AR REVIEWS IN ADVANCE10.1146/annurev.cellbio.18.032002.131219
(Some corrections may occur before final publication online and in print)

Annu. Rev. Cell Dev. Biol. 2002. 18:345–78
doi: 10.1146/annurev.cellbio.18.032002.131219

First published online as a Review in Advance on August 6, 2002

PROTEOLYSIS AND STEROL REGULATION

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Key Words sterols, SREBP, HMG-CoA reductase, ubiquitin, HRD pathway, ER

■ **Abstract** The mammalian cell continuously adjusts its sterol content by regulating levels of key sterol synthetic enzymes and levels of LDL receptors that mediate uptake of cholesterol-laden particles. Control is brought about by sterol-regulated transcription of relevant genes and by regulated degradation of the committed step enzyme HMG-CoA reductase (HMGR). Current work has revealed that proteolysis is at the heart of each of these mechanistically distinct axes. Transcriptional control is effected by regulated cleavage of the membrane-bound transcription factor sterol regulatory element binding protein (SREBP), and HMGR degradation is brought about by ubiquitin-mediated degradation. In each case, ongoing cell biological processes are being harnessed to bring about regulation. The secretory pathway plays a central role in allowing sterol-mediated control of transcription. The constitutively active endoplasmic reticulum (ER) quality control apparatus is employed to bring about regulated destruction of HMGR. This review describes the methods and results of various studies to understand the mechanisms and molecules involved in these distinct but interrelated aspects of sterol regulation and the intriguing similarities that appear to exist at the levels of protein sequence and cell biology.

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INTRODUCTION

The mevalonate pathway produces a large group of molecules known as isoprenoids, numbering in the thousands, with essential functions in many aspects of life (Lange et al. 2000, Sharkey 1996). The most well-known member of this Darwinian combinatoric library is cholesterol and its structural relatives. The centrality of cholesterol in lipid biochemistry and medicine has spawned an enormous amount of research directed toward understanding sterol homeostasis (Goldstein & Brown 1990, Haas et al. 2001, Kelley & Herman 2001, Vance & Van den Bosch 2000). From these studies, it has become clear that two apparently distinct strategies of control, transcriptional regulation of key genes and regulated degradation of HMG-CoA reductase, harness proteolysis. Despite their mechanistic independence, there are intriguing similarities between these processes when examined at the level of protein sequence. This review details the studies on these two modes of sterol regulation, emphasizing how proteolysis is a key molecular aspect of each and which aspects of cell biology pertain to each.

Regulation of Cellular Sterol Levels in Mammals

In mammals, regulation of cellular sterol content intertwines cellular ingestion and production: When cells need more sterol, they increase synthesis and uptake; when they need less, they decrease synthesis and uptake. Although important, these two aspects of regulation are the tip of a regulatory iceberg because the movement of sterols from dietary or synthetic sources into and out of the cell, the bloodstream,

and the body involves a remarkable number of potentially regulated processes (Buhman et al. 2001, Cohen et al. 1996, Edwards et al. 2002, Repa et al. 2000, Rigotti & Krieger 1999, Simons & Ikonen 2000).

Sterol synthesis is regulated by changing levels of pathway enzymes, principally (but not only) HMG-CoA reductase (HMGR) and HMG-CoA synthase (HMGS). Cellular uptake of cholesterol is adjusted by changing levels of low-density lipoprotein (LDL) receptor (LDLR), which mediates uptake of cholesterol-rich LDL particles from the extracellular space (Goldstein & Brown 1985). When cultured fibroblasts (and many other cell lines) are grown in sterol-rich medium or are treated with the potent cholesterol derivative 25-hydroxycholesterol, levels of LDLR and mevalonate pathway enzymes HMGS and HMGR drop (Brown et al. 1973, Goldstein & Brown 1974, Nakanishi et al. 1988). Conversely, in sterol-starved cells, levels of these proteins increase. Efforts to understand the underpinnings of these events has led to the parallel tales of proteolysis.

Proteolysis has two common meanings. It can refer to site-specific cleavage of a target protein, important in many processes including blood clotting (Goldsack et al. 1998), bacterial sporulation (Rudner et al. 1999), Notch signaling (Hartmann et al. 2001), Alzheimers disease (Dominguez et al. 2001), apoptosis (Creagh & Martin 2001), and sterol-regulated transcription. Proteolysis can also mean processive destruction of proteins to small peptides, important in the regulation of many proteins that include cyclins and their inhibitors (King et al. 1996), p53 (Fang et al. 2000, Huibregtse et al. 1995), transcription factors (Kornitzer et al. 1994, Treier et al. 1994), IκB (Chen et al. 1996), MCH-I (Beersma et al. 1993, Wiertz et al. 1996), damaged proteins (Brodsky & McCracken 1999), and metabolic enzymes including HMG-CoA reductase. This version of proteolysis is often called protein degradation, effected by a dedicated and separate set of cellular devices. These distinct meanings describe the two roles of proteolysis in sterol regulation.

STEROL-REGULATED TRANSCRIPTION: SREBP, SCAP, AND THE UNION OF PROTEOLYSIS AND MEMBRANE TRAFFIC

Early studies on sterol control of synthetic enzymes and LDLR indicated that transcription was a critical axis. The spectacular studies of sterol-regulated transcription represent a 30-year odyssey, the major contribution coming from the Brown and Goldstein laboratory (Brown & Goldstein 1996).

SREBP: Anchors Away, Full Transcription Ahead!

Sterol-regulated transcription of many genes is effected through an octamer *cis* sequence called the sterol regulatory element (SRE-1). Inclusion of SRE-1 in engineered reporter genes faithfully imparts sterol regulation in mammalian cells (Osborne 1991, Südhof et al. 1987). Although the details of sterol regulation of

HMGS, HMGR, and LDLR are not identical (Goldstein & Brown 1990, Osborne 1991), SRE-1 plays an important role in each.

A series of studies culminated in the isolation of SREBP (sterol regulatory element binding protein), which specifically binds SRE-1 (Sato et al. 1994; Wang et al. 1993, 1994; Yokoyama et al. 1993). cDNA isolation and sequencing demonstrated two distinct genes encoding SREBP-1 and SREBP-2. The SREBP-1 genes gives rise to SREBP-1a and SREBP-1c through alternate exon use (Hua et al. 1995). Taken together, the active SREBPs are responsible for transcription of a large battery of genes involved in both sterol synthesis and fat metabolism (Osborne 2000); in fact, SREBP-1c was independently isolated as adipocyte differentiation factor (ADD1) (Kim et al. 1995, Tontonoz et al. 1993). Although the three transcription factors have functional differences, all are proteolytically regulated by sterols in the same manner. In this discussion of the common sterol regulation, all forms are referred to as SREBP.

Full length forms of SREBP have a cytoplasmic N-terminal region homologous to basic helix-loop-helix leucine zipper (bHLHLZ) family of transcription factors, and this portion is the same size as the soluble protein first purified (Wang et al. 1993). However, the bHLHZ domain is followed by a segment with two transmembrane spans and then a large, cytoplasmic C terminus (Brown & Goldstein 1997, Sato et al. 1994). SREBP is originally produced as the full-length membrane-bound form (Figure 1*a*, see color insert), which is then cleaved to produce the active N-terminal region that migrates to the nucleus to direct gene transcription. Membrane-bound SREBP is mostly localized to the endoplasmic reticulum (ER) and must be cleaved to be transcriptionally active.

Sterol regulation of SRE-driven genes occurs through sterol regulation of SREBP processing (Wang et al. 1994). When cellular sterols are abundant, the cleavage of SREBP is halted, and the majority of the pool is found as the inactive, full-length ER form. Conversely, when sterols are low, processing is efficient and nearly all of the SREBP is found in the cell as the soluble, active nuclear form. The soluble form is rapidly degraded (Wang et al. 1994), ensuring that the steady state of the transcription factor will be tuned to current levels of cellular sterols.

SCAP, S1P, and S2P: The Apparatus and Mechanism of Sterol-Regulated Transcription

The soluble SREBP N terminus is liberated by the sequential cleavage of the full-length protein at two sites (Sakai et al. 1996). First, the lumenal loop between the two transmembrane domains is cleaved at site-1 (Duncan et al. 1997), which results in a membrane-anchored intermediate. This step is obligatory for release of the active transcription factor: Uncleavable site-1 SREBP mutants remain full-length. The second cleavage at site-2 occurs within the first transmembrane span (Duncan et al. 1998) and results in production of the active soluble protein. The first cleavage step is regulated by sterols: When sterols are abundant, the lumenal cleavage is blocked, which results in a buildup of the full-length SREBP. The

intramembrane second cleavage is sterol independent but absolutely requires the first step to proceed.

The Awesome Power of Mammalian Genetics

This heading makes reference to the oft-used descriptor of yeast's usual advantage in biomedical research (e.g., http://www.umanitoba.ca/faculties/medicine/biochem/gietz/). The discovery of the proteins involved in SREBP regulation demonstrates molecular complementation need not be restricted to that laudedeukaryote, which is a critical issue here because the SREBP pathway is not represented in *Saccharomyces cerevisiae* (Vik & Rine 2001). Two classes of mutant cell lines were used to clone components of the SREBP pathway, sterol-resistant lines that always process SREBP and sterol auxotrophs that fail to process SREBP (Goldstein et al. 2002).

