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A 'distributed degron' allows regulated entry into the ER degradation pathway

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Protein degradation is employed in both regulation and quality control. Regulated degradation of specific proteins is often mediated by discrete regions of primary sequence known as degrons, whereas protein quality control involves recognition of structural features common to damaged or misfolded proteins, rather than specific features of an individual protein. The yeast HMG-CoA reductase isozyme Hmg2p undergoes stringently regulated degradation by machinery that is also required for ER quality control. The 523 residue N-terminal transmembrane domain of Hmg2p is necessary and sufficient for regulated degradation. To understand how Hmg2p undergoes regulated degradation by the ER quality control pathway, we analyzed over 300 mutants of Hmg2p. Regulated degradation of Hmg2p requires information distributed over the entire transmembrane domain. Accordingly, we refer to this determinant as a 'distributed' degron, which has functional aspects consistent with both regulation and quality control. The Hmg2p degron functions in the specific, regulated degradation of Hmg2p and can impart regulated degradation to fusion proteins. However, its recognition is based on dispersed structural features rather than primary sequence motifs. This mode of targeting has important consequences both for the prediction of degradation substrates and as a potential therapeutic strategy for targeted protein degradation using endogenous degradation pathways.
Keywords: endoplasmic reticulum/HMG-CoA reductase/Hmg2p/protein degradation

Introduction

Cellular degradation pathways target and destroy specific proteins for purposes of physiological regulation and quality control (Hochstrasser, 1995; Kopito, 1997). In both circumstances, high specificity is essential and observed. However, these two roles for protein degradation have very different requirements for substrate selection. Regulated degradation occurs by specific recognition of a single protein. Quality control involves recognition of different proteins with common structural features.

The value in understanding these distinct modes of recognition is underscored by the importance of the cellular processes controlled or affected by each. For example, cell division is coordinated by the temporally regulated, targeted destruction of specific proteins (King *et al.*, 1996a; Hershko, 1997). On the other hand, the etiology of numerous clinical syndromes, including cystic fibrosis and neurodegenerative disorders, have molecular aspects best viewed as examples of protein quality control (Ward *et al.*, 1995; Johnston *et al.*, 1998).

Selective degradation of a protein for regulation requires its accurate identification. Often, a small region of a protein's sequence provides information that targets the protein for degradation. Such regions are known as 'degrons' (Varshavsky, 1991), and some examples include: the Deg1 sequence of the *MAT*α2 transcriptional regulator in yeast (Hochstrasser and Varshavsky, 1990; Johnson *et al.*, 1998), the destruction box of mitotic cyclins (Glotzer *et al.*, 1991; King *et al.*, 1996b; Hershko, 1997), the degradation motif of IκB (Whiteside *et al.*, 1995), the stability-regulating region of cMOS (Nishizawa *et al.*, 1993) and the N-terminal residue of some proteins (Bachmair and Varshavsky, 1989; Varshavsky, 1996). In most instances, a degron functions autonomously when included in the sequence of a heterologous protein, bringing about degradation of the fusion protein by the same mechanisms that operate in the original degradation substrate (Hochstrasser and Varshavsky, 1990; Glotzer *et al.*, 1991; King *et al.*, 1996b).

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

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

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

tase (HMGR) undergoes regulated degradation by ER-associated processes that target it to the proteasome for destruction (Inoue *et al.*, 1991; Meigs and Simoni, 1992; Hampton *et al.*, 1996a). HMGR is a key enzyme in the mevalonate pathway, from which sterols are synthesized, and is subject to feedback regulation as part of the cellular control of sterol synthesis (Edwards *et al.*, 1983; Nakanishi *et al.*, 1988; Chun *et al.*, 1990; Roitelman and Simoni, 1992; Hampton and Rine, 1994). At the same time, misfolded and mutant ER proteins, such as the membrane proteins CFTR ΔF508 and Sec61-2p (Ward *et al.*, 1995; Biederer *et al.*, 1996) or the soluble lumenal proteins α-antitrypsin and CPY* (Ciccarelli *et al.*, 1993; Finger *et al.*, 1993), are recognized by a quality control mechanism and degraded by ER-associated processes that target them to the proteasome.

This dual role for ER degradation has been analyzed by parallel genetic approaches in yeast. Some genes, termed *HRD* genes, required for regulated ER degradation of the HMGR isozyme Hmg2p (Hampton *et al.*, 1996a), are identical to genes required for degradation of the mutant protein CPY*, termed *DER* genes (Hiller *et al.*, 1996; Knop *et al.*, 1996; Bordallo *et al.*, 1998). Degradation of either protein requires ubiquitination that is strongly dependent on Ubc7p (Hiller *et al.*, 1996; Hampton and Bhakta, 1997), and a functional 26S proteasome (Hampton *et al.*, 1996a; Hiller *et al.*, 1996). Furthermore, at least two ER-resident membrane proteins, Hrd1p/Der3p and Hrd3p, are required for degradation of either substrate (Hampton *et al.*, 1996a; Bordallo *et al.*, 1998; D.Wolf, personal communication). Thus, the ER degradation pathways for these structurally and functionally distinct substrates employ common machinery.

The shared use of the ER degradation machinery for both quality control and regulated degradation substrates implies that a similar mode of recognition may be used for each type of substrate. However, only the degradation of Hmg2p (and related reporter proteins) is subject to regulation by the mevalonate pathway (Hampton and Rine, 1994; Hampton *et al.*, 1996b). Thus, the question arises as to how Hmg2p is distinguished from quality control substrates. Is Hmg2p a typical quality control substrate for which presentation to the structure scanning mechanism is regulated by the mevalonate pathway? Or, is Hmg2p a typical substrate for regulated degradation, which, by virtue of specific characteristic sequences, recruits proteins to deliver it to the shared ER degradation machinery?

To understand better how Hmg2p is recognized for degradation, we have studied the sequence features of Hmg2p important for this process. Specifically, we have performed a complete analysis of the 523 residue N-terminal transmembrane domain of Hmg2p, which is necessary and sufficient for regulated degradation (Hampton and Rine, 1994; Hampton *et al.*, 1996b). This domain is required for Hmg2p regulated degradation and is capable of transferring regulated degradation to other stable proteins when included as a heterologous sequence. We were particularly interested in the degree to which Hmg2p regulated degradation depended on discrete, modular determinants such as degrons, which often underlie regulated protein degradation, or on structural features, which are important in recognition of quality control substrates.

We have discovered that two lysines, located at distant positions along the linear sequence, were each essential and worked together to allow Hmg2p ubiquitination and degradation. The function of these lysines was highly specific, but strongly dependent on structural features of the Hmg2p N-terminal domain rather than any specific sequences. Our results indicated that Hmg2p, by virtue of its correctly folded structure, presents highly specific information used to program its degradation, through recognition of what we call a 'distributed degron'. The Hmg2p distributed degron incorporated features familiar to both regulated degradation and quality control. Like previously described degrons, the Hmg2p distributed degron was centrally involved in physiological regulation of Hmg2p stability and could autonomously program regulated degradation of otherwise stable proteins. However, the function of the Hmg2p distributed degron required information dispersed throughout its sequence, and specificity appeared to depend principally on structural features rather than specific stretches of sequence.

