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REVIEW ARTICLE

Control of cholesterol synthesis through regulated ER-associated degradation of HMG CoA reductase

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Abstract

Multiple mechanisms for feedback control of cholesterol synthesis converge on the rate-limiting enzyme in the pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase. This complex feedback regulatory system is mediated by sterol and nonsterol metabolites of mevalonate, the immediate product of reductase activity. One mechanism for feedback control of reductase involves rapid degradation of the enzyme from membranes of the endoplasmic reticulum (ER). This degradation results from the accumulation of sterols in ER membranes, which triggers binding of reductase to ER membrane proteins called Insig-1 and Insig-2. Insig binding leads to the recruitment of a membrane-associated ubiquitin ligase called gp78 that initiates ubiquitination of reductase. Ubiquitinated reductase then becomes extracted from ER membranes and is delivered to cytosolic 26S proteasomes through an unknown mechanism that is mediated by the gp78-associated ATPase Valosin-containing protein/p97 and appears to be augmented by nonsterol isoprenoids. Here, we will highlight several advances that have led to the current view of mechanisms for sterol-accelerated, ER-associated degradation of reductase. In addition, we will discuss potential mechanisms for other aspects of the pathway such as selection of reductase for gp78-mediated ubiquitination, extraction of the ubiquitinated enzyme from ER membranes, and the contribution of Insig-mediated degradation to overall regulation of reductase in whole animals.

Keywords: *Cholesterol metabolism; ubiquitination; 26S proteasome; ubiquitin ligase; sterol regulatory element-binding protein; Scap*

Introduction

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase catalyzes the conversion of HMG CoA to mevalonate, a rate-determining step in the synthesis of not only cholesterol, but also of nonsterol isoprenoids that are essential for normal cell function (Figure 1) (Goldstein and Brown, 1990). These molecules include ubiquinone and hemeA, which participate in aerobic cellular respiration, dolichol, which is required for the synthesis of N-linked glycoproteins, and the farnesyl and geranylgeranyl groups that become attached to various cellular proteins, increasing their membrane association. As the rate-limiting enzyme in cholesterol synthesis, reductase is the target of a complex, multivalent feedback regulatory system that is mediated by sterol and nonsterol end-products of mevalonate metabolism (Brown and Goldstein,1980). This complex regulatory system operates at transcriptional and post-transcriptional levels and guards against the overaccumulation of cholesterol while ensuring that essential nonsterol isoprenoids are constantly produced.

The complexity of the multivalent control of reductase was first revealed through the use of compactin (also known as ML-236B), a founding member of the statin family of competitive reductase inhibitors that was first isolated from the fungus *Penicillium citrinum* by Endo and co-workers in the 1970s (Endo *et al.*, 1976a; Endo *et al.*, 1976b). The activity of reductase is largely suppressed when cells are cultured under normal culture conditions (i.e. medium supplemented with fetal calf serum) and, as a result, cholesterol and nonsterol

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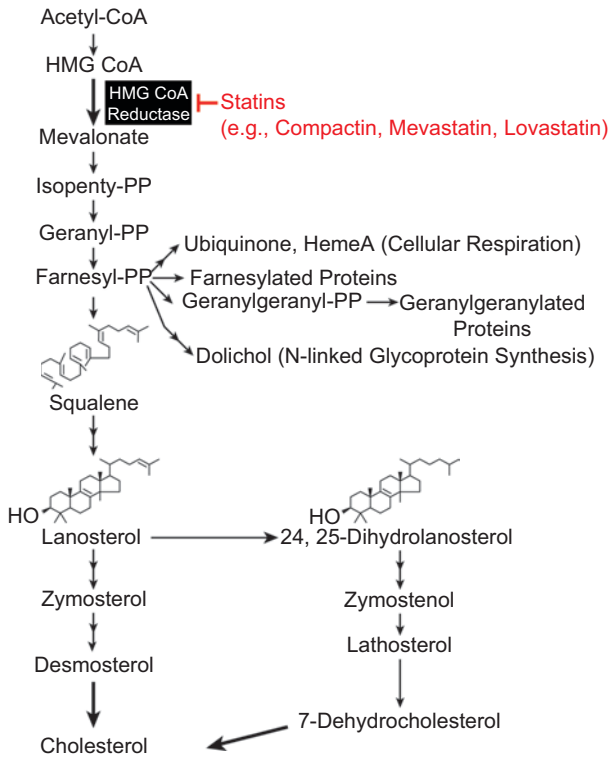

Figure 1. Schematic representation of the mevalonate pathway in animal cells. Statins, competitive inhibitors of HMG CoA reductase, are highlighted in red. The abbreviation "PP" (e.g. as in isopentyl-PP) designates pyrophosphate.

isoprenoids are produced at low rates. This suppression results from the receptor-mediated uptake of cholesterol-rich low-density lipoproteins (LDLs) present in the fetal calf serum of culture medium (Brown and Goldstein, 1986). Internalized cholesterol is utilized in the synthesis of cell membranes; excess cholesterol becomes esterified and stored in cytoplasmic lipid droplets as cholesterol esters. The sterol also suppresses reductase activity by inhibiting the enzyme's expression through the multivalent regulatory system. Subjecting cells to cholesterol deprivation through incubation in medium supplemented with lipoprotein-deficient serum plus compactin triggers a massive increase in the amount of reductase protein (Brown *et al.*, 1978). This compensatory increase in reductase results from the combined effect of three regulatory events: enhanced transcription of the reductase gene, enhanced translation of the reductase mRNA, and extended half-life of the reductase protein (Brown and Goldstein, 1980). Complete suppression of reductase in compactin-treated cells requires the addition of exogenous mevalonate together with LDL or oxysterols, oxygenated forms of cholesterol that are readily taken up by cells (Goldstein and Brown, 1990). Together, these findings formed the basis for the concept that multiple feedback mechanisms mediated by sterol and nonsterol end-products of mevalonate metabolism control the levels and activity of reductase.

Sterol and nonsterol isoprenoids inhibit reductase at different levels. For example, sterols inhibit the activity of sterol regulatory element-binding proteins (SREBPs), a family of membrane-bound transcription factors that enhance the uptake and synthesis of cholesterol by activating transcription of the genes encoding reductase and other cholesterol biosynthetic enzymes as well as the LDL-receptor (Horton *et al.*, 2002). Translation of reductase mRNA is blocked by a nonsterol isoprenoid (Nakanishi *et al.*, 1988). Although the identity of this regulatory product and its mechanism of action is unknown, the reaction may be mediated by the complex 5'-untranslated region of the reductase mRNA (Reynolds et al., 1985). Sterol and nonsterol isoprenoids combine to reduce the half-life of reductase protein in compactin-treated cells from 11–12 h to less than 1 h by accelerating its endoplasmic reticulum (ER)-associated degradation (ERAD) from membranes through a mechanism mediated by the ubiquitin-proteasome system (Inoue *et al.*, 1991; Ravid *et al.*, 2000; Sever *et al.*, 2003b).

