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HRD Gene Dependence of Endoplasmic Reticulum-associated Degradation

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Work from several laboratories has indicated that many different proteins are subject to endoplasmic reticulum (ER) degradation by a common ER-associated machinery. This machinery includes ER membrane proteins Hrd1p/Der3p and Hrd3p and the ER-associated ubiquitin-conjugating enzymes Ubc7p and Ubc6p. The wide variety of substrates for this degradation pathway has led to the reasonable hypothesis that the *HRD* (Hmg CoA reductase degradation) gene-encoded proteins are generally involved in ER protein degradation in eukaryotes. We have tested this model by directly comparing the *HRD* dependency of the ER-associated degradation for various ER membrane proteins. Our data indicated that the role of *HRD* genes in protein degradation, even in this highly defined subset of proteins, can vary from absolute dependence to complete independence. Thus, ER-associated degradation can occur by mechanisms that do not involve Hrd1p or Hrd3p, despite their apparently broad envelope of substrates. These data favor models in which the *HRD* gene-encoded proteins function as specificity factors, such as ubiquitin ligases, rather than as factors involved in common aspects of ER degradation.

INTRODUCTION

The endoplasmic reticulum (ER) is an important site of cellular protein degradation in eukaryotes. Both lumenal and integral ER membrane proteins undergo selective degradation for purposes of quality control or feedback regulation (Chun *et al.*, 1990; Klausner and Sitia, 1990). Accordingly, the ER degradation pathway plays an important role in normal and pathological processes, including cholesterol synthesis (Edwards *et al.*, 1983; Nakanishi *et al.*, 1988; Hampton and Rine, 1994), HIV biogenesis (Bour *et al.*, 1995), cystic fibrosis (Jensen *et al.*, 1995; Ward *et al.*, 1995), lipoprotein metabolism (Fisher *et al.*, 1997), and protein quality control (Hiller *et al.*, 1996; Kopito, 1997).

ER protein degradation is conserved between yeast and mammals, allowing genetic analysis of this process. In separate studies, yeast mutants deficient in degradation of the normal, ER-resident protein Hmg2p, an isozyme of HMG-CoA reductase (HMGR) (Hampton and Rine 1996b), and mutants deficient in ER degradation of CPY*, a misfolded protein that is retained in the lumen of the ER (Knop *et al.*, 1996; Bordallo *et al.*, 1998), have been isolated. The genes from these studies are referred to as *HRD* (Hmg CoA reductase degradation) and *DER* (degradation in the endoplasmic reticulum) genes, respectively. For either substrate, ubiquitination is required for subsequent degradation by the proteasome. Ubiquitination is effected by the ER-associated ubiquitin-conjugating enzymes, of which Ubc7p appears to play a major role (Hiller *et al.*, 1996; Hochstrasser, 1996; Hampton and Bhakta, 1997). Furthermore, integral ER membrane proteins Hrd1p/Der3p and Hrd3p are also required for degradation of both of these substrates (Hampton *et al.*, 1996a; Bordallo *et al.*, 1998; Plemper *et al.*, 1999).

These and subsequent studies on the *HRD/DER* genes have indicated a broad role for these genes in the ER-associated degradation of proteins (Plemper *et al.*, 1998). Thus, it has been reasonably suggested that the *HRD–DER* machinery, including the ER-associated ubiquitin-conjugating enzymes Ubc7p and Ubc6p, are components of a general degradation machinery for both lumenal and membrane-bound ER proteins. By this model, both Hrd1p and Hrd3p would be required along with the appropriate ubiquitin-conjugating enzymes and the proteasome for ER-associated degradation. In this work, we have examined the generality of this model using various ER-associated degradation substrates.

Many different types of proteins enter the ER degradation pathway. Substrates include normal ER residents such as HMGR (Hampton and Rine, 1994), ER-retained subunits of unassembled complexes such as components of the T cell receptor (Yu *et al.*, 1997; Yang *et al.*, 1998), proteins that are misfolded by virtue of mutations such as the product of the most common cystic fibrosis allele, CFTRΔ508 (Jensen *et al.*, 1995; Ward *et al.*, 1995), and normally stable proteins that have an autonomous "degron" engineered into the sequence (Hochstrasser and Varshavsky, 1990; Varshavsky, 1991). Because these well-known examples represent the gamut of

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ER-associated degradation substrates, we have evaluated the role of the *HRD* machinery on the degradation of yeast proteins that include representatives from each of these categories. To aid in comparisons, we have restricted our analysis to membrane proteins. Specifically, we have tested the involvement of the *HRD* pathway in the degradation of the normal, ER resident HMGR isozyme Hmg2p (Hampton and Rine, 1994), the unassembled Vph1p subunit of the vacuolar ATPase (Hill and Stevens, 1994, 1995), an ER-retained and degraded mutant of uracil permease, referred to as UP* (Galan *et al.*, 1998), and engineered mutants of each HMGR isozyme with the added Deg1 degradation signal (Basson *et al.*, 1988; Hochstrasser and Varshavsky, 1990; Hampton and Rine, 1994).

By the simplest model, all ER degradation substrates would be expected to show similar and equal dependence on genes that encode general components of the degradation apparatus. We have discovered that the *HRD* gene dependence of ER-associated degradation can vary widely, despite restricting our analysis to only ER membrane proteins. Some substrates absolutely required the *HRD* genes for ubiquitin-mediated degradation, some had partial dependency, and at least one substrate was degraded in a manner that appeared to be completely independent of the *HRD* genes, despite involvement of the ER-associated ubiquitin-conjugating enzymes. Furthermore, a partial requirement for *UBC7*/*UBC6* in the degradation of some of the proteins suggested that ER-associated degradation may in some cases involve UBCs distinct from these "canonical" ER ubiquitin-conjugating enzymes.

