.2. To those cultures indicated, lovastatin was added to a final concentration of $50\mu g/ml$ and the cultures were incubated for 60 min at 30°C. To those cultures indicated, zaragozic acid was added to a final concentration of $10 \mu g/ml$ and the cultures were incubated for 10 min at 30°C. Cells were pelleted by centrifugation and resuspended in 100 μ l SUME + protease inhibitors (10 mM PMSF, 100 $\mu g/ml$ TPCK, 100 $\mu g/ml$ leupeptin and 100 $\mu g/ml$ pepstatin) + N-ethyl maleimide (NEM, 5 mM). The cells were lysed by vortexing 3x1 min. Cell lysates were clarified by centrifugation and the lysates added to 500 μ l IP buffer + protease inhibitors + NEM. 40 μ l anti-HMG-R antibodies were added to the lysates and incubated at 4°C overnight. 60 μ l 10% w/v Protein A Sepharose beads were added to the lysate and the beads were incubated at room temperature for two hours. The beads were pelleted by centrifugation, and then washed once with IP buffer and twice with wash buffer. 40 μ l 2x USB was added and the beads were incubated at 55°C for 10 min.

Immunoprecipitates were immunoblotted as previously described (Hampton and Rine, 1994). Briefly, either 5 μ l or 35 μ l samples were resolved on 8% SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with the either the 9E10 anti-myc antibody or the 12CA5 anti-HA antibody, respectively.

F. Cross-linking Assays

The *in vivo* cross-linking assay was modified from the *in vitro* assay described (Marcusson *et al.*, 1994). Cells expressing the appropriate epitope-tagged proteins were

grown to log phase ($OD_{600}=1.0$) in 30 ml minimal media, harvested by centrifugation, and resuspended in XL buffer (Marcusson *et al.*, 1994) to a density of 6.0 OD_{600} units. Zymolase was added (final concentration 50µg/ml) and DSP (dithiobis(succinimidylproprionate)) was added in varying concentrations up to $400\mu g/ml$. Cells were incubated 30 minutes at 30° C. Spheroplasts were harvested by centrifugation and lysed by resuspension in 1/4x XL buffer plus 20μ M hydroxylamine to quench the cross-linking reaction. Lysed cells were harvested by centrifugation, resuspended in 300μ l SUME + protease inhibitors and vortexed for 3 minutes. Supernatants were clarified by centrifugation for 15 minutes. Supernatants were added to 1 ml immunoprecipitation buffer (IP buffer) (Hampton and Bhakta, 1997), containing 40 µl of anti-HMG-R antisera. Samples were incubated 16 hours at 4°C. Protein A sepharose CL-4B beads (100 μ l of a 10% wt/vol solution) were added and the samples were incubated for 2 hours. Beads were harvested by centrifugation and washed once with IP buffer and once with wash buffer (Hampton and Bhakta, 1997). Proteins were removed from the beads by addition of 50 µl 2xUSB (Hampton and Bhakta, 1997), and incubation at 55°C for 10 minutes.

Immunoblotting of precipitated proteins was performed as described above for the ubiquitination assays. Briefly, separate 5μ l and 35μ l volumes of each precipitated sample were resolved on 8% SDS-PAGE gels and transferred to nitrocellulose. The 12CA5 anti-HA antibody was used to detect HA-tagged proteins and a polyclonal anti-GFP antisera was used to detect GFP fusion proteins.

G. Flow cytometric Analyses

Strains for flow cytometric analysis were grown in supplemented minimal medium and analyzed while in log phase (OD_{600} between 0.2 and 0.5). Living cells were analyzed by flow microfluorimetry using a FACSscan (Beckton Dickinson, Palo Alto, CA) analytical flow microfluorimeter with settings for fluorescein-labeled antibody analysis. Data was analyzed with CellQuest software. Histograms were produced from 10,000 individual cells. To examine the effects of lovastatin or zaragozic acid on the steady-state levels of GFP reporters by flow cytometry, the drugs were added to early log-phase cultures ($OD_{600} < 0.2$) to the desired final concentrations and the cells were grown another 4 hours, so that final cultures were still in log phase.

Fluorescence microscopy on cells expressing GFP reporter fusions was performed with a Nikon Optiphot-2 microscope with epifluorescence illumination, using a 100X oil-immersion objective. GFP fluorescence was observed in living cells with a Nikon B2-A filter for fluorescein fluorescence with excitation at 450-490nm and long band pass emission. Images were captured and processed as described (Hampton *et al.*, 1996a,b).

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