The ER-associated degradation (ERAD) pathway

The ER is a major site of protein biogenesis with roughly 30% of all newly synthesized proteins becoming translocated across membranes into the lumen of the organelle (Huh *et al.*, 2003). Soon after their translocation, nascent polypeptides undergo folding and assembly through the assistance of a repertoire of ER-resident molecular chaperones (Buck *et al.*, 2007). Translocated proteins are also subject to co- and post-translational modifications such as N-linked glycosylation and disulfide-bond formation, which promote proper folding (Helenius and Aebi, 2004). Proteins that do not fold into their native conformations or fail to become incorporated into oligomeric complexes because of genetic mutation, cellular stress, or translational and transcriptional errors are selectively degraded in the cytosol by the 26S proteasome through a process known as ERAD (Jarosch *et al.*, 2003; Meusser *et al.*, 2005; Vembar and Brodsky, 2008). Efficient destruction of defective proteins is essential as they may lead to formation of toxic, insoluble aggregates or compete with functional counterparts for substrate binding and/or complex formation with interacting proteins. Many human diseases and pathologies are linked to known ERAD substrates, which further highlights the importance of the ERAD pathway (Aridor, 2007).

The highly conserved ERAD pathway is a multistep process that begins with the recognition of misfolded substrates, which appears to be carried out by a select set of molecular chaperones (Vembar and Brodsky, 2008). The variety of ERAD substrates can be enormous; potential

substrates can be either completely soluble within the lumen of the ER or integrated in membranes through one or more membrane-spanning segments. Thus, regions of these proteins that are located in the cytosol, within the ER lumen, and embedded in membranes must be stringently screened for misfolding (Carvalho *et al.*, 2006; Denic *et al.*, 2006). In the yeast *Saccharomyces cerevisiae*, detection of misfolded proteins engages three distinct ERAD pathways, depending upon the location of the misfolded region (Ahner and Brodsky, 2004). The ERAD-C pathway becomes engaged when misfolded cytosolic domains are detected, whereas detection of misfolded domains within the ER lumen engages the ERAD-L pathway. Key mediators of the ERAD-C and ERAD-L pathways are cytosolic and ER lumenal heat shock protein homologs (e.g. Hsp70, Hsp40, and Hsp90), which recognize hallmarks of misfolding, such as exposure of hydrophobic amino acid residues that are normally sequestered within the core of the folded protein (Buck *et al.*, 2007). A subset of misfolded glycoproteins present a single glucose moiety on their N-linked glycans, which promotes association with the lectin-like ER lumenal chaperones calnexin and calreticulin for additional rounds of folding cycles (Caramelo and Parodi, 2008). Prolonged association with calnexin/calreticulin leads to degradation of these substrates through the ERAD-L pathway. The third ERAD pathway, designated ERAD-M, is engaged through the detection of misfolded regions within the membrane. It has been reasonably postulated that ERAD-M substrates present hydrophilic amino acid residues within the hydrophobic environment of the membrane bilayer (Hampton and Garza, 2009). However, the precise mechanism through which these intramembrane lesions are recognized (perhaps through the action of unknown chaperones) and how this recognition engages the ERAD-M pathway is presently unclear. It is important to note that the three ERAD pathways have only been defined in yeast. Although mammalian cells can potentially present a much larger repertoire of misfolded proteins, it seems likely that some aspects of the yeast ERAD pathways, such as chaperone-mediated selection, are applicable to degradation of mammalian substrates. Moreover, these substrates would almost certainly be able to be classified as ERAD-L, ERAD-C, and ERAD-M.

Once selected for ERAD, it is generally accepted that substrates become dislocated from ER membranes into the cytosol where they are fully accessible to proteasomes for degradation. Most ERAD substrates become ubiquitinated, which ensures their efficient delivery to proteasomes, by ubiquitin-conjugating and ligating enzymes that transfer activated ubiquitin from the ubiquitin-activating enzyme (Pickart, 1997; Kostova *et al.*, 2007). The specificity of substrate ubiquitination is primarily determined by ubiquitin ligases (Deshaies and Joazeiro, 2009). It is assumed that chaperones not only mediate selection of ERAD substrates, but that they also mediate substrate selection by facilitating interactions with ubiquitin ligases through the actions of intermediary proteins or substrate selectors (Buck *et al.*, 2007). In addition to this, it is very likely that other mechanisms for selection of ERAD substrates for ubiquitination exist.

The final steps of the ERAD pathway constitute delivery of ubiquitinated substrates to proteasomes through reactions mediated in part by Valosin-containing protein (VCP)/p97, a member of the AAA (ATPases associated with diverse cellular activities)-ATPase superfamily (Ye *et al.*, 2001; Vij, 2008). VCP/p97 associates with ubiquitinated proteins through two substrate recruitment factors, Npl4 and Ufd1, which bind polyubiquitin chains. The ATPase activity of VCP/p97 is thought to drive extraction of ERAD substrates from ER membranes into the cytosol. In some cases, extraction is mediated by the 19S regulatory subunit of the proteasome, which also contains AAA-ATPase activity (Wahlman *et al.*, 2007). It is generally accepted that soluble ERAD substrates are transported across membranes into the cytosol through a protein-conducting channel formed by either Sec61, the major component of the translocation channel that imports polypeptides into the ER, or by the Derlin family of polytopic membrane proteins (Lilley and Ploegh, 2004; Ye *et al.*, 2004; Meusser *et al.*, 2005). Like their soluble counterparts, membrane-bound ERAD substrates are dislocated into the cytosol prior to proteasomal degradation. This has been demonstrated for substrates that contain one or more membrane-spanning segments (MHC Class I heavy chains and unpaired T-cell receptor subunits and cystic fibrosis transmembrane conductance receptor, Ste6p*, and connexins, respectively) (Wiertz *et al.*, 1996a; 1996b; Huppa and Ploegh, 1997; VanSlyke and Musil, 2002). Whether cytosolic dislocation of membrane-bound ERAD substrates requires a protein-conducting channel formed by Sec61 or Derlins is not known. It should be noted that some membrane-bound ERAD substrates appear to be degraded directly from membranes (Brodsky and Wojcikiewicz, 2009; Ikeda *et al.*, 2009). This degradation could be initiated at either end of the misfolded polypeptide or from an internal site following an endoproteolytic cleavage through a mechanism in which degradation and extraction are tightly coupled.

Following extraction, VCP/p97 appears to play another role in ERAD by facilitating delivery of substrates to proteasomes through interactions with a variety of ubiquitin regulatory X (UBX), ubiquitin-associated (UBA), and ubiquitin-like (UBL) domain-containing proteins (Schuberth and Buchberger, 2008). These proteins include Ufd2, an E4 enzyme that extends polyubiquitin chains (Koegl *et al.*, 1999), the deubiquitinating enzyme Otu1, and Rad23 and Dsk2, which simultaneously bind

to polyubiquitin chains and the proteasome (Raasi and Wolf, 2007).