Results

Lysine 6 was necessary for degradation of Hmg2p

Our earlier studies with Hmg2p mutants showed that the first 26 residues of Hmg2p were required for normal degradation (Gardner *et al.*, 1998). Accordingly, we performed a detailed analysis of this region. When residues 2–13 were deleted from Hmg2p, the resulting mutant was stable (Figure 1B, Δ2–13), indicating that this portion of Hmg2p contained a positive determinant of degradation. The Δ2–13 deletion removed the only two lysines (6 and 13) in the 26 residue tract (Figure 1A). We mutated each lysine separately to assess its role in degradation. Substitution of Lys6 with arginine completely stabilized Hmg2p (Figure 1B, K6R), despite the presence of nearby Lys13. In contrast, substitution of Lys13 with arginine (Figure 1B, K13R) or alanine (our unpublished data), had no effect on Hmg2p degradation. Therefore, it appeared that Lys6 was essential for Hmg2p degradation, whereas Lys13 was completely dispensable.

Hmg2p degradation can be hastened by addition of the squalene synthase inhibitor zaragozic acid (ZA) to cells. ZA causes a build-up of a mevalonate-derived molecule that stimulates Hmg2p ubiquitination and degradation (Hampton and Bhakta, 1997). Addition of ZA to cells expressing normal Hmg2p caused a decrease in the Hmg2p steady-state level due to stimulated degradation (Hampton and Bhakta, 1997; Gardner *et al.*, 1998; and Figure 1C, WT). In contrast, addition of ZA to cells expressing K6R-Hmg2p had no effect on the K6R-Hmg2p steady-state level (Figure 1C, K6R). Thus, the single K6R substitution rendered Hmg2p stable even when physiological signals for degradation were maximal.

The optical reporter Hmg2p–green fluorescent protein (GFP) was used to study further the effect of the K6R replacement. The GFP reporter facilitates quantitative examination of degradation through analysis of steady-state Hmg2p–GFP fluorescence. Alterations in Hmg2p stability brought about by physiological or genetic means are correctly reported by changes in Hmg2p–GFP steady-state fluorescence (Gardner *et al.*, 1998; Cronin and Hampton, 1999). When K6R was introduced into Hmg2p–

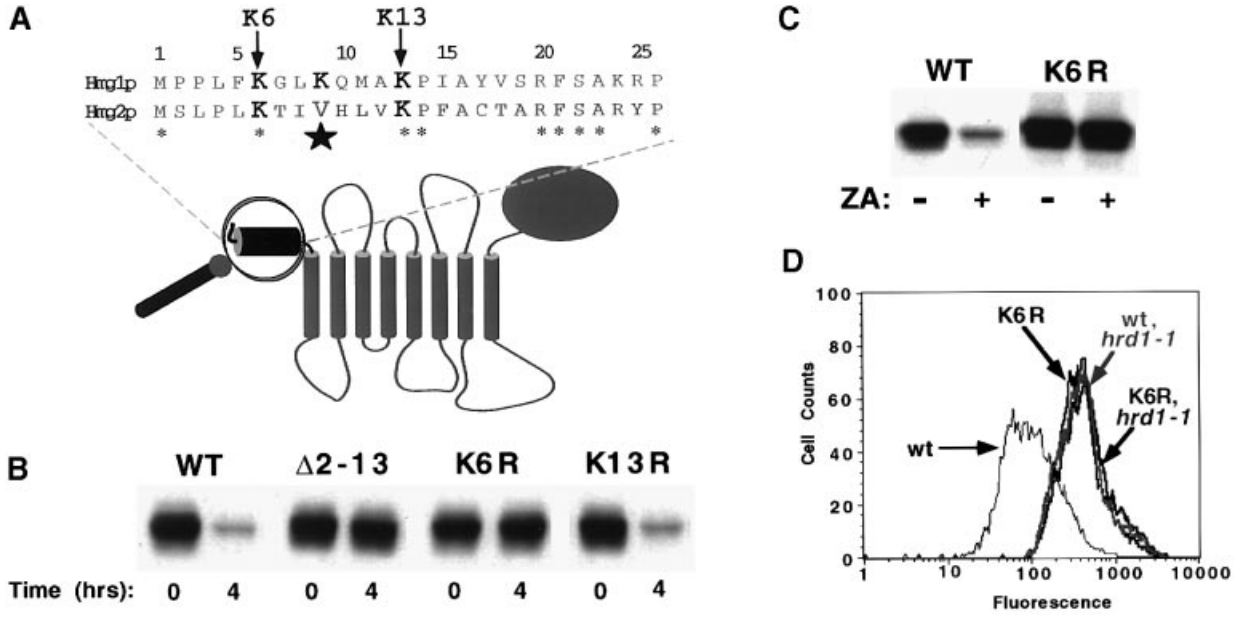

Fig. 1. Lys6 is required for Hmg2p degradation. (**A**) Sequence comparison of the first 26 residues of Hmg1p (top row) and Hmg2p (bottom row). Asterisks indicate conserved residues and position 9 is indicated with a large star. (**B**) Degradation of normal Hmg2p (WT), Hmg2p with residues 2–13 deleted (Δ2–13), Hmg2p with Lys6 replaced with arginine (K6R), or Hmg2p with Lys13 replaced with arginine (K13R). Otherwise identical strains expressing the indicated Hmg2p variant were subject to a cycloheximide–chase assay. Lysates for each time point indicated were made and immunoblotted to determine the level of protein. In all experiments in all figures, each Hmg2p variant had a single myc-epitope tag included in the sequence of the linker region to allow detection with the anti-myc 9E10 antibody. (**C**) Effect of ZA, which hastens Hmg2p degradation, on steady-state levels of Hmg2p (WT) or K6R-Hmg2p (K6R). Strains were treated for 4 h with 10 μg/ml ZA, then subjected to immunoblotting to determine the steady-state level of Hmg2p. (**D**) Effect of the *in cis* K6R mutation or the *in trans hrd1-1* mutation on Hmg2p–GFP. Flow cytometry analysis of *HRD1* or *hrd1-1* strains expressing normal Hmg2p–GFP (wt) or K6R-Hgm2p–GFP (K6R).

GFP, the resulting K6R-Hmg2p–GFP was stable in all assays (our unpublished data).

Cells expressing the stable K6R-Hmg2p–GFP had significantly increased fluorescence compared with cells expressing normal Hmg2p–GFP (Figure 1D, K6R versus wt), indicated by a rightward shift in the fluorescence histogram. We compared directly the stabilizing effect of the *in cis* K6R mutation with the stabilizing effect of the *in trans hrd1-1* mutation, which completely blocks normal Hmg2p–GFP degradation (Gardner *et al.*, 1998; Cronin and Hampton, 1999). The histogram of an *HRD1* strain expressing K6R-Hmg2p–GFP was superimposable with the histogram of an *hrd1-1* strain expressing normal Hmg2p–GFP (Figure 1D, K6R versus wt, *hrd1-1*). Furthermore, presence of the *hrd1-1* allele had no added effect on the fluorescence of a strain expressing K6R-Hmg2p–GFP (Figure 1D, K6R versus K6R, *hrd1-1*). These results indicated that the effect of the *in cis* K6R replacement was entirely due to an inability of K6R-Hmg2p–GFP to undergo *HRD*-dependent degradation.