SCAP: Making Sense of Sterols

The sterol-resistant, dominant 25-RA CHO mutant processes SREBP even in the presence of high LDL or the potent oxysterol 25-hydroxycholesterol (Chang & Limanek 1980). The responsible dominant gene was cloned from a 25-RA line cDNA library by its ability to impart sterol resistance to wild-type cells (Hua et al. 1996). The encoded protein was named SCAP (SREBP cleavage-activating protein) for its ability to stimulate SREBP cleavage at site-1. The isolated cDNA had a point mutation and a resulting single amino acid difference (D443N) from the wild-type SCAP protein. Interestingly, the identical mutation has been independently isolated in other sterol-resistant lines (Nohturfft et al. 1996), indicating a critical residue in SCAP regulation. Both D443N mutant and wild-type SCAP will stimulate site-1 cleavage of SREBP, but the mutant form is refractory to the effects of high sterols. SCAP is rate-limiting for SREBP cleavage, i.e., the more SCAP cDNA expressed, the more SREBP processing that occurs.

The central role of SCAP in SREBP regulation has been confirmed first by study of SCAP null CHO lines (Rawson et al. 1999) and then extended to the major site of mammalian cholesterol synthesis, the liver, using tissue-specific gene disruption (Matsuda et al. 2001). The results clearly indicate the key role of SCAP both in the sterol-regulated expression of genes and the broader arena of lipid metabolism.

The SCAP Protein and the Sterol-Sensing Domain

SCAP is a 1276 residue (1277 human) polytopic membrane protein, the bulk of which resides in the ER with both termini facing the cytosol (Figure 1*a*). The N-terminal region of the protein (∼730 aa) has most likely 8 transmembrane regions, with two large lumenal loops between spans 1 and 2 and spans 7 and 8, followed by a hydrophilic C-terminal region (Nohturfft et al. 1998b). The N-terminal transmembrane domain has homology to another sterol-regulated protein, HMG-CoA reductase (Hua et al. 1996). In particular, the region including

transmembrane spans 2–6 of SCAP are 25% identical and 55% similar to the same region in the 8 transmembrane-spanning HMGR molecule. The hydropathy plots and topology of these regions are similar, and they are oriented in the same manner in the ER membrane (Nohturfft et al. 1998b). This shared motif is called the sterol sensing domain (SSD), found in a number of proteins, each of which somehow involves sterols in their function (Osborne & Rosenfeld 1998). These include the Neimann Pick C1 (NPC1) protein (Carstea et al. 1997), involved in cholesterol movement in the secretory pathway; *patched* (Hooper & Scott 1989, Nakano et al. 1989); a *Drosophila* developmental receptor whose ligand, *hedgehog*, is covalently coupled to cholesterol (Porter et al. 1996); and *dispatched* involved in hedgehog presentation (Burke et al. 1999). The connection to HMGR is particularly interesting. HMGR is regulated by sterol-stimulated degradation (see below), and the HMGR SSD is included in the part of the molecule required for this function. The molecular actions of the SSD are not yet clear, but ongoing, detailed studies of SCAP function will undoubtedly help unveil the action(s) of this highly conserved motif.

The cytosolic C-terminal region of SCAP is hydrophilic and contains four WD40 repeats (Hua et al. 1996). These repeats are critical for the SREBP regulatory functions of SCAP. Direct interaction studies demonstrate that the WD40 repeats mediate interaction of SCAP with SREBP and that this interaction is required for SCAP to control the processing of SREBP (Sakai et al. 1997, 1998a).

The Site-1 and Site-2 Proteases: SREBP Gets the "HEIGH Five" After Making the First Cut

The two SREBP cleavages are each sequence specific. The Arg-X-X-Leu sequence of the lumenal loop is required for the site-1 cleavage of the distal Leu peptide bond (Figure 1*a*, *arrow 1*) (Duncan et al. 1997). The site-2 cleavage occurs within the first transmembrane region at an absolutely conserved Leu-Cys bond three amino acids distal to a required juxtamembrane sequence Asp-Arg-Ser-Arg (Figure 1*a*, *arrow 2*) (Duncan et al. 1998).

The M19 cholesterol auxotrophic CHO mutant cell line, specifically deficient in site-2 cleavage, was used to clone the site-2 protease (S2P) coding region (Rawson et al. 1997). Human fibroblast genomic DNA was transfected into the parent strain, and after three rounds of selection and retransformation, the resulting prototrophs were analyzed for human-specific DNA. Sequence information from this approach was then used to isolate a cDNA that encoded S2P.

S2P function requires a signature Zn^{2+} binding motif, His-Glu-X-X-His (HEIGH in S2P) found in over 30 zinc metalloproteases (Hooper 1994). Most metalloproteases are soluble, but S2P is part of a growing clan that is membrane anchored (Rudner et al. 1999): S2P is microsome associated, with four or five putative transmembrane domains (Zelenski et al. 1999). The HEIGH sequence at postion 170 is located in the midst of a transmembrane region, well-placed for the cleavage SREBP residue 484 (Duncan et al. 1998), also in the lipid bilayer.

A required Leu-Asp-Gly sequence in a separate hydrophobic stretch is analogous to remote required residues (usually an aspartate or a tyrosine) found in more traditional zinc proteases (Hooper 1994). So far it has not been possible to directly demonstrate in vitro proteolytic activity of S2P, although the genetic and molecular biological evidence is compelling.

Cloning site-1 protease (S1P) required isolation of a cholesterol auxotrophic SRD-12B line deficient in the first SREBP cleavage (Rawson et al. 1998). A reporter gene encoding a SCAP-regulated protein that releases secreted placental alkaline phosphatase (PLAP) when cleaved at site 1 was expressed in SRD-12B, along with excess SCAP to boost signal. The resulting reporter line was transformed with hamster cDNA library and screened for PLAP-secreting candidates, using "sib selection" to narrow the number of complementing plasmids to one (Sakai et al. 1998b).

S1P, also isolated as SKI-1 (Seidah et al. 1999), is a 1052 residue transmembrane protein, with an N-terminal signal sequence, a large hydrophilic region, and a single transmembrane span near the C terminus (Figure 1*a*). The hydrophilic region is unambiguously distinguishable as a subtilisin protease with a required catalytic triad Asp218, His249, and Ser414 (Dodson & Wlodawer 1998, Siezen & Leunissen 1997). Soluble recombinant S1P has selective protease activity against peptides with the lumenal sites of either SREBP-1 or SREBP-2 (Cheng et al. 1999a). S1P most closely resembles proteases from the Kex2/furin subfamily most often found in the Golgi.

Studies with transfected S1P indicate that it undergoes three proteolytic-processing steps: cleavage of the signal sequence (1–22) to produce inactive S1P-A; cleavage between residues 137 and 138 to active S1P-B; and cleavage between residues 186 and 187 to make active S1P-C (Espenshade et al. 1999). Both A and B are ER resident, whereas S1P-C is Golgi localized. Subsequent work indicates that the B form is almost undetectable when the S1P is expressed only from its native promoter (DeBose-Boyd et al. 1999), so that the normally active form is Golgi-localized S1P-C, which is reasonable in light of SCAPs mechanism of action.

SCAP Action: A Cell Biological Mechanism of Regulation

SCAP is required for SREBP cleavage, and sterols function to inhibit this positive action of SCAP. The key to sterol regulation came from detailed study of SCAP biochemistry. SCAP has three glycosylation sites on two lumenal loops of the polytopic N-terminal portion. Analysis of SCAP glycosylation revealed the surprising fact that the enzymatic sensitivity of the sugar modifications—a standard way to evaluate exposure to Golgi glycosidases—was dependent on cellular sterol levels (Nohturfft et al. 1998a, 1999). In cells grown in high sterols and not processing SREBP, SCAP glycosylation remains endoglycosidase H (endo H) sensitive, indicating ER retention. When sterol levels are lowered and SCAP-dependent cleavage of SREBP commences, SCAP acquires endo H resistance caused by Golgi modifying enzymes. Eventually in low-sterol conditions, the entire pool of SCAP

is converted to endo H resistance. However, the bulk of SCAP in either condition is ER localized, indicating that SCAP cycles between the ER and the Golgi, with the whole pool acquiring H resistance if sufficient time is allowed for all of the protein to make a Golgi visit. Because SCAP binds SREBP, these observations indicate that SREBP is similarly trafficked to the Golgi in a sterol-regulated manner. This idea has been confirmed by monitoring glycanase sensitivity in versions of SREBP-2 with engineered N-linked glycosylation sites (Duncan et al. 1997; M. Brown, personal communication).

The resulting model of SCAP action intimately ties proteolytic regulation to the secretory pathway (Figure 1*a*). When sterols are low, SCAP moves from the ER to the Golgi, and by virtue of its ability to bind SREBP through their C termini, takes the full-length transcription factor along for the ride. Once there, the Golgi-localized S1P-C cleaves at site-1, allowing then and only then cleavage by S2P and liberation of the active N-terminal transcription factor. Because the site-2 cleavage will only proceed subsequent to site-1 cleavage, this regulation is effective and tight. Although SCAP cycles back to the ER, it is not clear if once-cleaved SREBP travels back to undergo site-2 cleavage or if that step happens in the Golgi, or elsewhere. When sterols are high, SCAP trafficking halts, as does SREBP trafficking, and thus, processing. Because the soluble, active portion of SREBP is rapidly and constitutively degraded, elevated sterols bring about a rapid and effective cessation of SRE-1-mediated transcription.

This model has been scrutinized, and all experiments point to its veracity. Bringing active S1P-C to ER-localized SREBP will allow processing to proceed independently of sterols or SCAP (DeBose-Boyd et al. 1999). This has been demonstrated by treatment with brefeldin A (see also Ridgway & Lagace 1995), which causes aberrant mixing of Golgi and ER compartments and by the more involved approach of expressing an active version of soluble S1P-C with an ER-retention signal (KDEL).

Using a fully complementing SCAP-GFP, Nohturfft et al. have directly observed the predicted sterol-regulated trafficking of this reporter in cultured cells (Figure 1*b*) (Nohturfft et al. 2000). When the cells are in a sterol-rich medium, SCAP-GFP fluorescence is consistent with ER localization (Figure 1*b*, *top row*). Depriving the cells of sterols causes rapid (less than 2 h) accumulation of SCAP-GFP fluorescence in punctate structures that co-localize with Golgi-resident enzymes (Figure 1*b*, *bottom row*). Reintroduction of sterols causes the SCAP-GFP distribution to return to the reticulated ER staining.