MATERIALS AND METHODS

Materials and Reagents

Restriction enzymes, Vent DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). [^{35}S]methionine label NEG-772 Easy Tag EXPRESS was obtained from NEN Life Science Products (Boston, MA). Protein A-Sepharose CL-4B was obtained from Pharmacia Biotech (Piscataway, NJ). Amplify, ECL chemiluminescence immunodetection reagents, and Hyperfilm were from Amersham (Arlington Heights, IL). Renaissance Chemiluminescence Reagent Plus was obtained from NEN Life Science Products, and BioMax film was obtained from Kodak (Rochester, NY). Polyclonal anti-Vph1p antibody was a generous gift from Tom Stevens (University of Oregon). Rabbit polyclonal antibodies raised against either the C-terminal or N-terminal peptides from the Fur4p sequence were generously provided by Dr. Rosine Hageunauer-Tsapis (Institut J. Monod, Université Paris, Paris, France). The anti-myc 9E10 antibody was used as a cell culture supernatant obtained by growing the 9E10 hybridoma (American Type Culture Collection, Manassas, VA; CRL 1729) in RPMI 1640 culture medium (Life Technologies, Grand Island, NY) with 10% fetal calf serum. HMGR antibodies were prepared as described previously (Hampton and Rine, 1994). The anti-hemagglutinin (HA) 12CA5 antibody was an ascites fluid obtained from Babco (Berkeley, CA). The mouse monoclonal anti-ubiquitin antibody was obtained from Zymed (San Francisco, CA). All HRP-conjugated antisera and chemical reagents, including protease inhibitors, were obtained from Sigma (St. Louis, MO).

Molecular Cloning

The *DEG1::HMGR* fusions, encoding either Hmg1p or Hmg2p with the first 26 amino acids replaced with the N-terminal 67 amino acid residues of the Matα2 transcriptional regulator from *Saccharomyces cerevisiae* (Hochstrasser and Varshavsky, 1990), were synthesized by the PCR-based overlap extension method as described previously (Ho *et al.*, 1989; Gardner and Hampton, 1999). A list of primers used in the PCR reactions is available on request. The resulting fusion genes were cloned between the *Pst*I and *Tth*111I sites in pRH561 (Gardner *et al.*, 1998) or the *Pst*I and *Afl*II sites in pRH423 (Hampton and Bhakta, 1997) to produce pRH368 and pRH369, respectively. pRH368 and pRH369 contain coding regions for Deg1-Hmg1p and Deg1-Hmg2p, respectively. Deg1-Hmg1p consists of the entire N-terminal transmembrane region of Hmg1p (residues 1–524) fused to the linker and C-terminal catalytic regions of Hmg2p. pRH369 consists of the Deg1-Hmg2p coding region only. The regions produced by PCR were sequenced to verify error-free amplification.

Green fluorescent protein (GFP) fusions with the Deg1-HMGRs were made by replacing the *Tth*111I–*Kpn*I region in pRH368 with the *Tth*111I–*Kpn*I GFP-encoding fragment from pRH475 or the *Sph*I–*Sal*I region in pRH369 with the *Sph*I–*Sal*I GFP-encoding region from pRH469 (Hampton *et al.*, 1996a). pRH475 was prepared by replacing the *Msc*I/*Sal*I of pRH407 (Hampton *et al.*, 1996b) with the corresponding *Msc*I/*Sal*I fragment of pS65T-C1 (Clonetech, Palo Alto, CA) to introduce the S65T mutation into the GFP portion of the HMG1–GFP coding region. The resulting plasmids are pRH421, expressing the Deg1-Hmg1p–GFP protein, and pRH446, expressing the Deg1-Hmg2p–GFP protein.

pRH652 (2u, *URA3*) expressed the UP* protein (*FUR4–430Np*) and was also known as Yep352fF-430N (Galan *et al.*, 1998). The UP* coding region was excised from this plasmid with *Bgl*II and *Xma*I and subcloned into pRH687 (ARS/CEN, *URA3*) to allow expression from the GAPDH promoter (Hampton and Rine, 1994).

pRH379 contained an HA-epitope–tagged ubiquitin coding region expressed from the GAPDH promoter. It was constructed by subcloning the HA-Ub gene from pRH381 (Gardner and Hampton, 1999) into a 2μ, *URA3* vector.

pRH1184, bearing the *hrd1*Δ*::LEU2* allele, was constructed by subcloning a 3.1-kb *Bam*HI–*Eco*RI fragment of the *HRD1* gene into pBluescript KS II (Stratagene, La Jolla, CA) followed by replacement of the *HRD1 Stu*I–*Sph*I fragment with a PCR-amplified LEU2 gene. pRH1185, bearing the *hrd3*Δ*::LEU2* allele, was constructed by subcloning a 3.1-kb *Xho*I–*Spe*I fragment of the *HRD3* gene into pBluescript KS II followed by replacement of the *HRD3 Bsa*BI–*Nhe*I fragment with a PCR-amplified LEU2 gene. pRH1186, bearing the *ubc7*Δ*::LEU2* allele, was constructed by placement a 650-bp fragment containing a nonfunctional *ubc7* gene into pBluescript KS II followed by replacement of the *ubc7 Hpa*I–BsrGI fragment with a PCR-amplified *LEU2* gene.

The *ubc6*Δ*::kanMX* allele was generated using PCR amplification. *UBC6* genomic sequences were added to a pair of 20 nt primers designed to amplify the *kanMX* gene from pUG6 (Güldener *et al.*, 1996). Candidates for the null allele were confirmed by PCR analysis.