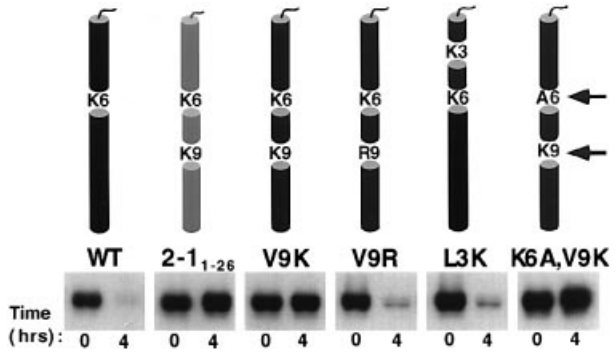

Fig. 2. Position 9 could not be a lysine. Degradation of the Hmg2p mutants indicated was directly compared by cycloheximide–chase assay. Cylindrical portion represents only the first 26 residues of Hmg2p (in magnifying glass in Figure 1A). Black color represents Hmg2p native sequence. The gray cylinder (second from left) represents the first 26 residues of Hmg1p present in the replacement, with the critical Lys9 and conserved Lys6 shown. Other cartoons represent Hmg2p with the point mutations indicated and Lys6 as shown.

Lys6 function had a singular sequence restriction

Previously, replacement of the Hmg2p N-terminal 26 residues with the corresponding tract from Hmg1p resulted in a stable protein (Gardner *et al.*, 1998; and Figure 2, $2\text{-}1_{1–26}$). However, Lys6 was preserved in this stable chimera (Figure 1A), indicating that some feature of the local context was also important. The stable chimera had 16 residues different from normal Hmg2p (Figure 1A), including eight non-conservative replacements. One of these was the replacement of valine 9 with a third lysine (Figure 1A, large star). This single lysine replacement was entirely responsible for the stabilizing effect of the 1–26 exchange. If only Lys9 in the stable chimeric protein was reverted to valine, the resulting protein was once again degraded (our unpublished data). Furthermore, if only residue 9 of normal Hmg2p was changed to a lysine, the resulting mutant was stable (Figure 2, V9K). Surprisingly, the stabilizing effect of the V9K replacement was highly specific for lysine. Replacement of position 9 in Hmg2p with an arginine, despite it also having a positive charge, had no effect on degradation (Figure 2, V9R). Replacement of residue 9 with several other residues also had no effect on Hmg2p degradation (our unpublished

data), indicating that stability was only caused by specific introduction of a lysine at that position.

The stabilizing effect of V9K was also highly specific for position 9. Replacement of residue 3, which was a similar distance from Lys6 to position 9, had no effect on degradation (Figure 2, L3K), nor did the replacement of residues 8, 7 or 2 with lysine (our unpublished data). Therefore, Hmg2p degradation was specifically disrupted by the presence of a lysine at position 9, and neither proximity nor charge appeared to explain the stabilizing effect.

One interpretation of this is that a lysine at position 9 interacted with the degradative machinery in a highly specific, albeit inhibitory manner in the presence of Lys6. We wondered if having only a single lysine at position 9, without the nearby Lys6, might once again allow degradation through this specific interaction. However, Hmg2p with this alteration was also stable (Figure 2, K6A,V9K). Thus, a lysine at position 9 could not, by itself, substitute for the essential degradative function of the normal lysine at position 6, but could only specifically inhibit degradation.

The above studies revealed a highly specific role for Lys6 in degradation. However, except for the no-lysine restriction on position 9, Lys6 function was quite insensitive to changes in its surrounding primary sequence. We have altered each residue in the tract individually, and no one residue was specifically required for regulated degradation except Lys6 (our unpublished data).

Distance from the ER membrane was critical for Lys6 function

Lysine 6 was critical for Hmg2p degradation and a lysine at position 9 instead of position 6 did not support degradation. This implied that the critical lysine must be a particular distance from the ER membrane to function in degradation. Accordingly, we added or removed residues between Lys6 and the first transmembrane span to determine whether an 'increased' or 'decreased' distance between Lys6 and the ER membrane would have a stabilizing effect.

All mutations that altered this distance had marked effects on Hmg2p degradation. Addition of three alanines between residues 18 and 19 of Hmg2p (Figure 1A) caused significant stabilization (Figure 3, +3A). In contrast, replacement of residues 17 and 18 in Hmg2p with alanines, which created a similar four-alanine tract but did not lengthen the N-terminus, had no effect on degradation (our unpublished data). Even addition of a single alanine between residues 18 and 19 had a strong stabilizing effect on Hmg2p (Figure 3, +1A). Moreover, removal of endogenous Ala16, thus shortening the N-terminal region by one residue, also had a strong stabilizing effect (Figure 3, –1A). However, addition of an alanine between residues 18 and 19 and simultaneous removal of Ala16, which preserved the distance between Lys6 and the ER membrane but included both separate stabilizing mutations, allowed normal Hmg2p degradation (Figure 3, +/–1A).

We tested other interpretations of these results. It was possible that the effects were due to a change in the overall length of the N-terminal region. However, addition of one or three alanines N-terminal to Lys6, between residues 1 and 2, had no effect on degradation (our unpublished data; and Figure 3, +3A-N). It was also possible that the distance between Lys6 and some critical region after the first transmembrane span was affected. However, addition of three alanines between residues 58 and 59, located immediately after the first transmembrane span, had no effect on Hmg2p degradation (Figure 3, +3A-tm). Thus, Hmg2p degradation required a precise distance between Lys6 and the start of the first transmembrane span at the surface of the ER membrane.

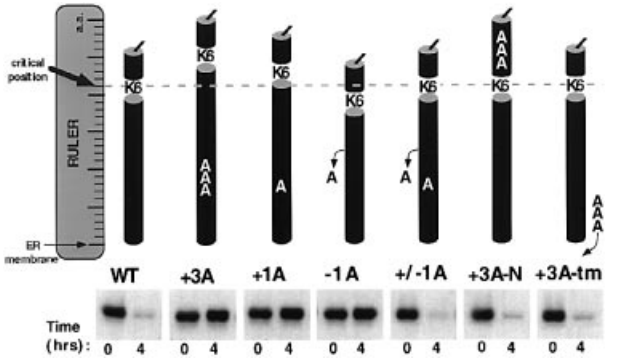

Fig. 3. A ruler for degradation: distance from the ER membrane was critical for Lys6 function. Strains expressing each variant of Hmg2p indicated, in which alanines were either inserted (+3A, +1A, +3A-N, +3A-tm, +/–1A) and/or removed (–1A, +/–1A) from the Hmg2p primary sequence, were assayed for Hmg2p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

In the above studies, it was important to ascertain whether the mutations that allowed degradation (e.g. K13R, +/–1A, V9R, etc.) were permissive for normal Hmg2p degradation or were causing structural aberrations that resulted in unregulated degradation. Physiological regulation of Hmg2p is quite sensitive to perturbations in the Hmg2p structure (Hampton *et al.*, 1996a; Gardner *et al.*, 1998; and see below). Accordingly, the degradation of each mutant was tested for physiological regulation by addition of lovastatin, which slows degradation through the regulatory mechanism, during the degradation assay (wild-type example in Figure 5 below). Each degraded mutant retained entirely normal regulation (our unpublished data), indicating that these mutations did not result in abnormal degradation.

Lys6 was required for Hmg2p ubiquitination

Covalent attachment of ubiquitin is a critical and regulated step in Hmg2p degradation (Hampton and Bhakta, 1997). Therefore, we examined the regulated ubiquitination of the stable Hmg2p mutants described above. Each Hmg2p variant (with a myc epitope tag) was expressed in a strain that also expressed HA-epitope-tagged ubiquitin (HA-Ub). Hmg2p ubiquitination was assayed by immunoprecipitation of Hmg2p, followed by immunoblotting with anti-HA antibody to detect covalently attached HA-Ub, or with anti-myc antibody to detect total precipitated Hmg2p (Gardner *et al.*, 1998). Regulation of ubiquitination was also tested by the addition of lovastatin ('Lov'), which decreases ubiquitination, or ZA, which increases ubiquitination (Hampton and Bhakta, 1997; Figure 4, WT).