The mechanism of sterol-regulated SCAP trafficking was studied in the same work with an in vitro assay for the budding of ER-derived secretory vesicles (Nohturfft et al. 2000). ER-derived cargo vesicles budded from microsomes of sterol-rich cells do not contain SCAP, whereas those from microsomes of sterol-depleted cells do. SREBP also shows sterol-inhibited entry into the ER-derived vesicles that is entirely dependent on SCAP. These studies indicate that sterols block the ability of SCAP to exit the ER (with SREBP in tow) as cargo in Golgi-bound vesicles. Because a growing body of work indicates Golgi processing

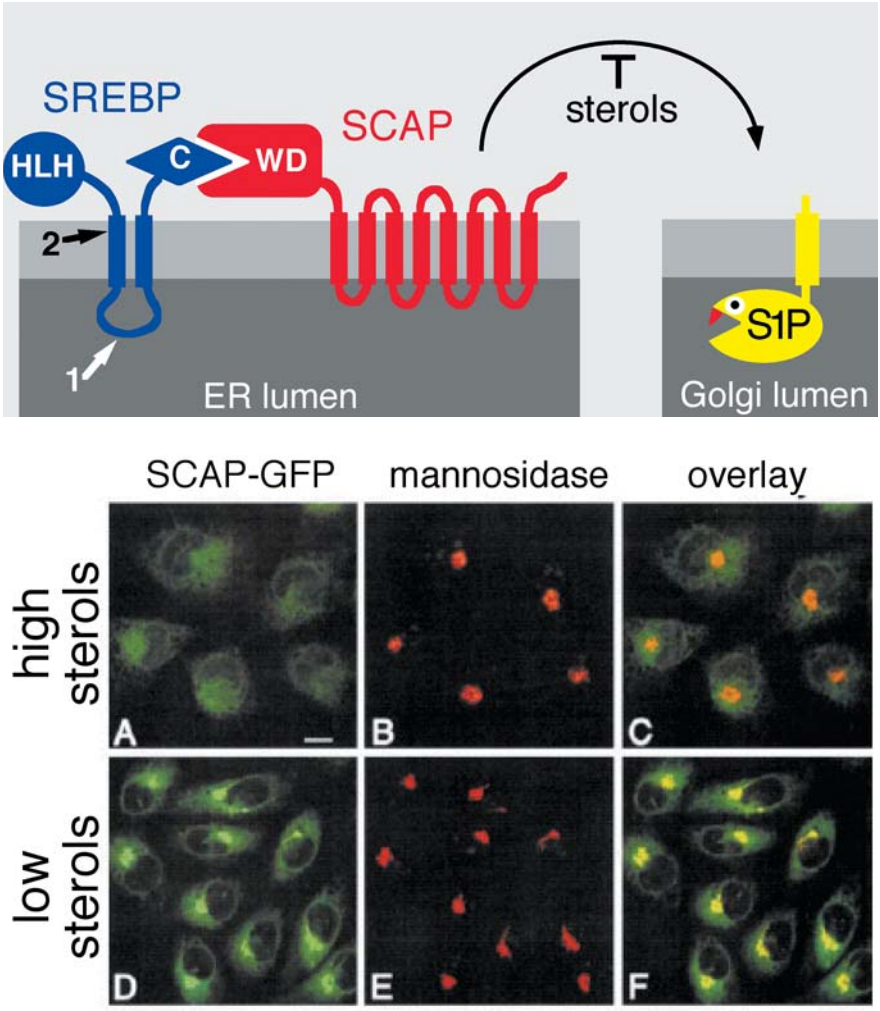

Figure 1 SCAP escorts SREBP to the Golgi, where site-1 protease (S1P) cleaves at lumenal site-1. Sterols inhibit this process. (*Top panel*) On SREBP, the site-1 and site-2 positions are indicated by arrows 1 and 2; HLH indicates the N-terminal transcription factor; C is the C-terminal region that interacts with SCAP. (*Top panel*) On SCAP, WD is the C-terminal region that binds to SREBP C region. (*Bottom panel*) Sterol-regulated trafficking of SCAP-GFP. In high sterols (*top row; A-C*) SCAP is ER-localized. When cells are deprived of sterols (*bottom row; D-F*) SCAP-GFP distribution changes to include significant Golgi occupancy, as indicated by Golgi marker mannosidase (*E*) and the overlap in signal indicated by the overlay (yellow is coincident) (from Nohturfft et al. 2000).

might sometimes involve removal of retrograde vesicles to allow maturation of the compartment (Bonfanti et al. 1998, Glick et al. 1997), caution is suggested in interpreting these in vitro results. Nevertheless, the combined collection of studies makes a strong case for a regulatory axis hitching a ride on the constitutively active secretory pathway.

Several experimental questions concerning the SCAP-traffic model remain. At present, SREBP has not been directly detected in the Golgi. Furthermore, the cellular site of S2P cleavage remains unclear. So far, the subcellular location of S2P has not been unambiguously determined (M. Brown, personal communication), which leaves open the location of site-2 cleavage. A related question concerns the fate of the remaining membrane-bound C-terminal regulatory region of SREBP—a significant issue because this portion of the molecule can block the interaction of SCAP with intact SREBP (Sakai et al. 1998a).

This mode of proteolytic regulation may seem at first glance unlikely. In fact, the S1P and S2P proteases are employed in an identical strategy for traffic-regulated cleavage of an entirely distinct protein, the transcription factor ATF6, in response to ER stress (Ye et al. 2000). Furthermore, there are clearly numerous cases of regulated cleavage of membrane proteins to effect regulation that span the biological range from bacillus sporulation to Alzheimer's disease (Brown et al. 2000, Rudner et al. 1999).

REGULATED DEGRADATION OF HMG-CoA REDUCTASE IN MAMMALS

The 3-hydroxy-3-methylglutaryl coenzyme A reductase, or HMG-CoA reductase (HMGR), catalyzes the NADPH-dependent reduction of HMG-CoA into mevalonic acid, which is the first large energy drop of the mevalonate pathway. In liver, HMGR is regulated in a coordinate fashion allowing levels over several-hundred-fold (Goldstein & Brown 1990, Nakanishi et al. 1988). One mode of control occurs through feedback regulation of HMGR degradation (Figure 2*a*, see color insert). When flux through the mevalonate pathway is high, the degradation of HMGR is high and levels of the enzyme tend to be low. When flux through the pathway is low, as when the catalytic activity of HMGR is blocked with statins, degradation of HMGR slows and levels of the enzyme tend to rise. Although HMGR-regulated degradation proceeds independently of the SREBP regulatory axis (Dawson et al. 1991), there are now intriguing similarities between these two axes of sterol regulation that hinge on mutual involvement of SSD domains.

Feedback regulation of HMGR stability was originally noted over 20 years ago (Bell et al. 1976; Edwards et al. 1983b; Faust et al. 1982; Sinensky et al. 1981, 1982). In pulse-chase experiments, HMGR degradation is accelerated by the presence of mevalonate or 25-hydroxycholesterol, even when added at the start of the chase period. Conversely, addition of HMGR inhibitor lovastatin slows the degradation of HMGR (drug targets shown in Figure 3; see color insert) (Edwards

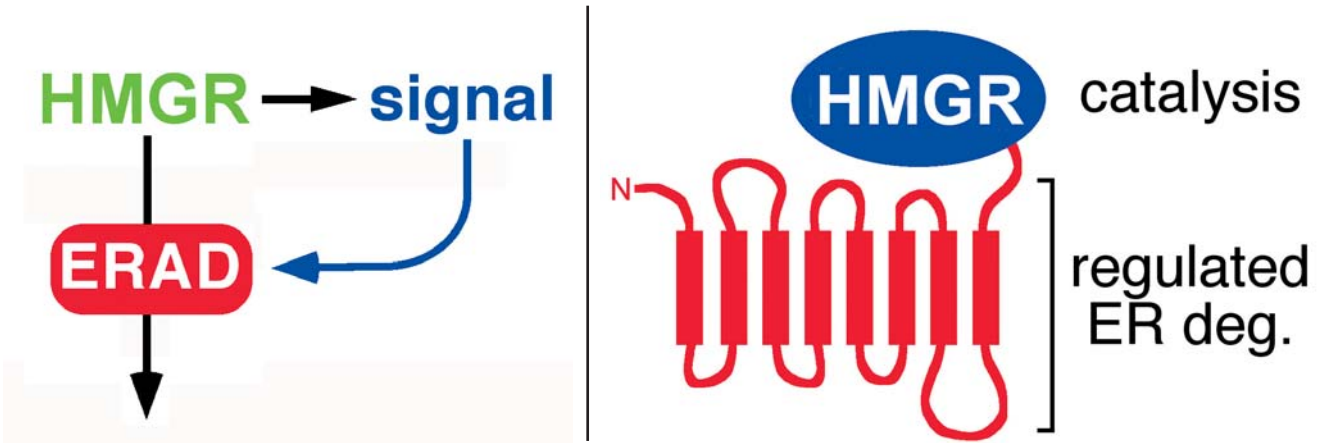

Figure 2 (*Left*) Feedback regulation of HMGR degradation by mevalonate pathway. Horizontal arrow represents mevalonate-derived signal production. ERAD; ER-associated degradation. (*Right*) Structure of HMGR, in mammals and yeast. Red portion is the N-terminal, ER transmembrane-spanning region and ER anchor. Blue portion is cytosolic catalytic domain required for enzyme activity.

et al. 1983a). In this way the half-life of mammalian HMGR can vary between 10 and less than 1 h, with significant variation in the range between cell or tissue types and protocols. If HMGR is expressed from a constitutively active heterologous promoter, regulated degradation can be studied separately from other modes of regulation (Chin et al. 1985, Chun et al. 1990, Inoue et al. 1991). Experiments of this sort demonstrate that regulated degradation alone contributes to HMGR control.