Yeast Strains and Media

All yeast strains were grown in minimal media with supplements at 30°C unless noted otherwise. *Escherichia coli* DH5α strains were grown in Luria broth + ampicillin (100 μg/ml) at 37°C. Yeast were transformed with plasmid DNA using the LiOAc method (Ito *et al.*, 1983).

Yeast strains mentioned in this study are summarized in Table 1. All strains, except those carrying 2u or ARS/CEN plasmids, were initially made by transformation of the desired plasmid into a parent strain. This strain was then crossed to strains carrying the appropriate mutations to ensure that the same single integrated copy of the plasmid was expressed. The genetic background for all strains, except RHY1951, RHY2094, RHY1904, and RHY1900, was *hmg1::LYS2 ade2-101 met2 lys2-801 his3*Δ*200*. RHY1951, RHY2094, RHY1904, and RHY1900 originated from MHY501 and MHY507 (Chen *et al.*, 1993) and are listed in Table 1. RHY918, the original *vma21::LEU2* disruption strain, was made using the disruption plas-

Table 1. Yeast strains

Strain	Genotype	Reference
RHY1611	*MAT**a** HMG2 ura3-52::URA3::1mycHMG2*	This study
RHY1626	*MAT**a** HMG2 ura3-52::URA3::1mycHMG2 trp1::hisG hrd1Δ::TRP1*	This study
RHY1628	*MAT**a** HMG2 ura3-52::URA3::1mycHMG2 hrd2-1*	This study
RHY1631	*MAT**a** HMG2 ura3-52::URA3::1mycHMG2 trp1::hisG hrd3Δ::TRP1*	This study
RHY1633	*MAT**a** HMG2 ura3-52::URA3::1mycHMG2 ubc7Δ::HIS3*	This study
RHY1723	*MAT**a** HMG2 ura3-52::URA3::1mycHMG2 ubc6Δ::KanMX*	This study
RHY871	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::LEU2::HMG2::GFP*	Cronin *et al.*, 2000
RHY880	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::LEU2::HMG2::GFP*	Cronin *et al.*, 2000
RHY1056	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::LEU2::HMG2::GFP ubc7Δ::URA3*	Cronin *et al.*, 2000
RHY1486	*MAT**a** hmg2::HIS3::1mycHMG2 ura3-52::LEU2::HMG2::GFP trp1::hisG hrd1Δ::TRP1 ubc7Δ::URA3*	This study
RHY566	*MATα hmg2::HIS3 ura3-52::6mycHMG2 leu2Δ*	This study
RHY918	*MATα hmg2::HIS3 ura3-52::6mycHMG2 leu2Δ vma21Δ::LEU2*	This study
RHY1032	*MAT**a** hmg2::HIS3 ura3-52::6mycHMG2 leu2Δ vma21Δ::LEU2 hrd1Δ::URA3*	This study
RHY1067	*MAT**a** hmg2::HIS3 ura3-52::6mycHMG2 leu2Δ vma21Δ::LEU2 hrd2-1*	This study
RHY1034	*MATα hmg2::HIS3 ura3-52::6mycHMG2 leu2Δ vma21Δ::LEU2 hrd3Δ::URA3*	This study
RHY1069	*MAT**a** hmg2::HIS3 ura3-52::6mycHMG2 leu2Δ vma21Δ::LEU2 ubc7Δ::HIS3*	This study
RHY1491	*MATα hmg2::HIS3 ura3-52::6mycHMG2 leu2Δ vma21Δ::LEU2 hrd1Δ::URA3 ubc7Δ::HIS3*	This study
RHY636	*MAT**a** hmg2::HIS3::URA3::1mycHMG1 ura3-52*	This study
RHY493	*MAT**a** hmg2::HIS3::URA3::Deg1-HMG1 ura3-52*	This study
RHY1467	*MAT**a** hmg2::HIS3::1mycHMG1 ura3-52* + pRH379 (2u *URA3* HA-Ub)	This study
RHY1460	*MAT**a** hmg2::HIS3::Deg1-HMG1 ura3-52* + pRH379	This study
RHY1948	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG1*	This study
RHY1949	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG1 leu2Δhrd1Δ::LEU2*	This study
RHY2079	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG1 hrd2-1*	This study
RHY1950	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG1 leu2Δhrd3Δ::LEU2*	This study
RHY2096	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG1 leu2Δ ubc7Δ::LEU2*	This study
RHY2097	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG1 ubc6Δ::KanMX*	This study
RHY1359	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG1-GFP*	This study
RHY1566	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG1-GFP trp1::hisG hrd1Δ::TRP1*	This study
RHY1568	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG1-GFP hrd2-1*	This study
RHY1570	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG1-GFP trp1::hisG hrd3Δ::TRP1*	This study
RHY1572	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG1-GFP ubc7Δ::HIS3*	This study
RHY1610	*MATα HMG2 ura3-52::URA3::Deg1-HMG2*	This study
RHY1613	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG2 trp1::hisG hrd1Δ::TRP1*	This study
RHY1615	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG2 hrd2-1*	This study
RHY1617	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG2 trp1::hisG hrd3Δ::TRP1*	This study
RHY1619	*MATα HMG2 ura3-52::URA3::Deg1-HMG2 ubc7Δ::HIS3*	This study
RHY1656	*MAT**a** HMG2 ura3-52::URA3::Deg1-HMG2 ubc6Δ::KanMX*	This study
RHY1374	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG2-GFP*	This study
RHY1575	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG2-GFP trp1::hisG hrd1Δ::TRP1*	This study
RHY1577	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG2-GFP hrd2-1*	This study
RHY1579	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG2-GFP trp1::hisG hrd3Δ::TRP1*	This study
RHY1581	*MAT**a** 1mycHMG2 ura3-52::URA3::Deg1-HMG2-GFP ubc7Δ::HIS3*	This study
RHY1951	*MATα HMG1 HMG2 ura3-52 his3-Δ200 lys2-801 trp1-1* + pRH652 (2u *URA3 FUR4-430N)*	This study
RHY2094	*MATα HMG1 HMG2 ura3-52 his3-Δ200 lys2-801 trp1-1 leu2-3,112 hrd1Δ::LEU2* + pRH652	This study
RHY1904	*MATα HMG1 HMG2 ura3-52 his3-Δ200 lys2-801 trp1-1 leu2-3, 112 hrd3Δ::LEU2* + pRH652	This study
RHY1900	*MATα HMG1 HMG2 ura3-52 his3-Δ200 lys2-801 trp1-1 leu2-3,112 ubc7Δ::LEU2* + pRH652	This study
RHY1216	*MAT**a** hmg2::HIS3 ura3-52::6mycHMG2* + pRH696 (ARS/Cen *URA3 Fur4-430N)*	This study
RHY1222	*MATα hmg2::HIS3 ura3-52::6mycHMG2 trp1::hisG hrd1Δ::TRP1* + pRH696	This study
RHY1218	*MAT**a** hmg2::HIS3 ura3-52::6mycHMG2 hrd2-1* + pRH696	This study
RHY1223	*MAT**a** hmg2::HIS3 ura3-52::6mycHMG2 leu2Δ hrd3Δ::LEU2* + pRH696	This study
RHY1221	*MATα hmg2::HIS3 ura3-52::6mycHMG2 ubc7Δ::HIS3* + pRH696	This study