All Hmg2p variants with stabilizing mutations showed

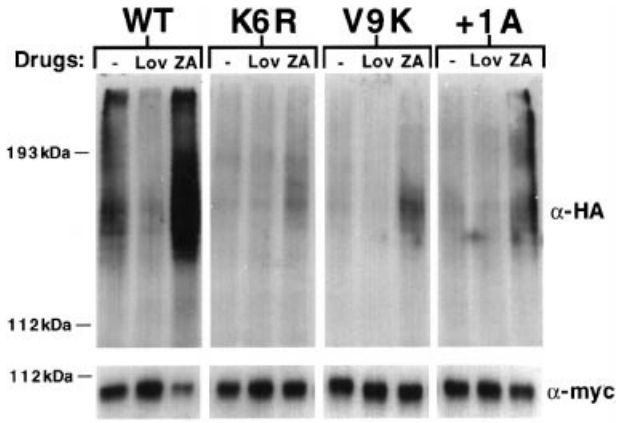

Fig. 4. Lys6 was required for Hmg2p ubiquitination. Ubiquitination assays of strains expressing the Hmg2p variant indicated were performed in the presence of no drug (–), lovastatin (Lov, 25 μg/ml) or zaragozic acid (ZA, 10 μg/ml). Upper panels are the result of anti-HA (α-HA) immunoblotting for covalently linked HA-tagged Ub. Lower panels are the result of parallel immunoblotting an aliquot (1/8 total volume) of the same immunoprecipitates with the 9E10 anti-myc antibody (α-myc) to assess total immunoprecipitated Hmg2p. Ubiquitinated Hmg2p is represented as a collection of five to eight bands with higher molecular weights than normal Hmg2p. Note that the total amount of Hmg2p precipitated in the WT ZA lane is less due to stimulated degradation, therefore the degree of ubiquitination in that lane is an underestimate.

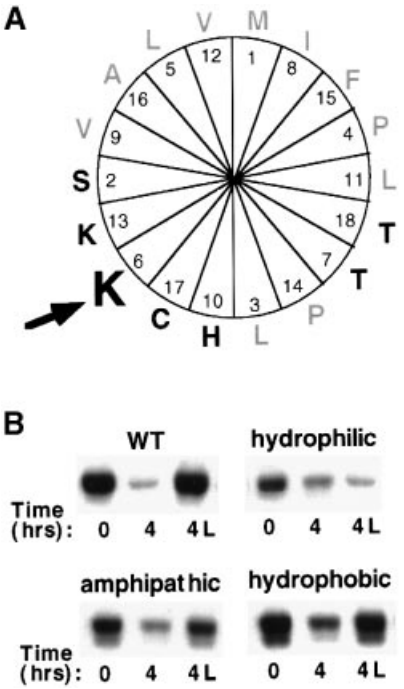

Fig. 5. Hydrophobicity of the Lys6-containing α-helix was important, not amphipathicity. (**A**) Helical wheel diagram of the region surrounding Lys6, residues 1–18. The hydrophobic residues are colored gray and the hydrophilic ones black. The arrow points to Lys6. (**B**) Effect of hydrophobicity/hydrophilicity alterations on regulated Hmg2p degradation. Each Hmg2p mutant was tested for regulated degradation by cycloheximide–chase assay in the presence or absence of lovastatin (25 μg/ml), which was added at the same time as the cycloheximide. The Hmg2p mutants tested include normal (WT), all hydrophobic residues replaced with alternate hydrophobic residues (amphipathic), all hydrophobic residues replaced with hydrophilic ones (hydrophilic) and all hydrophilic residues, except Lys6, replaced with hydrophobic ones (hydrophobic).

diminished ubiquitination. The stable K6R-Hmg2p had drastically decreased ubiquitination (Figure 4, K6R), even when signals for degradation were elevated by addition of ZA. The maximal difference between ubiquitination of normal Hmg2p and K6R-Hmg2p observed in the presence of ZA was estimated to be 20-fold (Figure 4, WT versus K6R).

Stabilizing mutations of Hmg2p that altered the context or position of Lys6 also resulted in decreased Hmg2p ubiquitination. For example, the V9K-Hmg2p showed decreased ubiquitination (Figure 4, V9K), as did the Hmg2p mutant with a single alanine inserted between residues 18 and 19 (Figure 4, +1A). All other stabilized mutants showed similarly lowered basal and stimulated ubiquitination (our unpublished data). If the K6R replacement was introduced into any of these stable mutants, the residual ubiquitination was decreased to that of the K6R mutant (our unpublished data). Thus, all stabilized Hmg2p mutants with Lys6 present were still subject to specific, albeit poor recognition of this critical lysine.

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

Residues 1–18 of Hmg2p, which contain Lys6, are predicted to adopt an amphipathic α-helical conformation (Basson *et al.*, 1986). When these residues were plotted on a helical wheel, Lys6 was on the hydrophilic face of the predicted helix (Figure 5A, black residues). We evaluated the importance of the amphipathic character of this region in Hmg2p degradation by altering the hydrophobicity/hydrophilicity. When the helical region was made completely hydrophilic by replacing all hydrophobic residues with hydrophilic ones (L3E, L5N, I8N, V9E, L11Q, V12T, F15Y, A16S), the resulting protein was subject to rapid, unregulated degradation. Addition of lovastatin, which strongly stabilizes normal Hmg2p (Hampton and Rine, 1994; and Figure 5B, WT, lane 4 versus 4L), had no effect on the degradation of the hydrophilic mutant (Figure 5B, hydrophilic, lane 4 versus 4L). Furthermore, degradation of the hydrophilic mutant was no longer dependent on Lys6 (see below). Surprisingly, when the region was made entirely hydrophobic by replacing all of the hydrophilic residues (except Lys6) with hydrophobic ones (S2A, T7G, H10Y, K13M, T18V), the resulting protein was still subject to normal, regulated degradation (Figure 5B, hydrophobic).

These results suggested that the hydrophobic face of the helical region, but not the amphipathic character, was important for regulated degradation. When the natural hydrophobic residues were replaced with alternate hydrophobic residues (L5F, I8L, V9I, L11M, V12A, F15I, A16V, C17A), Hmg2p regulated degradation was normal (Figure 5B, amphipathic). Thus, the hydrophobicity of the face, rather than any specific sequence, was important for regulated degradation. Since the hydrophilic mutant showed neither physiological regulation nor dependence on Lys6, the hydrophobic face was probably required for correct structure of the transmembrane region (see below).

Lysine 357 was also critical for Hmg2p degradation

The N-terminal 26 residue region with Lys6, although required for Hmg2p degradation, was not a traditional

degron because it was not sufficient to program degradation or ubiquitination of the stable Hmg1p (Gardner *et al.*, 1998), whereas the described degron Deg1 was (S.Kim, R.Gardner and R.Hampton, manuscript in preparation). Therefore, we conducted a systematic mutagenic search of the Hmg2p transmembrane region to identify other determinants required for regulated degradation. We were particularly interested in finding small, autonomous determinants of degradation.

We analyzed the entire Hmg2p transmembrane region by replacement of small regions of Hmg2p sequence with the corresponding sequence from the homologous (50% identical) and stable Hmg1p isozyme. We also created site-directed mutations in small regions in Hmg2p (six residues or less), which were left unaltered by homologous exchange. In each case where an altered degradation phenotype was observed, each individual change in a replacement was evaluated by producing the appropriate point mutations to determine whether the effect of the larger replacement was attributable to a single residue (see below). Over 300 Hmg2p mutants were produced in this analysis. In only a single case was the degradative effect of an alteration in Hmg2p sequence attributable to a change in a single residue.