In considering HMGR-regulated degradation, the pertinent questions are What features of the HMGR protein are involved? What cellular machinery is responsible? What are the signals and mechanisms that couple HMGR stability to the mevalonate pathway? Studies in mammalian cells and, more recently, in yeast have provided some of the answers.

The HMGR Protein: Anchor-Mediated Proteolysis Again?

HMGR is an essential enzyme in eukaryotes (Basson et al. 1988, Goldstein & Brown 1990) and most likely in archebacteria as well (Boucher et al. 2001, Lam & Doolittle 1992). Mammalian HMGR has three distinct structural regions in its 888 (human) amino acid sequence (Figure 2*b*): a ∼330 residue N-terminal transmembrane region, followed by a poorly conserved linker region of 100 residues, and then a widely conserved, C-terminal region responsible for the essential enzymatic activity (Chin et al. 1982, Liscum et al. 1985)

Mammalian HMGR is retained in the ER membrane by virtue of the eight span N-terminal transmembrane domain (Liscum et al. 1985, Olender & Simon 1992, Roitelman et al. 1992). This portion of the protein is critical for regulated degradation (Figure 2*b*). Replacement of the C-terminal catalytic region with β-galactosidase results in a protein (called HMGal) that undergoes regulated degradation as long as mevalonate pathway signals are provided (Chun et al. 1990, Chun & Simoni 1992). In contrast, the isolated soluble catalytic domain expressed from a truncated HMGR cDNA is fully functional as an enzyme but is not subject to stability control (Gil et al. 1985, Nakanishi et al. 1988). Originally the transmembrane region was considered necessary and sufficient for regulated degradation. However, it has recently been observed that mulitmerization of the native catalytic domain or of C-terminal fusion partners is required for maximal regulatory effects (Cheng et al. 1999b). When fusion partners are monomeric, the half-life of the resulting proteins in the absence of sterols is decreased. Nevertheless, the monomeric proteins still retain the same fold change in half-live in response to sterols.

The transmembrane domain of HMGR contains an SSD between membrane spans 2–6 (Hua et al. 1996). This region of the HMGR protein had been mapped as critical for sterol regulation of stability by making swaps between mammalian and the unregulated but homologous sea urchin HMGR (Kumagai et al. 1995). The cloning of SCAP then led to the realization that these two sterol-regulated proteins each have the SSD motif.

HMGR Degradation in the Endoplasmic Reticulum

Mammalian HMGR is a resident of the ER. Studies with brefeldin A (Chun et al. 1990, Inoue et al. 1991) or subcellular fractionation (Lecureux & Wattenberg 1994) indicate that regulated degradation of HMGR occurs in the ER, and this model has been strengthened by the observation that HMGR occurs in permeabilized cells (Correll et al. 1994, Meigs & Simoni 1992, Roitelman et al. 1991), under conditions where membrane traffic does not occur.

The ER is now a significant and heavily researched site of protein degradation (Bonifacino & Lippincott 1991, Bonifacino & Weissman 1998, Brodsky & McCracken 1999, Hampton 2000b, Hiller et al. 1996, Klausner & Sitia 1990, Nauseef 1999, Plemper & Wolf 1999, Sommer & Wolf 1997). There is a large and growing list of ER membrane-associated or lumenally located proteins that are degraded without exit from the organelle. Substrates are often damaged, misfolded, mutant, or misassembled proteins and include numerous alleles of normal proteins that cause disease by virtue of these folding problems (Le et al. 1990, Nauseef 1999, Ward & Kopito 1994, Ward et al. 1995). Accordingly, ER-associated degradation, referred to as ERAD (Werner et al. 1996), is viewed as a quality control pathway that destroys misfolded proteins in the lumen and membrane of this central protein processing center.

How is degradation of normal HMGR related to the ERAD pathway? Direct comparison of HMGR degradation to the ER-retained unassembled TCR-α subunit, a well-studied quality control substrate (Stafford & Bonifacino 1991, Yu et al. 1997), revealed non-overlapping response to some inhibitors (Inoue & Simoni 1992). Because HMGR is subject to regulation, differences between HMGR degradation and non-regulated substrates such as TCR-α could also reflect these unique regulatory aspects. In contrast, a mammalian cell mutant with deficiencies in HMGR degradation is also deficient in TCR-α degradation (Ravid et al. 1999). Thus the degree of separateness of HMGR degradation from other forms of ER quality control in the mammal is not resolved. In yeast it is clear that HMGR is degraded by a widely used ERAD pathway (see below).

Sterol Signals in Mammalian Cells

Treating cells with various sterols stimulates HMGR degradation (Bell et al. 1976, Chun & Simoni 1992, Fitzky et al. 2001, Panini et al. 1992), including cholesterol in LDL particles and pure oxysterols. The most commonly used oxysterol is 25-OH cholesterol, which stimulates HMGR degradation (and SCAP retention) at 0.1–5 μg/ml in cell culture medium. The mutual effectiveness of cholesterol or oxysterols has left open the question of what the actual sterol degradation signals may be.

Numerous oxysterols are produced in vivo. Coding regions for distinct enzymes that oxidize cholesterol to produce 24(S)-, 25-, and 27-hydroxycholesterol have been cloned, and analysis of the null mutants will be informative (Russell 2000). Oxysterols are also produced by the so-called alternate pathway (Figure 3; *blue*

panel). Normally, squalene is epoxidized at the 2,3 position, and then cyclized by oxidosqualene cyclase into lanosterol. 2,3 oxidosqualene can also undergo reoxidation to produce of 2–3:22,23 dioxidosqualene that is then cyclized to oxylanosterol and other downstream oxysterols. The alternate pathway occurs at low levels in mammalian cells (Peffley et al. 1998) and yeast (Field & Holmlund 1977, Fung & Holmlund 1976) and is drastically up-regulated by partial inhibition of the cyclase enzyme (Mark et al. 1996, Morand et al. 1997, Peffley et al. 1998). Products of this pathway can affect HMGR stability (Panini et al. 1992).

Sterol precursors of cholesterol (of which there are at least 19) are also potential signals and may be particularly important in inborn enzyme deficiencies that allow accumulation of normally low-level intermediates (Kelley & Herman 2001). This appears to be the case in Smith-Lemli-Opitz syndrome (SLOS), caused by a deficiency in one of the final enzymes in the cholesterol pathway, Δ^7-dehydrocholesterol reductase (DHCR7) (Fitzky et al. 1998). A mouse null model has 30- to 40-fold increases of the expected metabolite (Fitzky et al. 2001). In addition, the levels of tissue HMGR are significantly below normal and appear to be caused by the effectiveness of the aberrantly elevated sterol at stimulating HMGR degradation.

All these potential sterol signals may similarly play a role in SCAP regulation, although little is known about their roles. Interestingly, in the SLOS mouse HMGR degradation is stimulated, but SREBP-regulated genes appear normal. This could mean that different signals can affect SSD-containing proteins differently, despite the mutual action caused by more commonly used sterols. There are reports of compounds that specifically alter SREBP processing (Janowski et al. 2001), including one reported to cross-link to SCAP (Grand-Perret et al. 2001). As more molecules are discovered that alter these SSD-bearing proteins, it will be interesting to see the degree of specificity (or lack thereof) (see Seegmiller et al. 2002 and discussion in concluding section) for SCAP versus HMGR actions.

Non-Sterol Signals for HMGR Degradation

Signals from the pre-sterol part of the pathway (11 steps) also participate in controlling HMGR degradation (Figure 3, *left column*). Observing the effects of these early signals is difficult because addition of mevalonate, or use of early enzyme-blocking drugs to alter them also affects sterol production. By feeding cells abundant sterols through uptake of LDL and simultaneous use of high doses of lovastatin that block HMGR activity, or with cell lines deficient in pathway enzymes, it has been possible to observe an independent mevalonate-derived component of regulation (Nakanishi et al. 1988, Panini et al. 1989, Roitelman et al. 1991, Roitelman & Simoni 1992, Sinensky et al. 1982). The response to the early isoprenoid signal is sensitive to perturbations of cellular calcium, whereas the effect of sterols is not (Roitelman et al. 1991, Roitelman & Simoni 1992), indicating the two may act in different ways. The source of the mammalian signal is the 15-carbon pathway intermediate farnesyl pyrophosphate (FPP) (Figure 3). Results from two laboratories indicate that farnesol, the dephosphorylated product of FPP, is able to stimulate