mid pKH10b and confirmed by PCR and pH sensitivity (Hill *et al.*, 1994).

The *hrd1Δ::URA3* allele originated from a strain produced by replacement of *HRD1* with the disruption fragment in which the *URA3* gene was substituted for the *HRD1 Bst*E11 fragment, as described previously (Hampton *et al.*, 1996a). The *hrd3Δ::URA3* allele originated from a strain with the entire *HRD3* coding region replaced with *URA3* by PCR-mediated gene disruption as described previously (Hampton *et al.*, 1996a). In some cases, the *URA3* gene in the *hrd1Δ::URA3* and *hrd3Δ::URA3* alleles was replaced with the *TRP1* gene by a one-step gene replacement using *Sma*I-digested pUT11 (obtained from F. Cross, Rockefeller University). *hrd1Δ::LEU2*, *hrd3Δ::LEU2*, and *ubc7Δ::LEU2* alleles were constructed using the previously mentioned disruption plasmids pRH1184, pRH1185, and pRH1186, respectively. The *ubc7Δ::HIS3* allele was produced by PCR disruption. The *ubc7Δ::URA3* allele was constructed as described previously (Cronin *et al.*, 2000). All *hrd2-1* alleles originated from RHY402 (Hampton *et al.*, 1996a) and were introduced by crossing and subsequent sporulation to obtain the desired haploid progeny.

Degradation Assays

Cycloheximide–chase assays were performed as described previously (Gardner *et al.*, 1998). UP* samples were immunoblotted with antiserum generated against the last 10 residues of uracil permease (Silve *et al.*, 1991). Deg1-Hmg1p, Deg1-Hmg2p, and 1mycHmg2p samples were immunoblotted with 9E10 anti-myc antibody as described previously (Hampton and Rine, 1994).

Pulse–chase assays were performed by harvesting cells from log-phase cultures and resuspending them at 1 OD_{600} per milliliter in fresh minimal media with supplements without methionine. After 15 min of shaking at 30°C, cells were pulse-labeled with [^{35}S]methionine NEG-772 Easy Tag EXPRESS at 100 μCi/0.5 OD_{600} for 10 min. The chase period was initiated by addition of a stock solution of unlabeled methionine and cysteine at a final concentration of 50 μg/ml of each. At appropriate chase times, cells were harvested and resuspended in 100 μl SUME buffer + protease inhibitors (PI) (Gardner *et al.*, 1998); 100 μl of acid-washed glass beads were added, and the mixture was vortexed for 3 × 1 min. The mixture was clarified by centrifugation for 5 min, and 900 μl of IP buffer (100 mM Tris-HCl, 0.1% Triton X-100, 2 mM EDTA) and an appropriate quantity of specific polyclonal antiserum was added. Cultures were incubated at 4°C overnight. Protein A-Sepharose beads (100 μl, 10% wt/vol) were added to each sample and incubated for 1 h at 4°C. The beads were pelleted, washed three times with IP buffer + 0.1% SDS, and resuspended in 35 μl of 2× urea sample buffer (2× USB) (Gardner *et al.*, 1998). Samples were heated at 65°C for 5 min and loaded onto an 8% SDS-PAGE gel. Gels were treated with Amplify as directed, dried, and autoradiographed on Kodak BioMax film.

Membrane Fractionation

Localization of Deg1-Hmg1p in membrane fractions was performed similar to that described previously (Hampton and Rine, 1994). Briefly, ~8 OD_{600} of log-phase cells were harvested and resuspended in 200 μl of ice-cold lysis buffer (LB) (20 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 300 mM sorbitol) + PI + 200 μl of acid-washed glass beads. Samples were vortexed 6 × 1 min at 4°C with 30-s incubations on ice between each burst. Lysates were then withdrawn into another tube. The glass beads were washed two times with 100 μl of ice-cold LB + PI. Each wash was collected and placed with the withdrawn lysates to make up the crude lysate; 15 μl were withdrawn and added to 15 μl of 2× USB. The remaining crude lysate was spun two times for 5 s. The resulting supernatant was then spun for 30 min at 4°C to produce the final supernatant. The pellet remaining after the 30-min spin was resuspended in 275 μl of LB + PI and became the final pellet; 15 μl of 2× USB was added to both the final supernatant and final pellet. All samples were heated at 55°C for 10 min and immunoblotted as described above.