Current view of sterol-accelerated ERAD of HMG CoA reductase

Mammalian HMG CoA reductase consists of 887 or 888 amino acids that can be separated into two domains (Figure 2A). The N-terminal domain of reductase encompasses 339 amino acids; the region is embedded into ER membranes through eight membrane-spanning segments separated by short hydrophilic loops (Roitelman *et al.*, 1992). The 548 amino acid C-terminal domain projects into the cytosol where it exerts all of the enzymatic activity (Gil *et al.*, 1985; Liscum *et al.*, 1985). The membrane domain of reductase is highly conserved across mammalian species (Luskey and Stevens, 1985) and the region plays a key role in sterol-accelerated degradation of the enzyme as indicated by two early observations. First, the truncated, cytosolic C-terminal domain of reductase restores cholesterol synthesis when expressed in reductase-deficient Chinese hamster ovary (CHO) cells (Gil *et al.*, 1985). However, this protein is very stable and does not become rapidly degraded in the presence of sterols. The second observation stemmed from studies of a fusion protein between the membrane domain of reductase and soluble β-galactosidase. This reductase membrane domain-β-galactosidase fusion protein exhibits sterol-accelerated degradation that is similar to the wild type, full-length reductase (Skalnik *et al.*, 1988). Considered together, these key observations are consistent with a mechanism whereby the membrane domain of reductase senses levels of membrane-embedded sterols, triggering reactions that render the enzyme susceptible to proteolytic degradation.

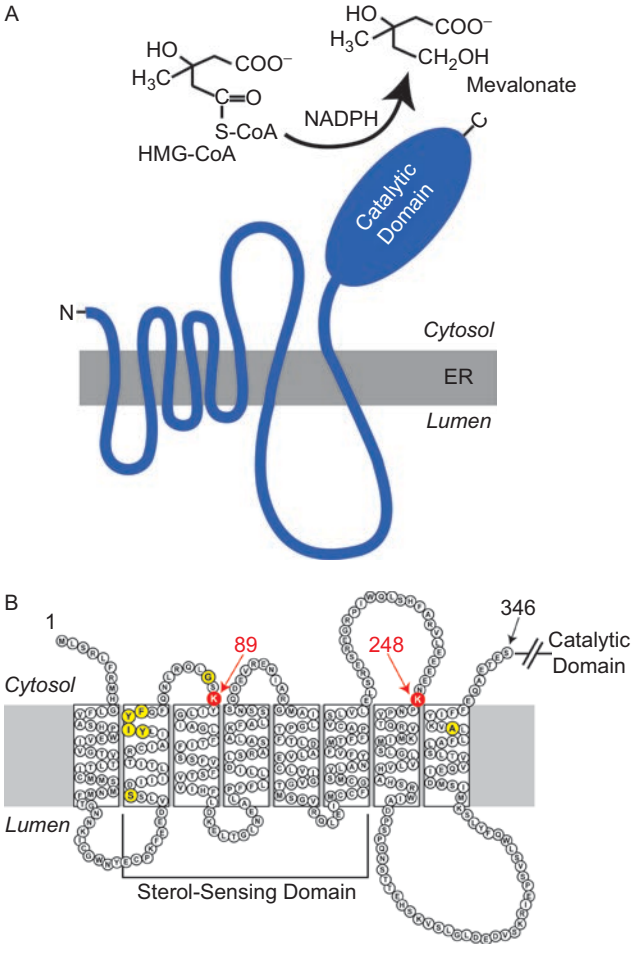

Figure 2. Domain structure of HMG CoA reductase. (A) As discussed in the text, HMG CoA reductase consists of two distinct domains: a hydrophobic N-terminal domain with eight membrane-spanning segments that plays a key role in sterol-accelerated degradation of the enzyme and a hydrophilic C-terminal domain that directs enzymatic activity. (B) Amino acid sequence and topology of the membrane domain of HMG CoA reductase. The lysine residues that are required for Insig-mediated, sterol-induced ubiquitination of HMG CoA reductase are enlarged, highlighted in red, and denoted by arrows. Sequences required for sterol-regulated binding of HMG CoA reductase to Insigs (YIYF, Ser-60, Gly-87, and Ala-333) are enlarged and highlighted in yellow.

The membrane domain of reductase contains a stretch of ~180 amino acids called the sterol-sensing domain (Figure 2B). This evolutionarily conserved domain comprises five of the eight membrane-spanning segments of reductase and is found in several other polytopic membrane proteins that are postulated to interact with sterols (Kuwabara and Labouesse, 2002). These proteins include the sterol-regulated escort protein Scap (Hua *et al.*, 1996), the lipid transport proteins Niemann Pick C1 (NPC1) and NPC1L1 (Loftus *et al.*, 1997; Altmann *et al.*, 2004), the Patched receptor for the cholesterol-modified morphogen Hedgehog (Eaton, 2008), and Dispatched, which mediates release of Hedgehog from cells (Burke *et al.*, 1999). The function of the sterol-sensing domain was first demonstrated for Scap, which binds to the SREBP transcription factors in the ER (Hua *et al.*, 1996). In sterol-deprived cells, Scap facilitates transport of SREBPs from the ER to the Golgi where active fragments of the transcription factor are released from membranes by proteolysis (DeBose-Boyd *et al.*, 1999; Goldstein *et al.*, 2006). The processed forms of SREBPs migrate to the nucleus and activate target gene expression, which leads to increased synthesis and uptake of cholesterol and other lipids (Horton *et al.*, 2002). When sterols accumulate in ER membranes, the membrane domain of Scap binds to one of two ER membrane proteins called Insig-1 and Insig-2 (Yabe *et al.*, 2002a; Yang *et al.*, 2002). Insig binding blocks incorporation of Scap-SREBP into COPII-coated vesicles that bud from ER membranes and deliver proteins to the Golgi (Nohturfft *et al.*, 2000; Sun *et al.*, 2007). Sequestration of Scap-SREBP complexes in the ER prevents proteolytic activation of SREBPs; expression of

SREBP target genes declines and consequently, cholesterol synthesis and uptake is suppressed.

The topology of Scap in ER membranes is similar to that of reductase. The protein is anchored to membranes through its N-terminal domain, which includes eight membrane-spanning segments (Nohturfft *et al.*, 1998b). The C-terminal domain projects into the cytosol and mediates association with SREBPs (Sakai *et al.*, 1997). The sterol-sensing domain of Scap comprises transmembrane helices 2–6 and exhibits 55% amino acid similarity and 25% identity with the corresponding region of reductase. The importance of the sterol-sensing domain in the regulation of Scap is highlighted by findings that three point mutations (Tyr-298 to Cys, Leu-315 to Phe, and Asp-443 to Asn) within the region abolish sterol-regulated Insig binding, thereby relieving sterol-mediated ER-retention of mutant Scap-SREBP complexes (Hua *et al.*, 1996; Nohturfft *et al.*, 1996; 1998a; Yabe *et al.*, 2002b; Yang *et al.*, 2002).

Insigs also bind to the sterol-sensing domain of reductase in a sterol-regulated fashion (Sever *et al.*, 2003b). This binding is disrupted by mutation of the tetrapeptide sequence YIYF, which is located in the second transmembrane segment of reductase (Figure 2B). Mutation of the YIYF sequence to alanine residues abolishes Insig binding and the mutant enzyme is no longer subjected to sterol-accelerated degradation (Sever *et al.*, 2003a). The first tyrosine of the YIYF tetrapeptide (Tyr-75) is equivalent to Tyr-298 of Scap, which is required for Insig–Scap binding (see above). When overexpressed, the sterol-sensing domain of Scap blocks sterol-accelerated degradation of reductase (Sever *et al.*, 2003b). This effect is ablated by the Tyr-298 to Cys mutation in the Scap sterol-sensing domain, indicating that Scap and reductase binding sites on Insigs overlap. At least three additional amino acids (Ser-60, Gly-87, and Ala-333; see Figure 2B) within the membrane domain of reductase are also required for Insig binding (Lee *et al.*, 2007). Even though Ser-60 and Gly-87 localize to the sterol-sensing domain of reductase, these residues are not present in the corresponding region of the Scap sterol-sensing domain. These observations emphasize the importance of detailed structural analyses of Scap-Insig and reductase-Insig complexes in future studies.