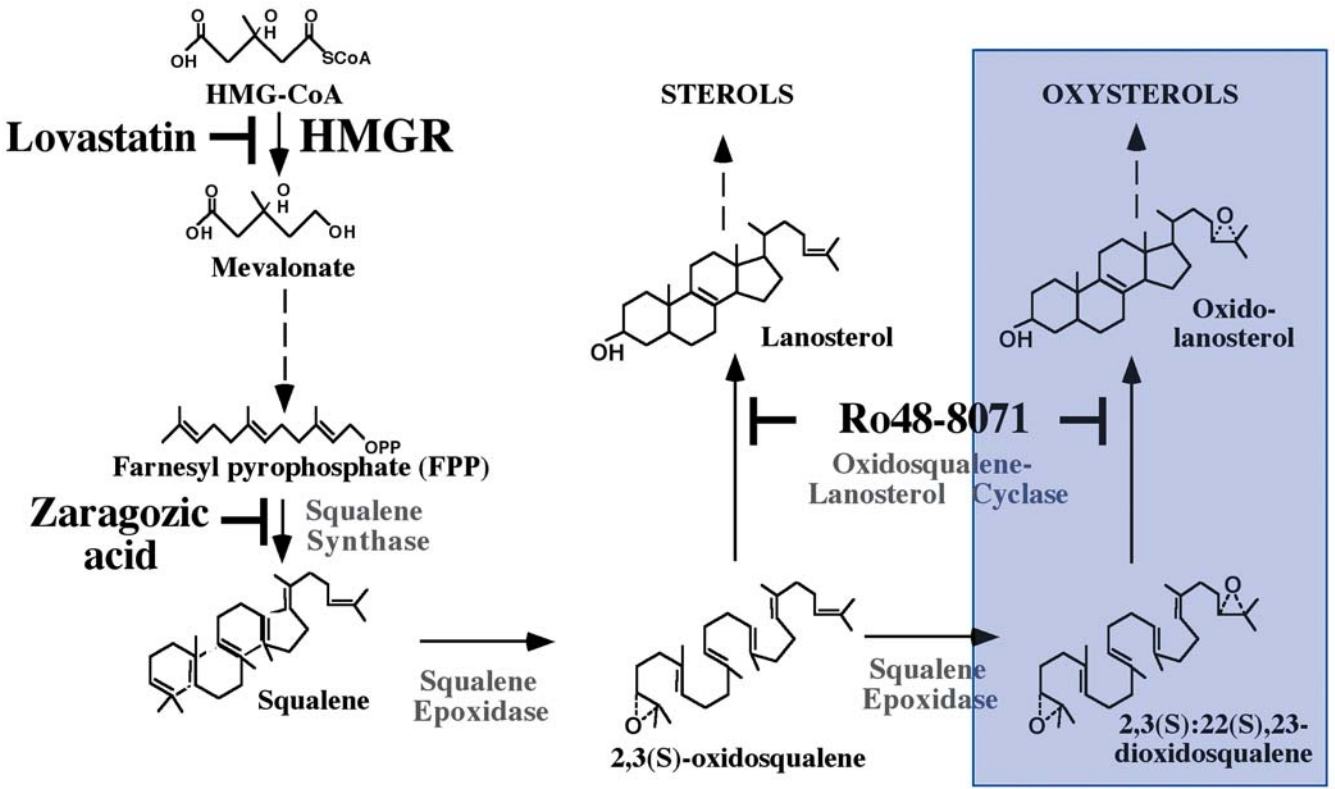

Figure 3 Mevalonate pathway, with oxysterol-producing alternate pathway, is highlighted in blue. Drugs lovastatin, zaragozic acid, and Ro48-8071 are shown with their enzymatic targets. Dotted arrow between mevalonate and farnesyl pyrophosphate (FPP) represents six enzymatic steps.

HMGR degradation in microsomal preparations in vitro (Correll et al. 1994, Meigs et al. 1996, Meigs & Simoni 1997). FPP was similarly implicated in whole-liver studies of HMGR stability, in which zaragozic acid, which inhibits squalene synthase causing FPP buildup, accelerates HMGR degradation (Keller et al. 1996), but other experiments in the same study indicate that farnesol may not be the causal agent. The identity of the non-sterol signal remains to be determined, but clearly FPP is important in mammalian HMGR degradation.

Mechanism of Mammalian HMGR Degradation

The proteasome is an abundant multiprotein complex dedicated to protein degradation (Bochtler et al. 1999, DeMartino & Slaughter 1999, Tanaka & Tsurumi 1997). Drugs such as lactacystin or MG132, which inhibit the proteasome, cause significant blockade of HMGR or HMGal degradation in intact cells (McGee et al. 1996, Ravid et al. 2000). Addition of sterols or mevalonic acid was not able to override the block to degradation, indicating that the 26S proteasome is at the end of the pathway of regulated degradation. Ubiquitin is the most common signal for 26S proteasomeal degradation (described in detail below). Nevertheless, studies implicating the proteasome in mammalian HMGR degradation were closely followed by work suggesting that ubiquitin was not involved in the mammalian pathway (McGee et al. 1996). These studies were done using the ts20 cell line hypomorphic (but not null) for ubiquitination and failed to reveal ubiquitin dependency. More recently, this question was revisited, and direct biochemical assay has confirmed that mammalian HMGR undergoes regulated ubiquitination (Ravid et al. 2000). The earlier conclusions of non-involvement of ubiquitin may stem from using a mutant cell line with residual activity.

ER-associated degradation is seen at another level of sterol regulation in mammals. The principle apolipoprotein in a number of blood lipoproteins, including LDL, is apolipoprotein B 100 (ApoB). ApoB is degraded in the endoplasmic reticulum by ERAD (Fisher et al. 1997), and the extent of this process is determined by the degree to which ApoB escapes degradation by successful assembly of the very-low-density lipoprotein particle that is produced in the liver secretory pathway (Ginsberg 1997). Although the role of ApoB degradation seems to be more directly one of quality control rather than sterol regulation, it nevertheless represents another crossroad between ER proteolysis and the physiology of cholesterol. Space limitations as opposed to conceptual dissonance restricts the treatment this process deserves.

HMGR DEGRADATION IN YEAST

Studies in *S. cerevisiae* show that regulated HMGR degradation is conserved across the billion year gap that separates them from liver cells. Yeast express two HMGR isozymes, Hmg1p and Hmg2p (Basson et al. 1986, 1988). They have structures similar to mammalian HMGR: a large N-terminal transmembrane region (525 residues Hmg1p, 524 residues Hmg2p), followed by a poorly conserved linker

region (~130 residues), and a highly conserved C-terminal catalytic domain. Either isozyme alone can provide the essential HMGR activity (Basson et al. 1986), as can expression of either catalytic domain as a soluble protein from a truncated coding region (Donald et al. 1997, Gardner et al. 1998). The transmembrane regions of Hmg1p and Hmg2p serve as ER anchors and are ~50% identical, whereas the catalytic regions are ~93% identical (Basson et al. 1988). The yeast HMGR transmembrane domains do have weak homology to the mammalian enzyme including some identities to the SSD, but the level of similarity is very low. Nevertheless, embedded in the yeast sequence is information for a similar mode of stability regulation.

Hmg2p Regulated Degradation: A Distant Mirror

The two similar yeast HMGRs have distinct degradative behaviors. Hmg2p undergoes rapid, regulated degradation, whereas Hmg1p is extremely stable, with a half-life in excess of 8 h. Hmg2p half-life is regulated, varying between 6 h and 5–10 min, depending on the level of mevalonate-derived signals (Hampton & Bhakta 1997, Hampton & Rine 1994, Gardner & Hampton 1999b). Because regulated Hmg2p is normally co-expressed with the more abundant and stable Hmg1p (Basson et al. 1986), the role of Hmg2p-regulated degradation is not clear. It may be important in anaerobiosis, where Hmg2p is dominant, and the early isoprenoids that regulate Hmg2p stability would tend to build up (Hampton et al. 1996a). Whatever the function, study of Hmg2p degradation has provided information about both HMGR and the ER degradation pathway.

Hmg2p degradation has numerous similarities to that of mammalian HMGR. Studies with appropriate yeast mutants indicate Hmg2p is degraded in the ER and with no requirement for vacuolar (yeast lysosomal) enzymes (Hampton & Rine 1994). Regulated degradation critically depends on the Hmg2p transmembrane domain. Replacement of the Hmg2p catalytic region with reporter proteins such as Suc2-His4c (Hampton & Rine 1994), or GFP (Cronin & Hampton 1999, Hampton et al. 1996c) results in a non-catalytic fusion that undergoes regulated degradation (In such experiments, regulatory signals must be provided by separately expressed HMGR.) The identical reporter fusions made with the Hmg1p transmembrane region are completely stable, yet still ER-localized. Similarly, the isolated catalytic domains, while completely functional, are stable and unregulated.

The Genetics of HMGR Degradation: HRD Genes and Ubiquitin

To discover yeast *HRD* genes required for HMG-CoA Reductase Degradation, we isolated mutants deficient in this process (Hampton et al. 1996b). A yeast strain was constructed so that degradation was the only mode of control over Hmg2p. In this strain, a single integrated copy of the *HMG2* gene was expressed from constitutively active *TDH3* (glyceraldehyde-3-phosphate dehydrogenase) promoter as

the sole source of HMGR. Thus, HMGR levels are determined by the steady state resulting from degradation and constitutive synthesis of Hmg2p. Deficient degradation caused by mutation results in increased steady-state levels of the Hmg2p and consequent resistance to the growth-inhibiting effects of the HMGR inhibitor lovastatin. The actual HRD selection includes an important technical detail. Normally regulated Hmg2p is stabilized by the selection concentration of lovastatin, causing up-regulation of the Hmg2p and consequently a very high background (1 versus 0.000l% mutant) of physiologically resistant, wild-type survivors. To avoid this pitfall, we used a variant of Hmg2p called 6myc-Hmg2p, with 300 residues from the transmembrane region replaced with six tandem copies of the hydrophilic myc epitope tag. 6myc-Hmg2p is catalytically active but undergoes completely unregulated degradation that is unaffected by lovastatin. Use of a parent strain expressing unregulated 6myc-Hmg2p results in 50 to 75% true mutants among the lovastatin-selected colonies. The resulting *hrd* mutants all stabilize the normally regulated Hmg2p protein as well.

The results of the first HRD selection immediately suggested a mechanism of degradation (Hampton et al. 1996b). *HRD2* encodes the RPN1 subunit of the yeast 26S proteasome, which is part of the 19S regulatory cap involved in substrate recognition and unfolding (Chu et al. 1994, DeMartino et al. 1994, Finley et al. 1998). Thus as in in the mammal, yeast HMGR degradation requires the 26S proteasome. We have subsequently isolated alleles of numerous proteasome genes as *hrd* mutants (N. Bays, unpublished observations).