Ubiquitination Assays

Hmg1p and Deg1-Hmg1p were assayed for ubiquitination as described previously (Gardner and Hampton, 1999). Ubiquitination of UP* was assayed similar to Hmg1p except that samples were immunoblotted with monoclonal anti-ubiquitin antibody instead of anti-HA antibody.

GFP Analysis

Strains expressing GFP fusion proteins were grown into log phase in minimal media plus supplements and analyzed using a FACScan (Becton Dickinson, Palo Alto, CA) analytical flow microfluorimeter with settings typically used for fluorescein-labeled antibody analysis. Data were analyzed using CellQuest software. Each histogram represents 10,000 individual cells.

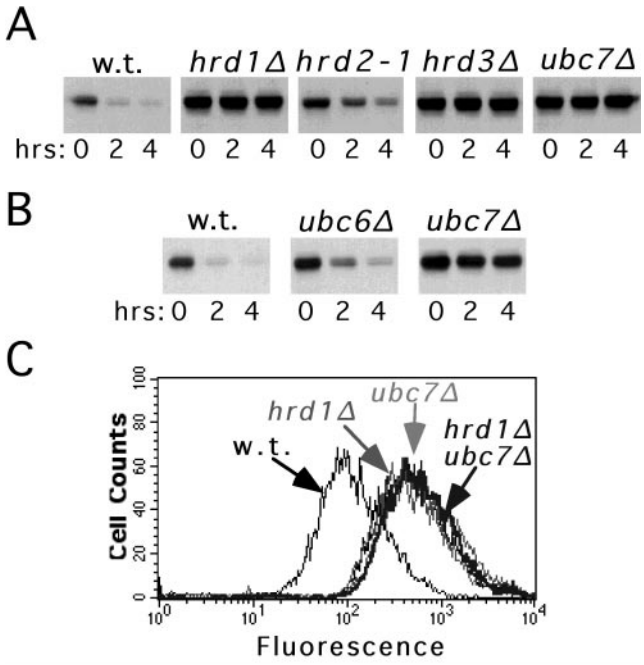

Figure 1. Hmg2p degradation is *HRD* dependent and *UBC6* independent. (A) Cycloheximide–chase assay of strains expressing 1myc-Hmg2p in a wild-type (RHY1611), *hrd1*Δ (RHY1626), *hrd2-1* (RHY1628), *hrd3*Δ (RHY1631), and *ubc7*Δ (RHY1633) genetic background. After addition of cycloheximide, lysates were prepared at the indicated times and immunoblotted with the 9E10 anti-myc antibody. (B) Cycloheximide–chase assay of strains expressing 1myc-Hmg2p in a wild-type (RHY1611), *ubc6*Δ (RHY1723), and *ubc7*Δ (RHY1633) genetic background. (C) Fluorescence histogram of strains expressing Hmg2p–GFP in a wild-type (RHY871), *hrd1*Δ (RHY880), *ubc7*Δ (RHY1056), and *hrd1*Δ*ubc7*Δ (RHY1486) genetic background. Strains were analyzed directly from early log-phase cultures. Each histogram represents 10,000 cells.

RESULTS

To evaluate the generality of *HRD* gene function in ER degradation, five distinct degradation substrates were analyzed for *HRD* dependence. Included were Hmg2p, a naturally degraded protein; Deg1-Hmg2p, a naturally degraded protein with an added sequence that specifically directs degradation by the ER ubiquitin–proteasome pathway; Deg1-Hmg1p, a normally stable ER membrane protein with the same added degron; Vph1p, a normal yeast protein that is degraded when not correctly assembled into a complex; and UP*, a protein that is retained in the ER and degraded by virtue of a mutation that inhibits proper folding.

Hmg2p: A Naturally Degraded Protein

The integral ER membrane protein, Hmg2p, is subject to *HRD*-dependent degradation that is regulated by the mevalonate pathway (Hampton and Rine, 1994; Hampton *et al.*, 1996a). It has been shown that Hmg2p degradation requires *HRD1*, *HRD3*, and *UBC7*; disruptions in any of these genes resulted in complete stabilization (Figure 1A) (Hampton *et al.*, 1996a; Hampton and Bhakta, 1997). A hypomorphic mu-

tation in the essential *HRD2*, which encodes a subunit of the 26S proteasome, stabilized Hmg2p to a lesser extent (Figure 1A) (Hampton *et al.*, 1996a). Interestingly, Hmg2p did not require *UBC6* for its degradation as was indicated in our earlier work (Hampton and Bhakta, 1997), which was confirmed in Figure 1B. The degradation of Hmg2p was slowed by less than twofold in the *ubc6*Δ strain, as seen by a less than twofold increase in the steady-state level and decrease in the degradation rate (Figure 1B, *ubc6*Δ), and confirmed by subsequent densitometric analysis (our unpublished results). Furthermore, the extreme stability of Hmg2p in a *ubc7*Δ mutant was not further enhanced by the added presence of the *ubc6*Δ mutation (our unpublished results).