Two major differences exist between the Insig-mediated regulation of Scap and that of reductase. Insig binding to Scap leads to its retention in the ER, whereas Insig binding to reductase causes it to become rapidly ubiquitinated and degraded. This discrepancy can be rationalized when considering the other major difference between Insig-mediated regulation of Scap and reductase: sterol specificity. Cholesterol directly binds to the membrane domain of Scap, triggering a conformational change in the protein that allows for Insig binding (Radhakrishnan *et al.*, 2004). In contrast, cholesterol does not potently induce rapid ubiquitination of reductase, even when added to sterol-deprived membranes *in vitro* (Song and DeBose-Boyd, 2004). Instead, the reaction is potently stimulated by the cholesterol synthesis intermediate 24,25-dihydrolanosterol both *in vitro* and in intact cells (Song *et al.*, 2005a) (Figure 1). It should be noted that lanosterol, the immediate precursor of 24,25-dihydrolanosterol (see Figure 1), was also found to stimulate ubiquitination of reductase. However, it was subsequently determined that this activity was attributable to small amounts of contaminating 24,25-dihydrolanosterol in the preparations of lanosterol used in the initial studies (Lange *et al.*, 2008). The specificity of reductase ubiquitination is remarkable considering that lanosterol and 24,25-dihydrolanosterol only differ in the degree of side-chain saturation. This suggests that the mechanism through which 24,25-dihydrolanosterol stimulates ubiquitination of reductase likely involves its direct binding to the enzyme. However, attempts to demonstrate direct binding of 24,25-dihydrolanosterol to reductase have so far been unsuccessful. Thus, the possibility that some other protein binds 24,25-dihydrolanosterol and induces reductase to bind Insigs cannot be excluded.

The findings described above not only help to explain how Insigs mediate sterol regulation of Scap and reductase through distinct mechanisms, but they also point to the production of 24,25-dihydrolanosterol as a key focal point in sterol regulation. The demethylation of lanosterol and 24,25-dihydrolanosterol has been implicated as a rate-limiting step in the sterol branch of the mevalonate pathway (Williams *et al.*, 1977; Gaylor, 2002). 24,25-Dihydrolanosterol suppresses its own synthesis by reducing flux through the mevalonate pathway via Insig-mediated degradation of reductase. Accumulation of lanosterol and 24,25-dihydrolanosterol is avoided because these sterols do not inhibit ER-to-Golgi transport of Scap-SREBP (Song *et al.*, 2005a). Thus, mRNAs encoding the enzymes that catalyze reactions subsequent to lanosterol synthesis remain elevated and lanosterol and 24,25-dihydrolanosterol are efficiently converted to cholesterol. As cholesterol begins to accumulate, Scap-SREBP transport to the Golgi is blocked, SREBP processing becomes inhibited, and the entire pathway is shut down. The physiologic relevance of 24,25-dihydrolanosterol as a regulator of reductase degradation is highlighted by the finding that oxygen deprivation causes the sterol to accumulate in cells (Nguyen *et al.*, 2007). At the same time, expression of both Insigs is enhanced through the action of the oxygen-sensitive transcription factor hypoxia-inducible factor (HIF)-1α. The accumulation of 24,25-dihydrolanosterol, coupled with HIF-mediated induction of Insigs, leads to rapid degradation of reductase, providing a link between oxygen sensing and cholesterol metabolism.

Targeting HMG CoA reductase for proteasomal degradation: sterol-regulated, Insig-mediated ubiquitination

An early clue as to the identity of the proteolytic machinery responsible for reductase degradation was provided by the observation that inhibitors of the proteasome block the reaction (Inoue *et al.*, 1991). This led to the finding that proteasome inhibition leads to the accumulation of ubiquitinated forms of reductase on ER membranes (Ravid *et al.*, 2000). A role for the ubiquitin-proteasome pathway is consistent with observations by Hampton and co-workers in *S. cerevisiae* (Hampton and Bhakta, 1997). Hmg2 is one of two reductase isozymes expressed in yeast; the protein is rapidly degraded when flux through the mevalonate pathway is high. Hmg1, the other reductase isozyme, is not subject to regulated degradation.

The genetic analysis of Hmg2 degradation led to the identification of genes encoding several components of the ubiquitin–proteasome pathway as mediators of the reaction (Hampton, 1998; Hampton and Garza, 2009). These genes are termed HRD genes, for HMG CoA reductase degradation, and include: Hrd1, a Really Interesting New Gene (RING) finger ubiquitin ligase with multiple membrane-spanning segments followed by a large cytosolic domain; Hrd2, a component of the 26S proteasome; Hrd3, the binding partner of Hrd1 that mediates substrate selection of the enzyme; and Hrd4, the yeast homolog of Npl4, one of at least two ubiquitin-binding substrate selectors for VCP/p97 (Vij, 2008). Like mammalian reductase, the membrane domain of Hmg2 is necessary and sufficient for accelerated degradation (Hampton *et al.*, 1996). However, degradation of Hmg2 is stimulated by nonsterol isoprenoids derived from mevalonate, but not by sterols (Garza *et al.*, 2009b; Hampton and Garza, 2009). The yeast Insig protein, called Nsg1, does not promote degradation of Hmg2. Instead, Nsg1 stabilizes Hmg2, even in the presence of degradative signals (Flury *et al.*, 2005). Despite these differences, regulated ubiquitination and subsequent ERAD of reductase is a common mechanism both yeast and mammalian systems use to limit synthesis of sterols.

Ubiquitination of mammalian reductase is obligatory for sterol-accelerated degradation of the enzyme and the reaction exhibits an absolute requirement for the action of Insigs. For example, reductase overexpressed in cells by transfection resists both sterol-accelerated ubiquitination and degradation (Sever *et al.*, 2003a). These processes are restored by co-expression of Insig-1 or Insig-2, suggesting saturation of endogenous Insigs by the overexpressed reductase. RNA interference (RNAi)-mediated knockdown of Insig-1 and Insig-2 mRNA or mutation of genes encoding both Insigs abrogates sterol-mediated ubiquitination of reductase and renders the enzyme refractory to accelerated degradation (Sever *et al.*, 2003a; Sever *et al.*, 2004; Lee *et al.*, 2005). Finally, mutation of the YIYF sequence as well as Ser-60, Gly-87, and Ala-333 in reductase abolishes Insig binding and markedly blunts the enzyme's sterol-accelerated ubiquitination (Sever *et al.*, 2003a; Lee *et al.*, 2007). The ubiquitination of reductase is also blocked by conservative substitutions of arginine for two cytosolically exposed lysine residues at positions 89 and 248 in the membrane domain of reductase (Figure 2B). While these mutations prevent reductase degradation, they do not block sterol-induced binding of the enzyme to Insigs. Thus, lysines 89 and 248 in reductase are implicated as sites of Insig-dependent, sterol-induced ubiquitination. The catalytic domain of reductase does not become ubiquitinated as indicated by the observation that mutation of lysines 89 and 248 blocks degradation in the context of the full-length protein. This is consistent with observations that the soluble catalytic domain is dispensable for regulated ubiquitination and degradation.