Ubiquitination is the most common mechanism for targeting proteins for proteasomal destruction. A covalently added polyubiquitin tag consisting of multiple copies of 7.6 kDa ubiquitin allows proteasomal recognition of degradation substrates. Hmg2p (or it degraded variants) undergoes ubiquitination as measured by immunoprecipitation of Hmg2p and subsequent immunoblotting for attached tag (Hampton & Bhakta 1997). Hmg1p or stable variants of Hmg2p do not. Furthermore, Hmg2p ubiquitination is regulated in a manner completely consistent with the physiological regulation: Decreasing degradation signals decreases ubiquitination, whereas increasing degradation signals increases ubiquitination (Gardner & Hampton 1999b, Gardner et al. 2001a, Hampton & Bhakta 1997). Thus the key to Hmg2p regulation lies in the mechanism of this critical tagging step.

Hrd1p and Hrd3p: An ER-Anchored Ubiquitin Ligase

Ubiquitination of a target protein is initiated by formation of an isopeptide bond between a lysine residues (or, in some cases, a particular one) on the target protein and the G76 C terminus of ubiquitin (Ciechanover & Schwartz 2002, Hochstrasser 1996). A polyubiquitin chain is then constructed by repeated addition of the next isopeptide-linked ubiquitin to the K48 residue of the previous one. The resulting chains can include hundreds of ubiquitin molecules, and this structure is specifically recognized by the 19S complex of the 26S proteasome (Thrower et al. 2000). Ubiquitination is catalyzed by an enzyme cascade. First, an ATP-dependent

ubiquitin-activating enzyme, or E1, forms a high-energy thioester adduct with ubiquitin. E1-charged ubiquitin is then transferred as a thioester adduct to one of a small collection (11 in yeast, ~ 50 in mammals) of ubiquitin-conjugating enzymes, called UBCs or E2. Finally, E2-bound ubiquitin is transferred as an isopeptide adduct to the target protein or the growing polyubiquitin chain by a ubiquitin ligase, E3, that is often a multi-protein complex (Deshaies 1999, Gieffers et al. 2001, Joazeiro & Weissman 2000, Page & Hieter 1999, Peters 1998, Tang et al. 2001). Thus the ubiquitin ligase plays a critical role in substrate ubiquitination. Hrd1p and Hrd3p are key components of the ER-associated E3 that ubiquitinates Hmg2p.

Hrd1p is a 510 residue ER-anchored membrane protein with a multi-spanning N-terminal region (residues 1–210) followed by a C-terminal globular domain with a RING-H2 motif between residues 349 and 399 (Bordallo et al. 1998, Hampton et al. 1996b) (Figure 4*a*, see color insert). The ER-anchored C-terminal domain is exposed to the cytosol (Gardner et al. 2000) where the enzymes of ubiquitination reside. The RING-H2 motif is a cysteine-rich zinc-binding module found in catalytic subunits of numerous ubiquitin ligases (Joazeiro & Weissman 2000, Lorick et al. 1999). Hrd1p is absolutely required and rate-limiting for ubiquitination and degradation of Hmg2p or other HRD pathway substrates (Bays et al. 2001a, Bordallo & Wolf 1999, Gardner et al. 2000). The purified Hrd1p RING domain catalyzes ubiquitination of itself and test proteins in vitro (Bays et al. 2001a). Point mutation of the ring (C399S) abolishes in vivo and in vitro Hrd1p activity. Chemical cross-linking, Hrd1p overexpression studies, and direct examination of null mutants indicate that both Ubc7p and Ubc1p participate in ER degradation of Hmg2p, with Ubc7p playing the more prominent role (Figure 4*a*) (Bays et al. 2001a). Ubc7p is anchored to the ER membrane by the small membrane protein Cue1p, which is also required for the HRD pathway (Gardner et al. 2001b).

Hrd3p is an 833 residue type I (N-terminal lumenal) ER membrane protein with a cleavable 20 residue N-terminal signal sequence single-transmembrane span (residues 768–789) near the C-terminus (Gardner et al. 2000, Hampton et al. 1996b). Thus the majority of the Hrd3p sequence is in the lumen of the ER (Figure 4*a*). Hrd3p has at least two functions in ER-associated degradation (Gardner et al. 2000). It is required for stability of Hrd1p: In the absence of Hrd3p, Hrd1p undergoes very rapid (half-life ~5–10 min) RING-H2 domain-dependent degradation, resulting in steady-tate levels of Hrd1p significantly below those of wild-type strains. The Hrd1p-stabilizing function of Hrd3p is important. Overexpression of Hrd1p suppresses the ERAD deficiency of a *hrd3*Δ null (Gardner et al. 2000, Plemper et al. 1999a). Hrd1p and Hrd3p form a stable 1:1 complex in the ER membrane. Cross-linking and co-immunoprecipitation studies indicate that Hrd3p directly interacts with the Hrd1p protein through the Hrd1p transmembrane region. This regulation of the Hrd1p cytosolic RING domain occurs by interaction of the Hrd3p lumenal region with the Hrd1p transmembrane domain. In other words, regulatory information is transmitted across the ER membrane from Hrd3p to Hrd1p (Gardner et al. 2000). This is intriguing because the degradation of ER substrates involves coordination of events and information on each side of the ER membrane.

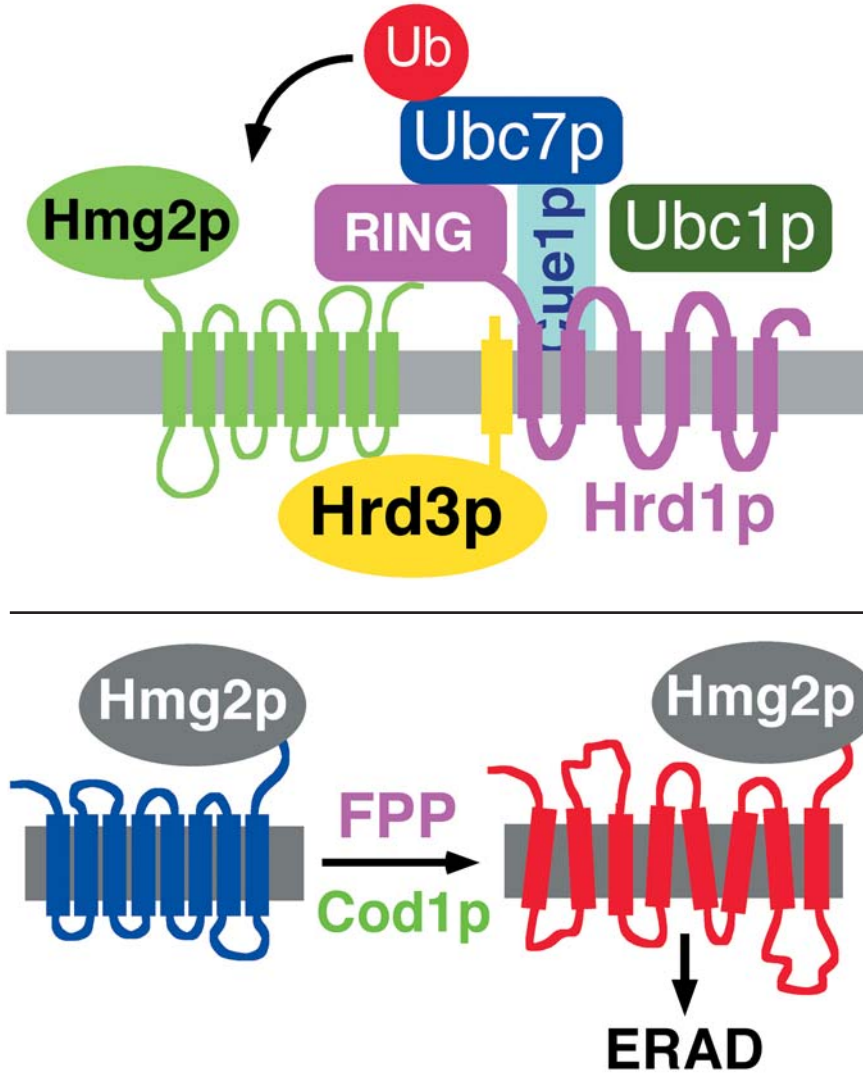

Figure 4 (*Top*) HRD complex ubiquitin ligase and partner ubiquitin conjugating enzymes. ER membrane is gray band. Top is cytosol, bottom is ER lumen. Note that Cue1p is an ER anchor for Ubc7p, as described in text. Hrd1p RING-H2 domain is indicated as RING. (*Bottom*) The structural transition model for regulated HRD pathway degradation. FPP levels must be appropriately high, and the *COD1* gene must be functional to effect the transition to a state that is recognized by the HRD complex.

Hrd3p appears to have a function independent of Hrd1p stability in Hmg2p degradation. A truncation allele of Hrd3p missing the first 356 residues of the lumenal domain (but with the signal sequence intact to allow proper localization) stabilizes Hrd1p but will not support degradation of Hmg2p (Gardner et al. 2000). Thus both Hrd3p and Hrd1p participate in ER degradation when expressed at normal levels in the cell.

RING domain ubiquitin ligases function in part by bringing together appropriate ubiquitin-charged E2s with the substrate targeted by the ligase. We directly tested this model by examining in vivo cross-linking of the principle E2 for Hmg2p degradation, Ubc7p, with HMGR (Gardner et al. 2001b). In vivo, Ubc7p cross-links with degraded Hmg2p or 6myc-Hmg2p, but not with stable Hmg1p. This interaction is regulated; when degradation signals are lowered, the Ubc7p/Hmg2p interaction disappears. Hmg2p interaction with Ubc7p requires both Hrd1p and Hrd3p, indicating that the HRD complex functions as predicted for a ubiquitin ligase (Figure 4*a*). Interestingly, these experiments could not detect any interaction specificity (as measured by in vivo cross-linking) between the Hrd1p/Hrd3p complex and degraded substrate. Although cross-linking was demonstrable, the ligase was just as prone to interact with stable Hmg1p as with Hmg2p. Perhaps this reflects the quality control function of the HRD complex (see below) that must query proteins of widely different sequence to detect hallmarks of misfolding.