We discovered that a lysine distant from position 6, at position 357, was also essential for regulated degradation. When Lys357 was replaced with arginine, the resulting mutant was stable (Figure 6B, K357R). K357R-Hmg2p stability was unaffected by increased signals for degradation caused by addition of ZA (Figure 6C, K357R).

The K357R replacement was introduced into Hmg2p–GFP to allow a more detailed analysis of its effects. K357R-Hmg2p–GFP behaved similarly to K6R-Hmg2p–GFP. The fluorescence of a strain expressing K357R-Hmg2p–GFP was significantly brighter than that of an identical strain expressing Hmg2p–GFP (Figure 6D, K357R versus wt). Furthermore, the brightness of the *HRD1* strain expressing K357R-Hmg2p–GFP was nearly the same as that of the *hrd1-1* strain expressing Hmg2p–GFP (Figure 6D, K357R versus wt,*hrd1-1*). Unlike the K6R mutant, the K357R mutant did show very slow degradation, such that a strain expressing K357R-Hmg2p–GFP was made slightly brighter (~1.25-fold) by the presence of the *hrd1-1* allele (Figure 6D, K357R versus K357R,*hrd1-1*).

Like Lys6, Lys357 was also required for normal ubiquitination of Hmg2p. The stable K357R-Hmg2p showed decreased ubiquitination that was unaffected by addition of ZA (Figure 6E, K357R). The double mutant (K6R,K357R) showed essentially the same low ubiquitination as either single mutant. Thus, Hmg2p ubiquitination was critically co-dependent on two specific lysines far separated in the primary sequence.

Lysine 357 function was also permissive of changes in local sequence that did not alter length. Within our collection of mutants, each residue in the neighborhood of Lys357 (position 340–399) has been altered without effect on regulated degradation (our unpublished data). Thus, no other single residue in the region of Lys357 was specifically required for regulated degradation. Since the only specific context requirement for Lys6 function was the lysine restriction at position 9, we tested similar mutations in the 'sequence neighborhood' of Lys357. Placement of lysines at position +3 or –3 to Lys357 did not affect Hmg2p regulated degradation (our unpublished data).

Similarly to Lys6, Lys357 function was also sensitive to changes in distance between Lys357 and the nearest transmembrane span. Insertion of one or three alanines between residues 351 and 352 N-terminal to Lys357, which resulted in a change in distance between the nearest predicted transmembrane span and Lys357, significantly stabilized Hmg2p (Figure 7, +1A and +3A). Similar insertions between residues 360 and 361, C-terminal to Lys357, did not effect degradation (Figure 7, +1A-C and +3A-C), indicating that the distance between Lys357 and the nearest transmembrane span was important for degradation. However, the stabilized mutants did show slow degradation (half-life ~3.5 h). This residual degradation was not subject to regulation nor was it dependent on Lys6 (our unpublished data), and was probably a result of concomitant structural alteration caused by the central position of the insertion site (see below).

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

Lysines 6 and 357 are separated by 350 residues in the linear sequence of Hmg2p. Both lysines were required for Hmg2p degradation, and loss of either by conservative replacement resulted in strong stabilization and loss of ubiquitination. Unlike typical degrons, the sequence regions containing Lys6 and Lys357 did not contribute independently to degradation.

In our search for sequence determinants of Hmg2p degradation, we observed numerous replacement mutations that stabilized Hmg2p but did not disturb the local sequence context around Lys6 and Lys357. Degradation experiments on three of these stabilizing mutants are shown in Figure 8A. In each case, separate regions of Hmg2p distant from each lysine were replaced with the corresponding tract from Hmg1p. Despite the preservation of each critical lysine and its local sequence, these mutants showed strong stabilization. Other regions of Hmg2p required for degradation were similarly found along the length of the Hmg2p transmembrane domain (see below). Thus, normal function of Lys6 and Lys357 was not solely determined through autonomous action of their local sequence and structure, but also required information from many parts of the Hmg2p transmembrane domain.

A different series of mutants indicated that Lys6 and Lys357 played a critical role in physiological regulation of Hmg2p degradation. Many structural variants of Hmg2p were still degraded in an *HRD*-dependent manner, but were no longer regulated by the mevalonate pathway. Examples include 6myc-Hmg2p (Hampton *et al*., 1996a), Δ40-Hmg2p (deletion of residues 146–186, our unpublished data), and hydrophilic-Hmg2p described in Figure 5 (our unpublished data). Because these unregulated mutants were still subject to *HRD*-dependent degradation, we tested whether their degradation was dependent on Lys6 or Lys357. In no case was there any effect of changing either (Figure 8B) or both (our unpublished data) lysines to arginine. In each of these diverse structural mutants, loss of regulation and loss of Lys6/Lys357 dependence coincided. From analysis of a number of other mutants,