A subset of Insig molecules is associated with a membrane-bound ubiquitin ligase called gp78 (Song *et al.*, 2005b). The enzyme consists of 643 amino acids and contains an N-terminal domain with 5–7 membrane-spanning segments that mediates association with Insigs. The C-terminal domain of gp78 projects into the cytosol and contains a RING finger domain that is required for ubiquitin ligase activity as well as binding sites for VCP/p97 and the ubiquitin-conjugating enzyme Ubc7 (Kostova *et al.*, 2007). The role for gp78 in sterol-accelerated degradation of reductase is illustrated by several observations. The overexpressed membrane domain of gp78 competes with the full-length enzyme for Insig binding and blocks sterol-accelerated degradation of reductase. Moreover, a mutant form of gp78 harboring inactivating mutations in the RING finger domain exhibits dominant-negative activity towards reductase degradation. Sterols trigger binding of gp78 to reductase, but only when Insigs are co-expressed. The specificity of this interaction is demonstrated by the finding that gp78 does not bind to Scap, regardless of the absence or presence of sterols. Finally, RNAi-mediated knockdown of gp78 blunts sterol-induced ubiquitination and degradation of endogenous reductase. This effect is specific inasmuch as knockdown of mammalian Hrd1, a membrane-bound ubiquitin ligase that resembles gp78 (see below), does not affect reductase ubiquitination. This result is consistent with the failure of Hrd1 to interact with Insig-1 as determined by co-immunoprecipitation experiments.

The pathway for Insig-mediated, sterol-accelerated ERAD of reductase is shown in Figure 3. The process is initiated by the sensing of membrane embedded sterols through direct or indirect interactions with the membrane domain of reductase. This reaction triggers binding of the Insig–gp78 complex to the membrane domain of reductase, resulting in transfer of ubiquitin from the E2

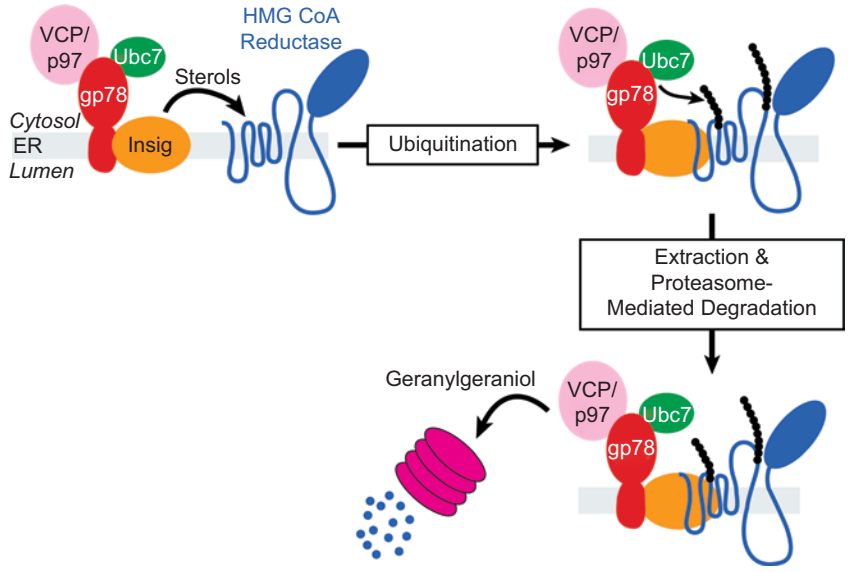

Figure 3. Current model for sterol-accelerated ERAD of HMG CoA reductase. Accumulation of certain sterols (e.g. oxysterols such as 25-hydroxycholesterol and the cholesterol synthesis intermediate 24,25-dihydrolanosterol) stimulates binding of Insigs to the membrane domain of HMG CoA reductase. Some of the Insig molecules are associated with gp78, a membrane-anchored ubiquitin ligase that associates with the ubiquitin conjugating enzyme Ubc7 and the AAA-ATPase VCP/p97. Ubc7 and gp78 combine to initiate the polyubiquitination of two cytosolic lysine residues in the membrane domain of HMG CoA reductase. This ubiquitination triggers extraction of HMG CoA reductase from ER membranes through the action of VCP/p97 and its associated cofactors; this step appears to be enhanced by the 20-carbon nonsterol isoprenoid geranylgeraniol through an undefined mechanism. Once extracted, HMG CoA reductase is delivered to proteasomes for degradation.

Ubc7 to lysines 89 and 248 in reductase. Ubiquitination marks reductase for recognition by the gp78-associated VCP/p97 that, together with its cofactors, somehow extract ubiquitinated reductase from ER membranes and delivers it to proteasomes for degradation. Although the 20-carbon nonsterol isoprenoid geranylgeraniol (GGOH) augments sterol-accelerated ERAD of reductase, the compound does not appreciably affect ubiquitination of reductase when added to cells (Sever *et al.*, 2003a). In addition, GGOH does not appear to augment *in vitro* ubiquitination of reductase in a specific manner (unpublished observations). This contrasts the situation in yeast, where it has been recently determined that the phosphorylated derivative of GGOH, GG-pyrophosphate, stimulates ubiquitination of Hmg2p (Garza *et al.*, 2009b). Thus, we postulate that GGOH augments degradation of mammalian reductase by enhancing the extraction of the ubiquitinated enzyme from ER membranes, facilitating its delivery to proteasomes for degradation (Sever *et al.*, 2003a). The possibility exists that GGOH is converted to GG-pyrophosphate and becomes incorporated into a protein that mediates the membrane extraction of ubiquitinated reductase. Geranylgeranylated proteins include the well-known Rab family of proteins that participate in various aspects of vesicular transport (Seabra *et al.*, 2002). Thus, a vesicle-mediated transport event may deliver ubiquitinated reductase from ER membranes to a specific organelle or subdomain of the ER where the protein is subsequently degraded.

Unresolved and remaining questions

Despite substantial advances over the past several years in the understanding of sterol-accelerated ERAD of reductase, many questions remain unresolved. For example, how does Insig binding lead to selection of reductase for gp78-mediated ubiquitination? How is ubiquitinated reductase extracted from ER membranes, and do nonsterol isoprenoids augment this reaction? How does sterol-accelerated ERAD contribute to regulation of reductase in the liver, the major site of cholesterol synthesis?