Mammalian HMGR undergoes regulated ubiquitination (Ravid et al. 2000), but the responsible ubiquitin ligases are not characterized. There are Hrd1p and Hrd3p homologues in mammalian genomes (Biunno et al. 2000, Donoviel et al. 1998, Fang et al. 2001), and these would be reasonable candidates. However, there are several ER degradation pathways that function with distinct ligases in yeast (Braun et al. 2002, Swanson et al. 2001, Wilhovsky et al. 2000), so the number of possible E3s in the mammal could be large. This will be an interesting avenue to explore in the next few years.

Retrotranslocation: Stuck in the Middle with Ub

The original HRD screen netted key parts of the ubiquitin ligase and a component of the proteolytic corral to which Hmg2p is "HRD-ed". But what about getting the protein out of the ER membrane? The movement of membrane-bound and lumenal ERAD substrates out of the ER to the cytosolic side of the ER or the cytosol for ubiquitination and 26S destruction is a common feature and represents a problem that is receiving much experimental attention. This process is generally referred to as retrotranslocation (Sommer & Wolf 1997). This reverse movement of ER degradation substrates was originally posited from genetic criteria (Hiller et al. 1996, Plemper et al. 1997) and has now been clearly demonstrated to occur (McCracken & Brodsky 1996, Plemper et al. 1999b, Werner et al. 1996) and to proceed continuously in lockstep with ER degradation in the course of normal cellular life (Friedlander et al. 2000, Hampton 2000b, Travers et al. 2000). An oft-cited candidate for mediating retrotranslocation is the translocation pore formed by Sec61

and partner proteins in both yeast and mammals. Because *SEC61* is an essential gene, a definitive genetic test of involvement in ERAD is not possible. There are most certainly cases in which Sec61p can be shown to broker the movement of molecules from the ER to the cytosol (Pilon et al. 1997), but a general role in ERAD has not been unambiguously demonstrated. Hypomorphic alleles of sec61 show defects in ERAD of various substrates (Plemper et al. 1997, 1999a); however, this complex is also required for establishment of most ER functions so such results must be interpreted with caution. A recently derived set of mutants, called sec61-R, reported to be specifically deficient in ERAD retrotranslocation (Zhou & Schekman 1999) had no discernable defect in degradation of Hmg2p, Hmg2p-GFP or unregulated 6myc-Hmg2p (R. Gardner & R. Hampton, unpublished observation). On the other hand, we have observed small defects (twofold) in the degradation rate of Hmg2p in a more traditional sec61-2 mutant. Thus the role of Sec61p in retrotranslocation of Hmg2p, although reasonable and likely, requires more experimental support.

A complex of particular interest in retrotranslocation is composed of the three proteins Npl4p/Cdc48p/Ufd1p, described in a remarkable number of recent studies in the degradation literature (Bays & Hampton 2002). With regard to Hmg2p degradation, the *HRD4* gene was discovered to be identical to *NPL4* (Bays et al. 2001b), and mutants in each complex member stabilize Hmg2p and other ERAD substrates. *HRD4*/*NPL4* mutants have a strong defect in Hmg2p degradation but no deficiency in regulated ubiquitination of Hmg2p, nor in proteasome function. These and the many other studies indicate that the complex is necessary for ubiquitinated ER proteins to be degraded by the proteasome and may indeed be involved in removal of both lumenal and membrane-bound proteins after ubiquitination (Bays et al. 2001b, Dai & Li 2001, Hitchcock et al. 2001, Jarosch et al. 2002, Meyer et al. 2000, Rabinovich et al. 2002, Ye et al. 2001) and/or dismantling complexes that include ubiquitinated species (Braun et al. 2002, Rape et al. 2001). Perhaps this is an ATP-driven motor that pulls retrotranslocating proteins out to the cytosol.

The Signals for Hmg2p Degradation

The mevalonate pathway signals regulating yeast Hmg2p degradation have been analyzed using drugs and molecular genetic methods. Many drugs are not readily permeable in yeast, thus the combination of methods is the most powerful. Inhibition of early pathway enzymes such as HMGR (with statins) or HMG-CoA synthase (with L625,699) leads to rapid decrease in Hmg2p ubiquitination and degradation. Conversely, inhibition of squalene synthase with zaragozic acid (Figure 3) causes a sharp increase in Hmg2p ubiquitination and degradation. The effects of zaragozic acid are fast and quite striking, causing many-fold increases in ubiquitination, and half-lives on the order of 5–10 min (Gardner & Hampton 1999b, Hampton & Bhakta 1997). These results are consistent with FPP (Figure 3), the substrate of squalene synthase, providing a signal for Hmg2p degradation, and genetic experiments bolster this model (Gardner & Hampton 1999b). Overexpression

of squalene synthase causes slowing of Hmg2p degradation and ubiquitination, whereas down-modulation of the same enzyme with a regulated promoter causes increased degradation and ubiquitination. Similar down-modulation of FPP synthase, the enzyme that generates FPP, decreases Hmg2p ubiquitination and degradation. None of these manipulations alters degradation of unregulated HRD pathway substrates, indicating that FPP or something derived from it serves as a degradation signal. These results have a pleasing similarity to the mammalian studies pointing to the importance of FPP.

Mammalian HMGR stability is strongly regulated by both sterols and an early isoprenoid. Yeast Hmg2p degradation relies more heavily on its FPP-derived signal. Perhaps this is why inhibition of squalene synthase causes such clear-cut results in yeast: The effect of increasing FPP-derived signals is not offset by loss of a downstream sterol signal. However, it is still possible that an abundant and non-labile yeast sterol signal exists so that pathway inhibitors only significantly alter FPP in the experimental time frame.

We have found a partial involvement for oxysterols in Hmg2p degradation (Gardner et al. 2001a). Inhibition of oxidosqualene cyclase with Ro48-8071 causes production of oxysterols, by the alternate pathway describe above, through buildup of dioxidosqualene that is then converted to oxylanosterol (Figure 3). Using these drugs and tandem genetic approaches, it appears that oxysterols can influence Hmg2p stability and appear to be needed for a maximal response to the FPP-derived signal. Thus in both yeast and mammal, sterol and FPP-derived signals together bring about destruction of HMGR, but the nature and importance of each may be different.

Sequence Features of Hmg2p Required for Regulated Degradation

The transmembrane domains of Hmg2p and Hmg1p are 50% identical, have identical hydropathy plots and topology, but behave very differently. Hmg1p is quite stable, whereas Hmg2p undergoes regulated degradation. An extensive analysis ($\sim$600 mutants total) was undertaken to discern sequence features responsible for these degradative differences (Gardner et al. 1998, Gardner & Hampton 1999a). Hmg2p regulated degradation is a conspiracy of many necessary conditions distributed across its transmembrane region, and Hmg1p has multiple alterations that affect the function of these determinants. We refer to the information in Hmg2p as a "distributed degron" to distinguish it from the more traditional, autonomously acting linear degrons (Laney & Hochstrasser 1999).

The only amino acids indispensable for regulated degradation are two lysines spaced far apart on the transmembrane region, at position 6 and 357 (Gardner & Hampton 1999a). Conservative replacement of either with arginine causes profound stabilization and loss of ubiquitination. The sequence context of either lysine is fairly relaxed, implying that they are not parts of a specific linear sequence that directs degradation. However, the structural context of these lysines is critical and

includes length and amphipathicity requirements for function. K6 and K357 are absolutely critical for regulation of Hmg2p degradation. Loss of either stabilizes Hmg2p no matter what the level of FPP-derived signal was. In contrast, very subtle aberrations in Hmg2p sequence that render degradation unregulated also remove the dependence on K6 and K357. Thus these two lysines mediate the regulation of Hmg2p stability, but the mechanism is not yet clear. The simplest possibility is that they are ubiquitination sites. However, numerous unregulated Hmg2p variants undergo HRD-dependent degradation that is not dependent on these lysines, indicating that other sites of attachment can be used. Perhaps the correctly folded Hmg2p presents K6 and K357 in a regulated manner for the initial ubiquitination, after which other lysines can serve as sites of modification.

Fishing for COD: The Genetics of Hmg2p Regulation

Hmg2p is the only HRD pathway substrate regulated by the mevalonate pathway. We are seeking any genes that may be involved in this highly specific process. We call these *COD* genes for control of degradation. Their encodees may include enzymes that create the signal, proteins that respond to the signal, transporters that deliver it to the Hmg2p transmembrane domain, proteins that interact with Hmg2p and render it signal-responsive, proteins needed for ER functions involved in regulation, and proteins we have not thought of (the awesome power of genetics). There are two general classes of regulatory deficiencies: mutants that fail to stabilize Hmg2p even when signals are low (constitutive degradation) and mutants that fail to degraded Hmg2p even when signals are high (constitutive stability) and the HRD pathway is intact.

So far we have focused our attention on the constitutive degradation phenotype, that is, mutants that do not stabilize Hmg2p in the presence of lovastatin. We searched for such mutants using a reporter strain engineered to co-expresses Hmg2p as the only source of HMGR, and the normally regulated, optical reporter Hmg2p-GFP (Cronin et al. 2000). The two coding regions are expressed from strong, constitutive TDH3 promoters at distinct loci. The desired *cod* mutants that can not stabilize Hmg2p would show separate phenotypes owing to the effects on each regulated protein. Lovastatin normally causes upregulation of Hmg2p through regulated stabilization, so *cod* mutants deficient in Hmg2p stabilization will have a greater sensitivity to lovastatin because the drug does not upregulate its own target. Similarly, the Hmg2p-GFP reporter will not be upregulated by lovastatin, and the mutants will be less fluorescent than the wild-type on low doses of the drug. These phenotypes were scored in series to isolate *COD1*. [Author's note: *COD1* should not be confused with the identically named genes reported nearly two years after these studies were published (Whyte & Munro 2001).]