The stabilization that resulted from null mutations in *hrd1*, *hrd3*, and *ubc7* was quite strong. Thus, it appeared that loss of any of these genes resulted in complete stability, as would be predicted if Hmg2p was degraded by a single pathway requiring these genes. To further test this model, we used the optical reporter Hmg2p–GFP to quantitatively evaluate the contribution of the *HRD1* and *UBC7* genes in Hmg2p degradation. The Hmg2p–GFP reporter protein undergoes bona fide regulated degradation in a manner identical to Hmg2p (Hampton *et al.*, 1996a,b; Cronin and Hampton, 1999). Changes in the Hmg2p–GFP degradation rate caused by regulatory or genetic alterations are faithfully reported as changes in the whole-cell fluorescence, which is monitored by flow cytometry. The reproducibility of this technique allows accurate detection of very subtle differences in the Hmg2p–GFP degradation rate, indicated by differences in the fluorescence histograms (Gardner *et al.*, 1998, Gardner and Hampton, 1999).

Otherwise isogenic strains expressing Hmg2p–GFP and single or double null alleles of *hrd1* and *ubc7* were compared by flow cytometry. As observed previously, either null allele, *hrd1*Δ or *ubc7*Δ, stabilized Hmg2p and resulted in an increase in cellular fluorescence, indicated by a rightward shift of the fluorescence histograms in the presence of the mutations (Figure 1C). Either null allele alone had an identical effect on the position of the fluorescence histogram. Furthermore, the presence of both the *hrd1*Δ and *ubc7*Δ alleles in the same strain had no additional effect on the fluorescence histogram of the resulting strain. The histogram of the strain containing both null alleles was superimposable with strains containing either single null allele (Figure 1C). This lack of additivity indicated that both genes were involved in the same pathway for Hmg2p degradation. Similar analysis of *hrd3*Δ strains indicated that *HRD3* and *HRD1* are also nonindependent, as predicted from earlier studies (Hampton *et al.*, 1996a), and that *HRD3* also does not independently contribute to *UBC7*-dependent degradation of Hmg2p (our unpublished results).

These results with the *hrd1*Δ, *hrd3*Δ, and *ubc7*Δ alleles, taken in isolation, implied a single mechanism for ubiquitin-mediated ER protein degradation that involved the membrane-bound *HRD* gene-encoded proteins and *UBC7*. We extended this analysis to several substrates that represented other scenarios in which ER degradation plays a role.

Deg1-Hmg2p: A Retargeted Protein

In many cases, proteins are targeted for ubiquitination and proteasomal degradation by recognition of small, autonomous degradation signals called degrons (Hochstrasser and Varshavsky, 1990; Varshavsky, 1991). When such sequences are added to heterologous proteins, the resulting fusions are often directed to the degradation pathway specified by the added signal. An example of such an autonomous degron is the Deg1 sequence found in the MATα2 transcriptional regulator. This 67 amino acid residue sequence, when fused to β-galactosidase, is sufficient to target this normally stable fusion partner for *UBC7*/*UBC6*-dependent degradation (Chen *et al.*, 1993).

The Deg1 fusion can also target normally stable ER membrane proteins such as Sec62p (Mayer *et al.*, 1998). Deg1-mediated degradation of soluble proteins requires *UBC6* and *UBC7* but not *HRD1* (Bordallo *et al.* 1998). Thus, we tested whether Deg1-directed ER membrane protein degradation would similarly be *HRD* gene independent, or, alternatively, whether the *HRD* genes would be required as in the case of normal Hmg2p.

We constructed a fusion gene in which the Deg1 coding sequence replaced the first 26 codons of the 1myc-Hmg2p coding sequence. The resulting protein was very rapidly degraded (Figure 2A) and not subject to regulation by the mevalonate pathway (our unpublished results). Deg1-Hmg2p degradation was significantly dependent on *UBC7*, but only partially dependent on *HRD1* and *HRD3* (Figure 2A). Pulse–chase analysis of Deg1-Hmg2p revealed that the half-life of the protein in the presence of the *hrd1*Δ or *hrd3*Δ alleles was only twofold greater than in the presence of the wild-type alleles (Figure 2B), whereas the half-life of Deg1-Hmg2p in the *ubc7*Δ background was significantly greater. Furthermore, degradation of Deg1p-Hmg2p exhibited a higher dependency on *UBC6* than normal Hmg2p (Figure 2C). Thus, in contrast to normal Hmg2p, the degradation of Deg1-Hmg2p had a significant component of *UBC7*-dependent degradation that was independent of both *HRD1* and *HRD3* and was partially dependent on *UBC6*.

Curiously, strains containing the *hrd2-1* allele also stabilized Deg1-Hmg2p, but additionally showed a 60-kDa immunoreactive fragment (Figure 2A, arrowhead) that has not been observed with Hmg2p in the same strain (our unpublished results). This fragment included the epitope tag in the linker region, and the Hmg2p catalytic region (our unpublished results), and was thus analogous to the C-terminal fragment produced from another Deg1-tagged ER membrane protein, Deg1-s62p, in the presence of a compromised proteasome (Mayer *et al.*, 1998). Because the Deg1-Hmg2p C-terminal fragment was not observed in the strongly stabilizing *ubc7*Δ null mutant, it most likely reflected some feature of proteasomal degradation or processing of Deg1-Hmg2p.

Flow cytometric analysis of strains expressing a GFP-reporter version of this protein, Deg1-Hmg2p–GFP, showed that the effects of the *hrd*Δ and *ubc7*Δ alleles on cellular steady-state fluorescence exactly recapitulated the effects as measured by cycloheximide–chase or pulse–chase assays (Figure 2D). Specifically, the fold change in steady-state fluorescence caused by a particular mutation was exactly the same as the change in half-life caused by that mutation. Thus, flow cytometric analysis provided information on Deg1-Hmg2p degradation that was equivalent to that provided by the pulse–chase or cycloheximide–chase analysis.