Selection of reductase for gp78-mediated ubiquitination

A model for ERAD of proteins with misfolded lumenal domains (ERAD-L substrates) is beginning to emerge from studies conducted in yeast (Carvalho *et al.*, 2006; Denic *et al.*, 2006). The yeast Hrd1 enzyme mediates ubiquitination of ERAD-L and ERAD-M substrates; another membrane-bound ubiquitin ligase, Doa10, mediates ubiquitination of ERAD-C substrates. Hrd1 exists in a large, multiprotein complex containing its cofactor Hrd3, the cytosolic ubiquitin-conjugating enzyme Ubc7 and its membrane anchor Cue1, the polytopic ER membrane protein Der1 and its recruitment factor Usa1, the UBX domain-containing protein Ubx2, which mediates recruitment of the AAA-ATPase cdc48, and the Hsp70 chaperone Kar2 bound to the lectin Yos9 (Figure 4). The

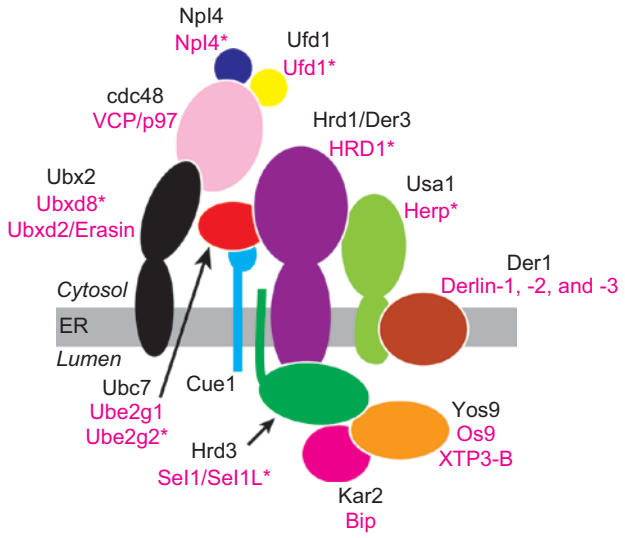

Figure 4. The *S. cerevisiae* Hrd1 ubiquitin ligase complex. Schematic representation of the Hrd1 complex in yeast that includes factors involved in substrate selection (Kar2 and Yos9), ubiquitination (Ubc7 and Cue1), and recruitment of cdc48 (Ubx2) and Der1 (Usa1). Yeast proteins are shown in black and their mammalian homologs are shown in magenta. Hrd1 complex components required for Insig-mediated degradation of HMG CoA reductase in *Drosophila* S2 cells are denoted by asterisks.

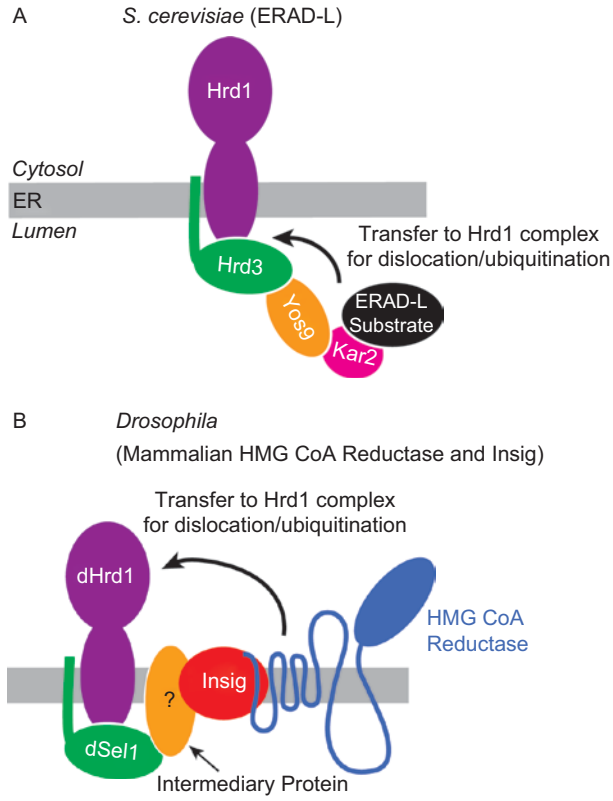

Figure 5. Proposed model for Insig-mediated selection of mammalian HMG CoA reductase for ubiquitination/degradation. (A) As discussed in the text, Hrd1-mediated degradation of proteins with misfolded lumenal domain (ERAD-L substrates) in yeast begins with their recognition by the chaperone Kar2, which associates with the lectin-like protein Yos9. These substrates are then transferred to the Hrd1 complex through a mechanism that is mediated by interactions between Yos9 and Hrd3. In subsequent steps, ERAD-L substrates become dislocated into the cytosol, ubiquitinated, and presented to proteasomes for degradation through the actions of other Hrd1 complex components shown in Figure 4. (B) Reconstitution experiments reveal that *Drosophila* Hrd1 and Sel1 (the Hrd3 homolog) are required for Insig-mediated, sterol-accelerated degradation of mammalian HMG CoA reductase in S2 cells. By analogy to the model present in A, this degradation may involve a mechanism whereby Insigs bridge HMG CoA reductase to the dHrd1 complex through interactions with an unknown intermediary protein(s) that plays a role similar to that of Yos9 in degradation of ERAD-L substrates in yeast.

mammalian genome encodes for homologs of all members of the yeast Hrd1 complex, except for Cue1. Sucrose gradient centrifugation experiments indicate that mammalian Hrd1 is present in a large, multiprotein complex (Schulze *et al.*, 2005). However, complete delineation of components of the mammalian Hrd1 complex has not been determined.

The model for Hrd1-mediated degradation of glycosylated ERAD-L substrates begins with their recognition by the chaperone Kar2, which in turn associates with the ER lumenal lectin Yos9. These substrates are then presented to the Hrd1 complex for dislocation and ubiquitination through a mechanism mediated by interactions between Yos9 and Hrd3, which forms a 1:1 stoichiometric complex with Hrd1 (see Figure 5A). A similar model appears to apply to Hrd1 mediated degradation of ERAD-L substrates in mammalian cells (Christianson *et al.*, 2008). As selectors for ERAD-L substrates, Kar2 and Yos9 sense hallmarks of protein misfolding such as the exposure of hydrophobic amino acid residues or the presence of mono-glucosylated N-linked glycans. Considering this, selection of ERAD-M substrates for Hrd1-mediated ubiquitination/degradation should involve intramembrane protein–protein interactions. By analogy to the model for ERAD-L substrate recognition/selection, it is reasonable to speculate the existence of molecular chaperones that somehow recognize hallmarks of misfolded intramembrane regions such as exposure of hydrophilic amino acid residues within the membrane (Hampton and Garza, 2009) and intermediary proteins that bridge the ERAD-M substrate to the Hrd1 complex through Hrd3-mediated interactions.