Cod1p and the Calcium Connection

In *cod1* mutants, including the viable null, Hmg2p degradation is unresponsive to any manipulation of FPP: Hmg2p is degraded at a reasonable rate (∼1 h half-life)

and neither lovastatin (to slow) nor zaragozic acid (to hasten) has any effect (Cronin & Hampton 1999). Cod1p is a P-type ATPase, first isolated as Spf1p in an unrelated screen (Suzuki & Shimma 1999). Because this class of proteins often pumps ions across membranes, we examined the role of calcium. Indeed *cod1* mutants are suppressed by addition of sufficient calcium to the growth medium, and no other ion tested does this. Similarly, depriving wild-type cells of calcium causes the *cod1* regulatory defect. These results implicate Ca^{2+} in the Hmg2p response to the FPP-derived signal. Intriguingly, earlier experiments with manipulating calcium in cultured mammalian cells similarly showed a role for calcium in the response to the FPP-derived degradation signal (Roitelman et al. 1991).

Cod1p is an ER-resident protein and is required for normal ER functions, including the maintenance of misfolded proteins and proper glycosylation (Cronin et al. 2002). Loss of *COD1* results in up-regulation of a variety of calcium-regulated genes. Before the discovery of *COD1*/*SPF1*, only the Golgi P-type ATPase Pmr1p was thought to be involved in secretory pathway function. Although Cod1p and Pmr1p have some overlapping functions, they are clearly responsible for different processes (Cronin et al. 2002). Most importantly, a *pmr1*Δ null has no deficiency in Hmg2p regulation (Cronin & Hampton 1999). Although Cod1p has numerous phenotypes that all point to calcium, purified Cod1p ATPase activity is unaffected by calcium or by any other ion (Cronin et al. 2002). This is a common indicator of transport substrate, thus this lack of response to any ion allows the possibility that Cod1p is transporting a non-traditional species. Whatever Cod1p pumps, the high conservation of this protein in all metazoans indicates it will play an interesting role in ER function and perhaps in mammalian HMGR regulation.

Sterol Regulation and Protein Quality Control: ERAD Gets a Day Job

Concurrent studies on other ERAD substrates in our and other laboratories indicate that the HRD pathway in yeast is responsible for the degradation of numerous misfolded proteins (Sommer & Wolf 1997). ER degradation of lumenal, misfolded carboxypeptidase Y mutant CPY* requires *DER3*, which is identical to *HRD1* (Bordallo et al. 1998), *HRD3* (Plemper et al 1999a), *UBC7* (Hiller et al. 1996), and to a lesser extent *UBC1* (Freilander et al. 2000). Other misfolded HRD substrates include Sec61-2 (Bordallo et al. 1998), 6myc-Hmg2p (used in the HRD selection) and misfolded versions of Hmg2p (Gardner & Hampton 1999a, Hampton et al. 1996b), the mutant transporters Pdr5p* (Plemper et al. 1998) and UP*, and misassembled Vph1p (Wilhovsky et al. 2000). This function operates continuously. Loss of the HRD ligase causes increased levels of naturally made unfolded proteins and can be lethal when these pools are independently elevated (Friedlander et al. 2000, Travers et al. 2000). In fact, the HRD genes are upregulated by ER stress as a cellular tactic to manage misfolded ER proteins (Travers et al. 2000).

Regulated Production of a Quality Control Substrate

How does normal, natural Hmg2p undergo physiologically regulated entry into the HRD quality control pathway? Hmg2p entry into the HRD pathway occurs after the protein is fully folded (Hampton & Bhakta 1997), and the entire pool of Hmg2p is subject to regulation (Gardner & Hampton 1999a, Hampton & Rine 1994). A simple model of stability control is that Hmg2p undergoes a regulated transition to a structure that is recognized as a HRD quality control substrate (Figure 4).

In this model, a variable fraction of Hmg2p would be in this unfolded state, and this fraction would be greater when degradation signals are high. To test this idea, we took a lesson from cystic fibrosis. The ER-resident CFTR-Δ508 variant of the cystic fibrosis transmembrane regulator (CFTR) is so slow to fold that it is nearly all degraded by ERAD before reaching the mature conformation that is trafficked to the plasma membrane. It has been shown that treating Δ508 expressing cells with glycerol, a chemical chaperone that enhances folding of proteins (Welch & Brown 1996), will cause significant appearance of functional, folded CFTR-Δ508 protein on the cell surface (Brown et al. 1996, Sato et al. 1996). If a similar misfolded state is involved in Hmg2p degradation, then one would predict that chemical chaperones would stabilize the Hmg2p. Treatment of living yeast cells with 10% glycerol causes significant, rapid stabilization of Hmg2p or Hmg2p-GFP (Gardner et al. 2001b). Glycerol has no effect on the function of the HRD pathway itself, nor on the stable Hmg1p isozyme. Furthermore, glycerol does not act through altering degradation signals and thus appears to directly affect Hmg2p or Hmg2p-GFP stability. The degree of stabilization caused by glycerol is the same as that caused by the regulatory action of lovastatin, and the two treatments together cause no added effect. Although these experiments represent a starting point for mechanistic studies of Hmg2p regulation, they indicate that in conditions that promote Hmg2p degradation, it behaves like a misfolded protein.

We have attempted to observe the change in Hmg2p physical state using limited proteolysis of Hmg2p in microsomes derived from cells with high or low degradation signals (Gardner et al. 2001b). The effects of lovastatin and zaragozic acid on the proteolytic susceptibility of microsomal Hmg2p are consistent with a change in physical state caused by altered signals. However, many of the Hmg2p epitopes detected in those studies are exposed to the proteolytic treatment, causing significant loss in detection. We are currently developing molecular tools to obviate these technical hurdles.

Normally multimeric mammalian HMGR has similarly been proposed to undergo a regulated structural transition, to a monmeric state in conditions that favor degradation, This was suggested by study of HMGR with interacting or noninteracting reporter fusions (Cheng et al. 1999b). Many quality control substrates are proteins that are missing their interaction partners, so this model could also be an example of HMGR undergoing regulated change to a structure (monomer) recognized by the quality control apparatus. If true, this would be a highly conserved example of a cellular quality control pathway being harnessed to control levels of a normal protein, an idea with both basic and biomedical implications.

Common Ground in Two Worlds of Regulation: The SSD

Both SCAP and HMGR have SSDs and respond to sterol levels. Are these domains doing something common in these (and other) SSD proteins? Because the SSD-containing regions are not very similar and have a more constrained set of amino acids owing to the biophysical restrictions of being in transmembrane spans, it is not clear how important is the low degree of similarity. On the other hand, very specific point mutants, such as D443N mentioned above, have very significant sterol-related phenotypes. It is worth mentioning that HMGR itself has this conserved D mutated to valine (Hua et al. 1996).

The most oft-mentioned possibility is that the SSD binds sterols. One group has discovered sterol-like drugs that selectivity alter the function of SCAP and appear to interact with the SSD, although these studies have not exhausted all controls for specific interaction (Grand-Perret et al. 2001). Another possibility is that the SSDs are involved in binding effector proteins, and these proteins determine the function of the protein. Perhaps sterols alter this interaction by binding to the SSD or to the interacting protein, or by altering the state of the membrane. Alternatively, the SSD could respond to changes in the ER membrane caused by sterols or other lipid signals. The growing number of SSD-containing proteins under intense study will certainly spawn a large amount of information in short order.

There is an instructive and simple way to view SCAP and HMGR regulation in similar ways, despite the apparent differences in their actions. Sterols (somehow) cause SCAP to be retained in the ER. Sterols (or the FPP-derived signal in yeast) cause HMGR to undergo ER degradation. These two outcomes sound different. However, the ER quality control system effects two responses to misfolded proteins: retention or degradation. The actual result of quality control recognition depends on the specific protein and can vary from one substrate to another (e.g., Gardner & Hampton 1999a, Halaban et al. 2000, Krause et al. 2000, Loayza et al. 1998). Perhaps the common theme is that both SCAP and HMGR undergo a signal-dependent transition to quality control substrate in the ER. In the case of SCAP, ER retention ensues; in the case of HMGR, ER degradation occurs.

The SSD is found in a variety of organisms, including those such as *Drosphila*, that do not synthesize sterols of any sort. In flies, the appropriate players in the SREBP pathway are present, and direct analysis shows that dSREBP undergoes the familiar processing mediated by dSCAP. However, SCAP-dependent regulation is regulated by an entirely distinct lipid, palmitate (!), the unsaturated 16-carbon fatty acid (Seegmiller et al. 2002). Subsequent analysis with RNAi blockade of various lipid metabolic enzymes indicates that the phospholipid phosphatidylethanolamine may be the actual lipid that mediates the regulatory effects of added palmitate (Dobrosotskaya et al. 2002). Clearly, these experments expand the possible models of SSD action and are guaranteed to clarify the correct questions to ask about this widely represented motif. Whether the first S stands for sterol or sometimes sterols, or state of membrane or signal or some other protein, the final answers will be broadly important, useful, and riddled with molecular aesthetics and pleasant surprises.

ACKNOWLEDGMENTS

I thank all members of the Hampton laboratory past and present for their interest, energy, discussions and discoveries. Together they have made our science home a most special place. See them at http://www.hamptonlab.ucsd.edu. I also thank Mike Brown, Peter Edwards, Mark Hochstrasser, Randall Johnson, David Katzman, Vivek Malhotro, Axel Nohturfft, Adam Richman, and David Rudner for input, information, opinions, discussions, and time. I dedicate this article to the memory of my parents Robert and Harriet, who wouldn't care much about its content but would be happy about its existence. This work is supported by the National Institutes of Health grant number 2 RO1 DK51996-06.

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