The results with Deg1-Hmg2p implied that a substantial component of *UBC7*-dependent degradation was indepen-

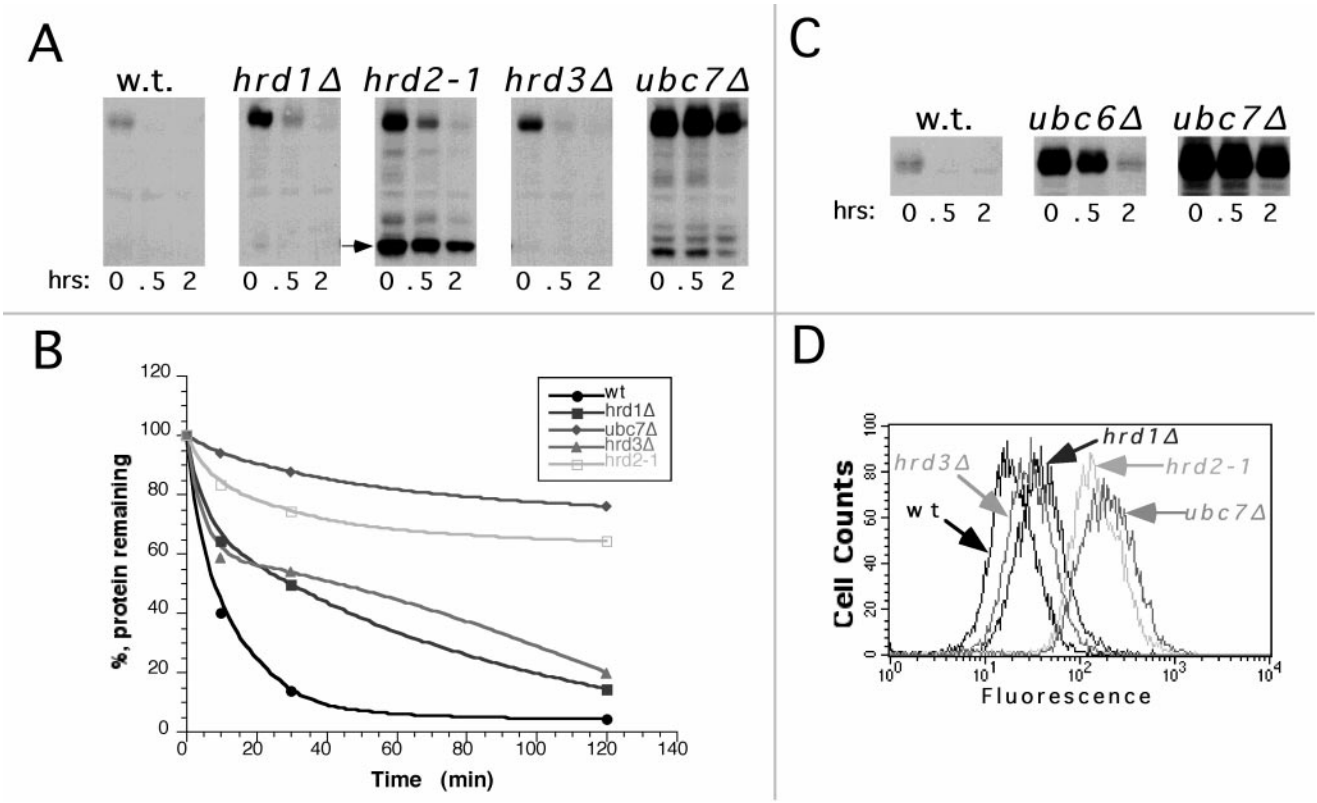

Figure 2. Deg1-Hmg2p was completely dependent on *UBC7* but only partially dependent on *HRD1*, *HRD3*, and *UBC6*. (A) Cycloheximide–chase assay of strains expressing Deg1-Hmg2p in a wild-type (RHY1610), *hrd1*Δ (RHY1613), *hrd2-1* (RHY1615), *hrd3*Δ (RHY1617), and *ubc7*Δ (RHY1619) genetic background. After addition of cycloheximide, lysates were prepared at the indicated times and immunoblotted with the 9E10 anti-myc antibody. An arrow marks the 60-kDa proteolytic fragment seen in *hrd2-1* strains. (B) Pulse–chase analysis of the identical strains in A. Cells were pulse-labeled with ^{35}S-Express for 10 min and chased for the indicated times. Deg1-Hmg2p was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The levels of Deg1-Hmg2p for each time point were determined by densitometric analysis of the autoradiograms. (C) Cycloheximide–chase assay of strains expressing Deg1-Hmg2p in a wild-type (RHY1610), *ubc6*Δ (RHY1656), and *ubc7*Δ (RHY1619) genetic background. (D) Fluorescence histogram of strains expressing Deg1-Hmg2p–GFP in a wild-type (RHY1374), *hrd1*Δ (RHY1575), *hrd2-1* (RHY1577), *hrd3*Δ (RHY1579), and *ubc7*Δ (RHY1581) genetic background. Strains were analyzed directly from early log-phase cultures. Each histogram represents 10,000 cells.

dent of *HRD1/HRD3*, because the effect of a *ubc7*Δ allele was much greater than the effect of the *hrd*Δ alleles. It was possible that the small *HRD* gene-dependent component of Deg1-Hmg2p degradation was due to the recognition of *HRD* gene-specific degradation determinants present in both Hmg2p and the Deg1-targeted fusion. Therefore, we tested the effect and *HRD* gene dependency of Deg1-dependent targeting on a normally stable ER membrane protein that does not undergo *HRD* gene-dependent (or any other sort of) ER degradation.