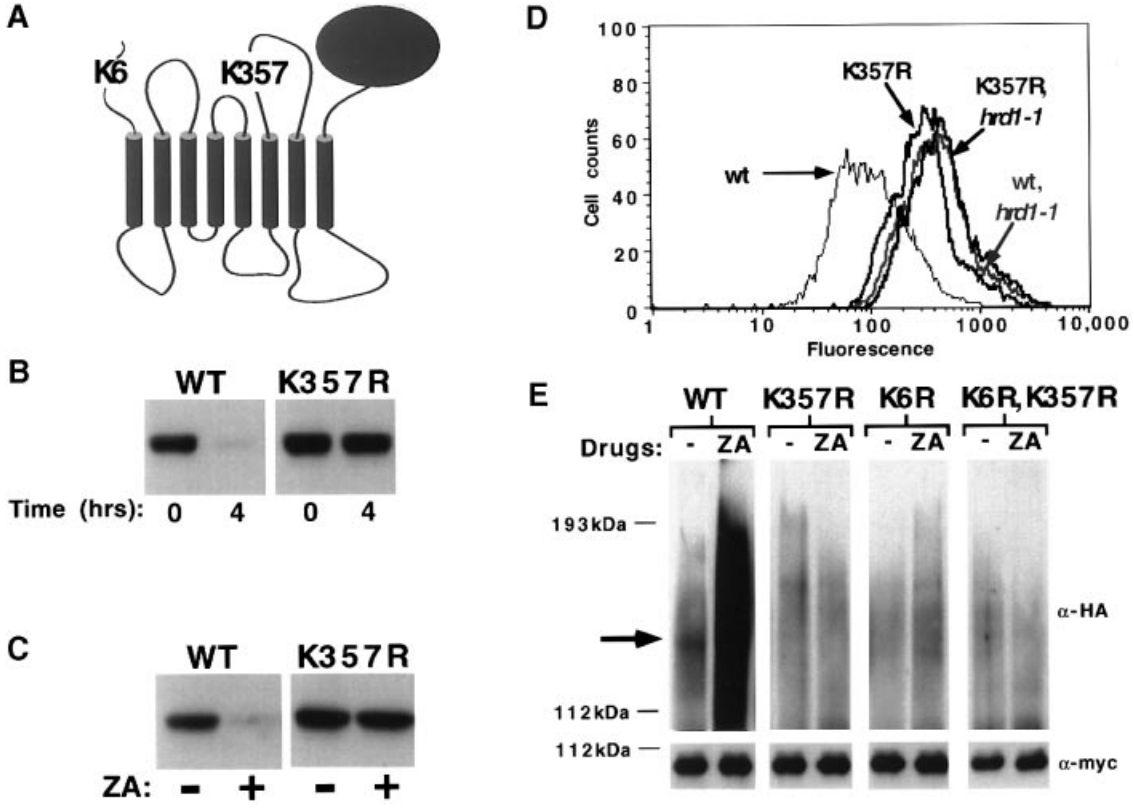

Fig. 6. Lys357 was also required for degradation. (**A**) Cartoon of the relative location of Lys357 in Hmg2p. (**B**) Strains expressing either normal Hmg2p (WT) or K357R-Hmg2p (K357R) were assayed for Hmg2p degradation by cycloheximide–chase assay. (**C**) Effect of ZA, which stimulates Hmg2p degradation, on steady-state levels of each variant. Strains expressing either form of Hmg2p were incubated for 4 h in the presence of 10 μg/ml ZA. (**D**) Effect of the *in cis* K357R mutation or the *in trans hrd1-1* mutation on Hmg2p–GFP steady-state fluorescence. Strains expressing either normal Hmg2p–GFP (wt) or K357R-Hmg2p–GFP (K357R) were compared by flow cytometry. Each protein was expressed in both *HRD1* and *hrd1-1* strains. (**E**) Each critical lysine was required for Hmg2p ubiquitination. Ubiquitination assays in the absence or presence of ZA (10 μg/ml) were performed in otherwise identical strains expressing normal Hmg2p (WT), K357R-Hmg2p (K357R), K6R-Hmg2p (K6R) and the double mutant (K6R,K357R) as described in Figure 4.

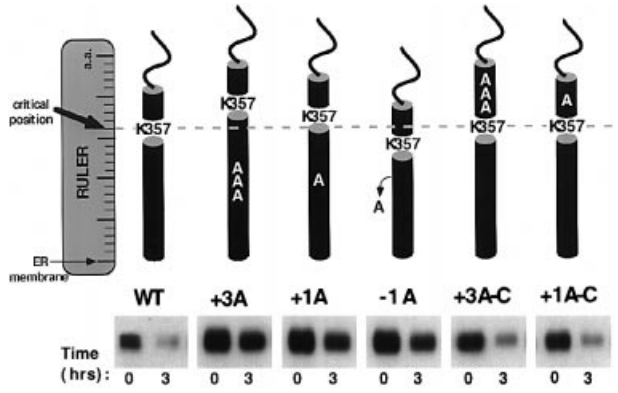

Fig. 7. A second ruler for degradation; correct distance from the ER membrane was required for Lys357 function. Strains expressing each variant of Hmg2p indicated, in which alanines were either inserted (+3A, +1A, +3A-C, +1A-C) or removed (–1A), were assayed for Hmg2p degradation by cycloheximide–chase assay. Cylinders represent the normal residues surrounding Lys357. Distance of Lys357 from the ER membrane is depicted schematically by the 'ruler' and the dotted line. Top of the diagram is the C-terminal side of Lys357, and the bottom is the N-terminal side. The bottom of each black cylinder is the predicted juxtamembrane residue, Asp342.

we have similarly been unable to uncouple regulation from Lys6/Lys357 dependence.

These data demonstrated that Lys6/Lys357 function was sensitive to a variety of alterations of the Hmg2p transmembrane domain distant from either lysine. Analysis of the large number of primary mutants derived by exchange with Hmg1p or by semi-conservative alteration of small regions of Hmg2p (usually six amino acids; described in the last section), allowed us to map the regions that were important for degradation or its regulation (Figure 8C). Alteration of the black regions resulted in partial or full stabilization. These include the three mutants in Figure 8A. Partially stabilized mutants remained subject to regulation by mevalonate pathway signals. In contrast, alteration of the checkered regions resulted in degradation that was no longer regulated, or poorly regulated, by signals from the mevalonate pathway.

The wild-type sequences of the sensitive regions of Hmg2p, both black and checkered (Figure 8C), were subjected to site-directed mutagenesis to evaluate the role of each individual change within a given replacement. In no case, except for Lys6 and Lys357, could the effect of a small replacement be attributed to alteration of a single residue. In all cases, replacement of three to six residues was needed to observe either of the two classes of effect, slowed degradation or poor regulation. The number and wide distribution of these sensitive regions was consistent with the idea that the entire transmembrane domain, including information from both sides of the ER membrane, was involved in regulated degradation of Hmg2p. Since no single residue change mimicked the altered,