A role for an intermediary protein and/or chaperone in the degradation of ERAD-M substrates may be suggested by studies of Hmg2, one of few ERAD-M substrates that have been studied in detail. When certain intermediates of mevalonate metabolism accumulate, Hmg2 acquires features of a misfolded protein and becomes degraded through a mechanism mediated by Hrd1 (Gardner and Hampton, 1999; Shearer and Hampton, 2005). Binding of apparently misfolded Hmg2 to the Hrd1 complex requires the presence of Hrd3, which contains a large lumenal domain, a single membrane-spanning segment,

and a short cytosolic tail (Gardner *et al.*, 2001). Two consequences of Hrd3 deletion are a block in degradation of Hmg2 and auto-ubiquitination followed by proteasomal degradation of Hrd1 (Gardner *et al.*, 2001; 2001). Hrd1 stability and degradation of Hmg2 is restored by expression of the lumenal domain of Hrd3. These processes are dissociable; in Hrd3-deficient yeast, the lumenal domain of Hrd3 lacking the N-terminal half stabilizes Hrd1, but cannot support Hrd1–Hmg2 complex formation and Hmg2 ubiquitination/degradation. These findings suggest that the N-terminal half of the lumenal domain of Hrd3 mediates associations involved in substrate selection (possibly through binding to an intermediary protein or chaperone), whereas the C-terminal half of Hrd3 mediates interactions with Hrd1. Importantly, a recent study suggests that Hrd1 plays a direct role in selection of Hmg2 as an ERAD-M substrate (Sato *et al.*, 2009). Mutation of several hydrophobic amino acid residues in the membrane domain of Hrd1 impairs the ability of the enzyme to initiate ubiquitination of Hmg2, but not of ERAD-L substrates. These results led to the conclusion that the hydrophilic intramembrane residues in Hrd1 engage in noncovalent interactions with hydrophilic residues in the membrane domain of Hmg2 that become exposed in the presence of degradative signals. An important question for future studies is whether an intermediary protein bridges Hmg2 to Hrd1 through Hrd3-mediated interactions.

Sterol-accelerated degradation of mammalian reductase has been recently reconstituted in *Drosophila* S2 cells, which lack a recognizable INSIG gene and cannot synthesize sterols *de novo* (Clark and Bloch, 1959; Clayton, 1964). Although S2 cells express a homolog for reductase, the enzyme is not subjected to sterol-accelerated degradation (Brown *et al.*, 1983; Gertler *et al.*, 1988). Regulated degradation of mammalian reductase in S2 cells precisely mirrors the reaction that occurs in mammalian cells with regard to the absolute requirement for the action of mammalian Insigs, sterol specificity, sensitivity to proteasome inhibition, and augmentation by nonsterol isoprenoids (Nguyen *et al.*, 2009). These findings indicate that Insig-mediated recognition/selection of reductase and subsequent proteasome-mediated degradation of the enzyme occur through a mechanism that is mediated by highly conserved components of the general ERAD pathway.

In yeast, the membrane-bound Hrd1 and Doa10 are the only ubiquitin ligases known to mediate ubiquitination of ERAD substrates (Kostova et al., 2007). It is becoming increasingly evident that the number of ERAD ubiquitin ligases in mammalian cells far exceeds that in yeast. Thus, it is not surprising that in addition to Hrd1 and Teb4 (the Doa10 homolog in mammals), other ubiquitin ligases such as Trc8, CHIP, as well as gp78 have been implicated in the ERAD pathway of mammalian cells. It has been estimated that more than 50 uncharacterized RING finger proteins contain membrane-spanning segments (Kostova *et al.*, 2007); it seems likely that some of these proteins play key roles in ERAD. The ERAD pathway has not been well-studied in *Drosophila* cells; however, the reconstitution of reductase degradation in S2 cells points to the existence of a *Drosophila* ubiquitin ligase that can bind Insigs and initiate ubiquitination of mammalian reductase. Indeed, the *Drosophila* genome contains homologs for Hrd1 (dHrd1), Trc8 (dTrc8), and Teb4 (dTeb4).

The RNAi-mediated knockdown of dHrd1, but not dTrc8 and dTeb4, abolishes sterol-accelerated degradation of mammalian reductase in S2 cells (Nguyen *et al.*, 2009). This finding is significant considering that mammalian Hrd1 and gp78 belong to a subfamily of membrane-bound ubiquitin ligases. Both proteins contain a hydrophobic N-terminal domain with multiple membrane-spanning segments followed by a cytosolic C-terminal domain with a RING finger motif that directs ubiquitin ligase activity (Kostova *et al.*, 2007). Hrd1 and gp78 are organized in ER membranes with similar topologies and their membrane domains share approximately 50% amino acid homology. Importantly, the membrane domains of these enzymes do not bear significant sequence homology with the corresponding regions of other membrane-bound ubiquitin ligases such as Teb4 and Trc8. Thus, gp78 and Hrd1 can be considered as a subfamily of membrane-bound ubiquitin ligases that mediate degradation of reductase in yeast, *Drosophila*, and mammalian cells.

Reconstitution experiments in S2 cells reveal that a subset of dHrd1 complex components including dSel1 (Hrd3 homolog), dHerp (Usa1 homolog), dUbxd8 (Ubx2 homolog), dNpl4, and dUfd1 are required for regulated degradation of reductase (see Figure 4). These findings are similar to those reported in yeast where a subset of the Hrd1 complex components, namely Hrd1 and Hrd3, are required for ERAD-M substrates such as Hmg2 (Carvalho *et al.*, 2006; Denic *et al.*, 2006). However, the reconstitution experiments reveal that dHerp may play a role in reductase degradation that is distinct from recruitment of Derlins. In mammalian cells, Herp binds to a family of proteins called ubiquilins, which contain an N-terminal UBL domain that binds to proteasomes and a C-terminal UBA domain that binds polyubiquitin chains (Kim *et al.*, 2008). The yeast equivalent of ubiquilin, Dsk2, is known to participate in ERAD by guiding ubiquitinated substrates to the proteasome (Ko *et al.*, 2004; Walters *et al.*, 2004). Whether Herp mediates degradation of reductase in *Drosophila* and mammalian cells through a mechanism involving ubiquilins remains to be determined.

Based on the current understanding of how ERAD substrates are selected for ubiquitination and degradation, a model for Insig-mediated selection reductase in *Drosophila* cells is presented in Figure 5B. The key

feature of this model is the recruitment of reductase to the dHrd1 complex through interactions mediated by dSel1 (the Hrd3 homolog), Insig, and an as-of-yet to be identified intermediary protein(s). The proposed intermediary protein(s) is presumed to bridge reductase to the dHrd1 complex through interactions with both Insig and dSel1; a similar mechanism applies to Yos9-mediated degradation of ERAD-L substrates (see Figure 5A). In the model of Figure 5B, Insig plays the role of the intramembrane chaperone that initiates recognition of reductase as ERAD-M substrate. Considering that Insig-mediated degradation of reductase in S2 cells is incredibly specific, it seems very likely that the proposed intermediary protein(s) are conserved components of the general ERAD pathway involved in selection of a subset of dHrd1 substrates. However, the possibility exists that dSel1 plays an indirect role in reductase degradation by stabilizing dHrd1. Thus, exciting avenues for future work will be to define the mechanism for Insig-dependent, Hrd1-mediated degradation of reductase in S2 cells and identify the proposed intermediary protein that mediates the reaction.