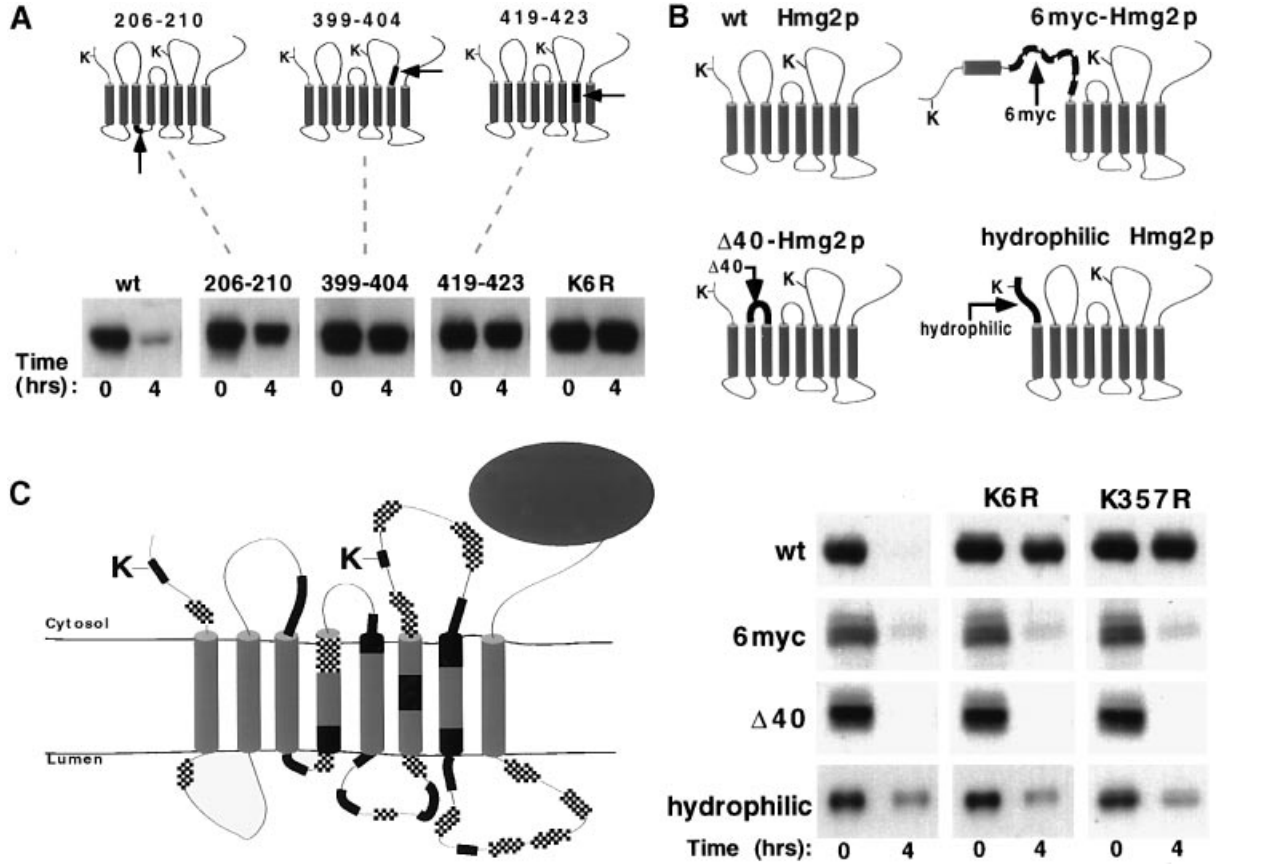

Fig. 8. Function of Lys6 or Lys357 required the correct structure of the transmembrane region. (**A**) Other regions of Hmg2p were required for degradation. Numbers for each mutant denote the residues of Hmg2p that were replaced with the corresponding regions from Hmg1p. Degradation of each mutant was assessed by cycloheximide–chase assay. Cartoons depict the location of each mutation relative to Lys6 and Lys357. (**B**) Several structurally perturbed mutants, all subject to unregulated degradation, were tested for Lys6 and Lys357 dependence by introducing the appropriate K6R or K357R replacement and assessing degradation by cycloheximide–chase assay. Cartoons depict the effects of each mutant and their location relative to each lysine. (**C**) Regions of Hmg2p required for correct regulated degradation. Hmg2p mutants were made either by replacing small regions of Hmg2p with corresponding regions from the stable, homologous (50% identical) Hmg1p, or by replacing the remaining unchanged residues of Hmg2p with semi-conservative residues. Regulated degradation of each mutant was assessed by cycloheximide–chase assay in the presence and absence of appropriate drugs. Mutation of the black regions resulted in partial or full stabilization. Alteration of the checkered regions resulted in degradation that was no longer regulated by signals from the mevalonate pathway.

regulated degradation phenotype of the small region mutations, it appeared that these three to six residue changes exerted their effect through alteration of the Hmg2p tertiary structure, rather than recognition of specific sequences.

Discussion

ER degradation of Hmg2p is tightly regulated and isozyme specific, yet occurs by the action of cellular machinery responsible for the destruction of a diverse array of misfolded proteins. We have explored the features of Hmg2p that were important for degradation in order to understand these seemingly dichotomous aspects of the process.

We have discovered two lysines, Lys6 and Lys357, located at distant points along the linear sequence, which were each critical for degradation. Lysines 6 and 357 did not independently contribute to the regulated degradation of Hmg2p. Both were essential and their degradative function required a correct structural context. Their participation in regulated Hmg2p degradation was dependent on information widely distributed throughout the 523-residue transmembrane domain. Numerous alterations of Hmg2p in regions distant from Lys6 and Lys357 strongly stabilized Hmg2p (Figure 8C, black patches), indicating that Lys6 and Lys357 did not function autonomously. These lysines were also intimately involved in the regulation of Hmg2p degradation. A variety of structurally compromised Hmg2p mutants were subject to unregulated degradation in an *HRD*-dependent fashion that no longer required either lysine. In all cases tested, degradation that was not regulated by the mevalonate pathway was also not dependent on Lys6/Lys357. Thus, these lysines worked together in a synergistic manner within the correct structure of Hmg2p to allow normal regulated degradation. From our analysis, we propose that Hmg2p presents specific information for degradation within a ‘distributed degron’, in which the structure of the entire transmembrane domain is required to present the information that allows the cell to control Hmg2p stability uniquely.

A distributed degron

The action of the Hmg2p distributed degron combines aspects of regulation and quality control. Like more traditional degrons, the Hmg2p distributed degron is

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

The Hmg2p distributed degron is different from previously described degrons in that it requires structural information distributed over an entire protein domain of 523 residues. These individual determinants are not autonomous and can not program the degradation of stable proteins when included as heterologous sequences (Gardner *et al.*, 1998; R.Gardner and R.Hampton, unpublished observations). In contrast, typical degrons are composed of single, short sequences that can act autonomously and program the degradation of other stable proteins when included as a heterologous sequence, as is the case for the well defined degron of the yeast *MAT*α2 transcriptional regulator, Deg1 (Hochstrasser and Varshavsky, 1990; Johnson *et al.*, 1998; Mayer *et al.*, 1998). Within the Deg1 sequence there are specific sequence requirements and structural features critical for degradative function (Johnson *et al.*, 1998), but they are restricted to a small, independently folding region.

Some degrons act by binding molecules that protect them from recognition by the degradation machinery or that actively promote degradation (Murakami *et al.*, 1992; Johnson *et al.*, 1998). The Hmg2p distributed degron contained regions necessary for Hmg2p degradation, which are present on both sides of the ER membrane. This suggests that there may be two or more binding regions within the distributed degron for proteins or molecules involved in Hmg2p degradation. Proteins known to be required for Hmg2p degradation are located in both the lumen (Hrd1p/Der3p and Hrd3p; Hampton *et al.*, 1996a; Bordallo *et al.*, 1998) and the cytosol (Ubc7p and Hrd2p; Hampton *et al.*, 1996a; Hampton and Bhakta, 1997). Perhaps those regions required for Hmg2p degradation form distinct lumenal or cytosolic binding sites for the respective proteins to interact with Hmg2p. We are in the process of examining interactions between Hmg2p and the *HRD* proteins to assess whether the stabilizing mutations affect these interactions. Alternatively, the dispersed elements of the distributed degron may allow the formation of an emergent structure, such as a protein surface, which allows recognition by the degradation or regulatory apparatus and is not reducible to small, autonomous sequence determinants.

Alterations in Hmg2p structure resulted in a variety of degradative phenotypes including stability, poorer recognition of degradative signals or enhanced recognition of degradative signals (Gardner *et al.*, 1998; this work). The sensitivity of Hmg2p degradation to structural changes may be important in the regulation of stability. It is quite possible that physiological control of Hmg2p degradation is brought about by induced changes in the structure of the Hmg2p distributed degron of a similar magnitude to those we have introduced by mutation.

Lys6/Lys357: gateway to the HRD pathway

How do Lys6 and Lys357 participate in the function of the distributed degron? One possibility is that they interact with a regulatory molecule, resulting in the alteration of the Hmg2p structure from one that is stable to one that is recognized as a quality control substrate. Alternatively, since lysines are the usual sites for addition of ubiquitin to proteins (Finley and Chau, 1991; Jentsch, 1992), it is possible that Lys6 and/or Lys357 serve as the initial ubiquitination sites in processive degradation of Hmg2p. It is possible that these two lysines may be required for establishment of a tertiary structure required for Hmg2p regulated degradation. Subtle alterations in the position of these lysines would change this structure and so alter interaction with the machinery of regulated degradation. Perhaps fluctuations in the isoprenoid content of the ER membrane, determined by mevalonate pathway production, affect the precise juxtaposition of the two critical lysines, resulting in degradation when they are in the correct position.

By any model, it is clear that Lys6 and Lys357 serve in the specific recognition of Hmg2p for regulated degradation. However, these lysines are not needed for *HRD*-dependent degradation of many variants, such as 6myc-Hmg2p. In addition, other regions in Hmg2p required for normal degradation, such as residues 166–186 and 421–497, were similarly not required for *HRD*-dependent degradation of the unregulated variants Δ40-Hmg2p and 6myc-Hmg2p (our unpublished observations). Thus, Hmg2p has dispersed structural information necessary for its unique targeting as a substrate for regulated degradation, but not generally required for *HRD*-dependent degradation. Identifying the molecules that mediate Lys6/Lys357-dependent degradation will clarify how the Hmg2p distributed degron allows specific recognition of Hmg2p.

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

Implications of a distributed degron

The existence of a distributed degron has important implications in understanding protein degradation at the ER surface, and possibly in general. Many models of protein degradation specificity presume discrete autonomous regions of the protein, degrons, which interact with proteins responsible for degradation. Such discrete, modular degrons can be revealed by primary sequence analysis. Their absence in a particular protein sequence might be considered evidence of independence from the associated degradation pathway. However, our studies have demonstrated that highly selective, regulated degradation can also depend on information that is neither readily

apparent at the level of primary sequence nor detectable by homology. The prevalence of distributed degrons in the spectrum of protein degradation is not yet known, but the existence of Hmg2p indicates that it is unwarranted to assume that regulated degradation must always proceed by recognition of discrete, small regions of primary sequence. In this regard, it is worth noting that mammalian HMGR and yeast Hmg2p have the same enzymatic function, undergo regulated degradation that is in many ways similar, yet have little or no primary sequence homology in the transmembrane regions. Perhaps similar structural features that are transparent to primary sequence comparison mediate their similar degradative behaviors.

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

Materials and methods

Materials and reagents

All enzymes were obtained from New England Biolabs (Beverly, MA). Other chemical reagents were obtained from Sigma (St Louis, MO). ECL chemiluminescence immunodetection reagents were from Amersham (Arlington Heights, IL). Lovastatin and zaragozic acid were generously provided by Merck (Rahway, NJ). The 9E10 anti-myc antibody was used directly from a hybridoma (ATCC CRL 1725) supernatant. The anti-HA antibody was an ascites fluid obtained from Babco (Berkeley, CA). Affinity-purified goat anti-mouse HRP-conjugated antiserum was obtained from Sigma. Protein A–Sepharose CL-4B was obtained from Pharmacia Biotech (Piscataway, NJ). Sequencing was performed using the T7 Sequenase v 2.0 sequencing kit from Amersham and a model SA sequencing apparatus (Life Technologies, Madison, WI).

Site-directed mutagenesis and DNA cloning

The overlap extension method (Ho *et al.*, 1989) was used to create site-specific mutations in *HMG2*. All PCR amplifications were performed as described previously (Gardner *et al.*, 1998). A list of primers used in the PCRs is available upon request.

Final PCR products were cloned as follows: PCR products with mutations created in codons 1–228 were cloned between the *Afl*II and *Sph*I sites in *1mycHMG2*. PCR products with mutations in codons 228–370 were cloned between the *Sph*I and *Bgl*II sites in *1mycHMG2*. PCR products with mutations between codons 371 and 523 were cloned between the *Bgl*II and *Nru*I sites in *1mycHMG2*. All *HMG2* mutants were tested for complementation of mevalonate auxotrophy in yeast lacking both endogenous HMGR genes. Mutations in codons 1–26 were introduced into the *hmg2::GFP* reporter gene by replacement of the *Sph*I–*Sal*I region of the mutant *hmg2* coding regions with the *Sph*I–*Sal*I GFP-containing region from pRH469 (Gardner *et al.*, 1998). Mutations in codons 353–370 were introduced into the *hmg2::GFP* reporter gene by replacement of the *Bgl*II–*Sal*I region of the mutant *hmg2* coding regions with the *Bgl*II–*Sal*I GFP-containing region from pRH469.

pRH1100 is an Ade$^+$ selectable, ARS-CEN vector containing a triple HA-epitope-tagged ubiquitin expressed from the GAPDH promoter. Construction of pRH1100 was as follows: the 900 bp *Eco*RI–*Cla*I fragment from YEp112 (Hochstrasser *et al.*, 1991), which contains the HA-Ub gene, was cloned between the *Eco*RI and *Cla*I sites in pBluescript KS II (Stratagene, La Jolla, CA) to yield pRH381. The coding region for a triple HA-epitope tag was PCR amplified and cloned between the *Eco*RI and *Bgl*II sites in pRH381 to yield pRH964. The 4.2 kb *Pst*I–*Cla*I region from pRH423 (Gardner *et al.*, 1998) was replaced with the 900 bp *Pst*I–*Cla*I region from pRH964 to yield pRH988, resulting in placement of the 3HA-Ub gene under control of the GAPDH promoter. The 3HA-Ub-containing, 2.2 kb *Pvu*II–*Sac*I fragment from pRH988 was cloned between the *Sma*I and *Sac*I sites in pASZ11 (Stotz and Linder, 1990) to yield pRH1100.

Strains and media

Growth and transformation of *Escherichia coli* or yeast strains were performed as described previously (Gardner *et al.*, 1998). The lithium acetate (LiOAc) method was used to transform yeast with plasmid DNA (Ito *et al.*, 1983).

Mevalonate auxotrophic yeast strain RHY468 (**a** *his3Δ200 lys2-801 ade2-101 ura3-52 met2 hmg1::LYS2 hmg2::HIS3*) was used as a host strain for all plasmids expressing the enzymatically active HMGR mutants as described (Gardner *et al.*, 1998). Each integrating plasmid containing a mutant *HMG2* gene was introduced into the recipient strain by targeted integration at the *Bam*HI site of the *hmg2Δ::HIS3* genomic locus, followed by selection for mevalonate prototrophy on yeast extract/peptone/dextrose (YPD) medium. In all cases, the mutant plasmids were able to restore mevalonate prototrophy.

Plasmids with mutant *hmg2::GFP* fusions were transformed into strain RHY519 (*MAT***a** *his3Δ200 lys2-801 ade2-101 ura3-52::hmg2cd met2 hmg1::LYS2 hmg2::HIS3*). RHY519 expressed a soluble, enzymatically active Hmg2p catalytic domain as the sole source of HMGR activity (Gardner *et al.*, 1998). Thus, all GFP-derived optical reporter fusions were expressed in strains with identical mevalonate pathway activity. GFP fusion plasmids were introduced into RHY519 by targeted integration at the *Stu*I site of the *ura3-52* allele followed by selection for Ura$^+$ prototrophy.

As in previous studies, all Hmg2p proteins, both normal and mutant, were expressed from the strong, constitutive GAPDH promoter in order to separate degradation from other possible modes of regulation (Hampton and Rine, 1994; Hampton *et al.*, 1996a; Gardner *et al.*, 1998). In addition, all *HMG2* coding regions had a single copy of the myc-epitope coding sequence inserted between codons 618 and 619 located in the poorly conserved *HMG2* linker region (Hampton and Bhakta, 1997). Presence of this single myc epitope had no effect on the essential HMGR activity or regulated, *HRD*-dependent degradation of Hmg2p (Hampton and Bhakta, 1997; Gardner *et al.*, 1998).

Assays

Degradation of Hmg2p and its variants was assessed by cycloheximide–chase assay as described previously (Gardner *et al.*, 1998).

Hmg2p ubiquitination was assayed in a manner similar to that described previously (Hampton and Bhakta, 1997; Gardner *et al.*, 1998), except that no $CuSO_4$ was needed for the induction of tagged ubiquitin. Strains expressing individual Hmg2p variants were transformed with pRH1100, which expressed a triple HA-epitope-tagged ubiquitin from the constitutive GAPDH promoter. Transformants were selected for Ade$^+$ prototrophy.

Flow cytometric analysis was performed as described previously (Gardner *et al*., 1998). Living cells were analyzed by flow microfluorimetry using a FACScalibur™ (Beckton Dickinson, Palo Alto, CA) flow microfluorimeter with settings for fluorescein-labeled antibody analysis. Histograms were produced from 10 000 individual cells and were plotted with log fluorescence (arbitrary units) on the horizontal axis and cell number on the vertical axis.

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