Studies of the yeast ERAD pathway have clearly established that the Hrd1 and Doa10 complexes contain an array of conserved factors involved in processes ranging from the selection and recruitment of substrates to the extraction of ubiquitinated substrates from membranes and their delivery to proteasomes (Figure 4) (Vembar and Brodsky, 2008). Although not completely defined at the molecular level, complexes of mammalian ERAD ubiquitin ligases are likely to exist. In fact, mammalian Hrd1 associates with gp78 and appears to mediate its proteasomal degradation (Ye *et al.*, 2005; Shmueli *et al.*, 2009). However, it is presently unknown whether gp78 and Hrd1 combine to mediate degradation of ERAD substrates or whether they share common subunits. The complete definition of mammalian Hrd1 and gp78 complexes, and determination of the role of these proteins in Insig-mediated, sterol-accelerated ERAD of reductase are obvious areas of future investigation. The original experiments demonstrating sterol-regulated binding of reductase-Insig to gp78 relied on co-immunoprecipitation of the proteins from detergent lysates of cells (Song *et al.*, 2005b). It is presently unknown whether binding between reductase-Insig and gp78 is mediated by direct interactions between gp78 and Insig. Thus, the possibility of the existence of an intermediary protein that bridges this interaction cannot be excluded, further emphasizing the importance of defining the gp78 ubiquitin ligase complex.

It should be noted that an ER membrane protein called SPFH2 can be recovered in a multiprotein complex that includes gp78, VCP/p97, and inositol trisphosphate receptors (IP_3Rs), polytopic membrane proteins known to undergo regulated ERAD (Brodsky and Wojcikiewicz, 2009). RNAi-mediated knockdown of SPFH2 prevents ubiquitination and subsequent degradation of IP_3Rs; degradation of other ERAD substrates is also blunted in SPFH2-knockdown cells. Whether SPFH2 plays a role as an intermediary protein in the ERAD of IP_3Rs and other substrates mediated by gp78 or other ERAD ubiquitin ligase remains to be determined.

Mechanism for extraction of ubiquitinated reductase from ER membranes

The striking feature of reductase degradation is that the catalytic and membrane domains are degraded together as a unit. That is to say, the soluble catalytic domain is not released from membranes during the degradation process (Gil *et al.*, 1985). Ubiquitination is obligatory for reductase degradation, occurring on two cytosolic lysine residues in the membrane domain. As mentioned earlier, mutation of these lysines abolishes detectable ubiquitination of reductase, even in the context of the full-length protein. Moreover, the membrane domain of reductase confers sterol-accelerated degradation when fused to a normally stable soluble protein such as β-galactosidase, GFP, or luciferase (Skalnik *et al.*, 1988; and our unpublished observations). Thus, the question arises as to how ubiquitination of the membrane domain renders the entire reductase protein susceptible to proteasomal degradation. Ubiquitinated reductase could be degraded by proteasomes directly from ER membranes or completely extracted from the membrane into the cytosol prior to proteolysis. Evidence in favor of the latter scenario is beginning to accumulate. Recent studies have shown that in both yeast and mammalian systems, full-length reductase becomes dislocated from ER membranes into the cytosol (Garza *et al.*, 2009a; Leichner *et al.*, 2009). However, these observations raise additional questions as to the mechanism for cytosolic dislocation of reductase. Does the reaction require a protein-conducting channel? If so, does Sec61, one of the Derlin proteins, or a novel protein form this channel? It has been proposed that the multiple membrane-spanning segments of ERAD ubiquitin ligases form the channel through which ERAD substrates are dislocated (Nakatsukasa and Brodsky, 2008; Nakatsukasa *et al.*, 2008). Finally, an intriguing hypothesis has been put forth in which ERAD substrates become dislocated from ER membranes into the cytosol through lipid droplets. Lipid droplets are cytosolic organelles traditionally regarded as storage depots for neutral lipids such as triglycerides and cholesterol esters. A role for lipid droplets in ERAD is suggested by (1) the accumulation of the ERAD substrate apolipoprotein B-100 on lipid droplets when proteasome activity is blocked (Ohsaki *et al.*, 2006); and (2) identification of several chaperones as well as VCP/p97 and its membrane receptor Ubxd8 as lipid droplet-associated proteins (Liu *et al.*, 2004;

Bartz *et al.*, 2007). Other open questions regarding dislocation of reductase pertain to how the solubility of its membrane-spanning segments is maintained during dislocation, how the enzyme is delivered to proteasomes following dislocation, and whether dislocation involves a vesicular budding or fusion event that is mediated by a geranylgeranylated protein. Answers to these questions may be provided through rigorous examination of reductase degradation *in vitro*.

Contribution of sterol-accelerated degradation to overall regulation of reductase in vivo

Prior to the availability of anti-reductase antibodies and cDNA probes, indirect methods such as measurement of enzymatic activity were used to study the regulation of reductase in livers of whole animals. These key studies demonstrated that a multivalent feedback regulatory system similar to that described in cultured cells operates in the liver to control the levels and activity of reductase (Endo *et al.*, 1979; Kita *et al.*, 1980; Singer *et al.*, 1984). The significance of this regulatory system is highlighted by the effectiveness of statins, which potently inhibit reductase, in lowering plasma LDL-cholesterol and reducing the incidence of coronary heart disease in humans (Scandinavian Simvastatin Study, 1994; Heart Protection Study Collaborative, 2002). However, statin-mediated inhibition of reductase disrupts feedback regulation of the enzyme and induces a compensatory increase in the levels of reductase. Notably, this compensatory increase has been observed in the livers of patients undergoing statin therapy (Reihner *et al.*, 1990). The accumulation of reductase becomes progressively harder to inhibit, evoking the need for higher doses of statins to maintain their LDL-cholesterol lowering effects. Studies of genetically altered mice suggest that disruption of Insig-mediated degradation accounts, in part, for the compensatory increase in reductase that accompanies statin treatment. In livers of mice deficient in Insig-1 and Insig-2, reductase protein accumulates to a level > 100-fold than that in wild type animals (Engelking *et al.*, 2005). This accumulation is presumably attributable to the contribution of both transcriptional and post-transcriptional regulation of reductase. However, it should be noted that reductase protein accumulates disproportionately to its mRNA in the absence of Insigs. A remarkably similar increase in the amount of reductase protein occurs in Insig-deficient CHO cells (Lee *et al.*, 2005), indicating that the response is a common feature of many cell types. Studies focused on Insig-mediated degradation are required to precisely determine the contribution of protein stability to the overall regulation of reductase and its impact on cholesterol metabolism at the level of the whole animal.

The further elucidation of mechanisms for sterol-accelerated degradation of reductase will have important implications for both basic science and clinical medicine. From the basic science perspective, reductase represents an ideal model for the ERAD of polytopic membrane proteins. Degradation of reductase is a highly specific reaction that only occurs in Insig-expressing cells that have been subjected to sterol treatment. The sterol-dependence of reductase degradation is a valuable control that guards against artifactual degradation that may occur once various steps of the reaction are reconstituted *in vitro*. From a clinical point of view, detailed understanding of reductase degradation may lead to the development of drugs that accelerate this process and counteract the accumulation of the enzyme that occurs during statin treatment. These new drugs may provide an important alternative or adjuvant to statin therapy. Finally, understanding mechanisms for reductase degradation may provide valuable insight into the ERAD of other clinically relevant polytopic membrane proteins such as mutant forms of the cystic fibrosis transmembrane conductance receptor (cystic fibrosis; Ward *et al.* 1995), connexins-32 (X-linked Charot-Marie-Tooth disease; VanSlyke *et al.* 2000), and polycystin-2 (autosomal dominant polycystic kidney disease; Liang *et al.* 2008).

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Declaration of interest

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