Deg1-Hmg1p: A Degron-targeted Stable ER Protein

To evaluate the effect of Deg1 on a normally stable ER membrane protein, we used the extremely stable HMGR isozyme, Hmg1p, as a fusion partner. Hmg1p also resides in the ER and is functionally redundant with Hmg2p (Basson *et al.*, 1988) but is strikingly stable (Hampton and Rine, 1994; Gardner *et al.*, 1998; Gardner and Hampton, 1999). In particular, we fused Deg1 to a composite reporter protein consisting of the Hmg1p transmembrane domain fused to the myc epitope-tagged linker and catalytic domain of Hmg2p. This protein has been shown to be as stable as native Hmg1p and is easily detectable with an anti-myc monoclonal antibody (Gardner *et al.*, 1998). We refer to the resultant fusion protein as Deg1-Hmg1p because the membrane region entirely determines the degradation behavior of yeast HMGR and its related reporter (Hampton and Rine, 1994; Hampton *et al.*, 1996b).

The Deg1 coding sequence was used to replace the first 26 codons of the coding region of the Hmg1p transmembrane domain. The stability of the resulting Deg1-Hmg1p fusion was compared with the unmodified protein by a cycloheximide–chase experiment. In a 4-h cycloheximide–chase experiment, Deg1-Hmg1p was completely degraded, whereas the parent protein without Deg1 (referred to as Hmg1p) was totally stable (Figure 3A).

The Deg1-Hmg1p fusion protein was also assayed for ubiquitination, by coexpressing HA-tagged ubiquitin with either Hmg1p construct. From these strains, each Hmg1p

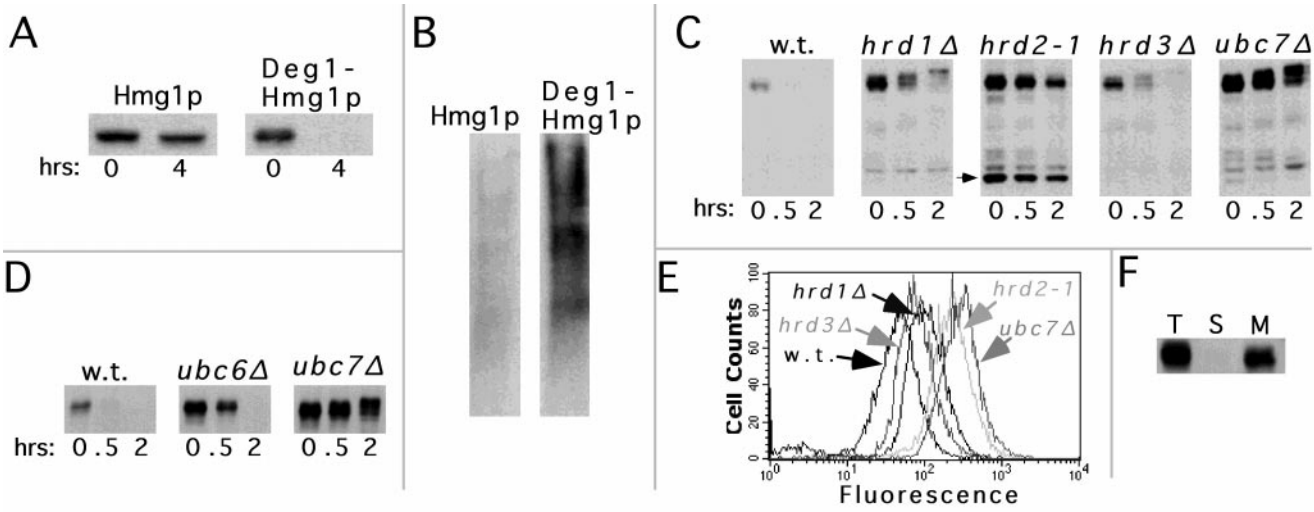

Figure 3. Deg1-Hmg1p degradation was completely dependent on *UBC7* but only partially dependent on the *HRD* genes and *UBC6*. (A) Degradation of Deg1-Hmg1p. Results of cycloheximide–chase assay of strains expressing Hmg1p (RHY636) and Deg1-Hmg1p (RHY493) are shown. After addition of cycloheximide, lysates were prepared at the indicated times and immunoblotted with the 9E10 anti-myc antibody. (B) Ubiquitination of Deg1-Hmg1p. Cultures of strains coexpressing an HA-tagged ubiquitin with either Hmg1p (RHY1467) or Deg1-Hmg1p (RHY1460) were lysed, and either Hmg1p or Deg1-Hmg1p was immunoprecipitated with antibodies raised against HMGR. Ubiquitination of the proteins was assayed by immunoblotting with the 12CA5 anti-HA antibody. (C) Cycloheximide–chase assay of strains expressing Deg1-Hmg1p in a wild-type (RHY1948), *hrd1*Δ (RHY1949), *hrd2-1* (RHY2079), *hrd3*Δ (RHY1950), and *ubc7*Δ (RHY2096) genetic background. Arrowhead marks the 60-kDa proteolytic fragment seen in *hrd2-1* strains. (D) Cycloheximide–chase assay of strains expressing Deg1-Hmg1p in a wild-type (RHY1948), *ubc6*Δ (RHY2097), and *ubc7*Δ (RHY2096) genetic background. (E) Fluorescence histogram of strains expressing Deg1-Hmg1p–GFP in a wild-type (RHY1359), *hrd1*Δ (RHY1566), *hrd2-1* (RHY1568), *hrd3*Δ (RHY1570), and *ubc7*Δ (RHY1572) genetic background. Strains were analyzed directly from early log-phase cultures. Each histogram represents 10,000 cells. (F) Membrane association of Deg1-Hmg1p. Membrane fractionation of strains expressing Deg1-Hmg1p (RHY1948) is shown. Lysates were spun for 30 min at 4°C to produce the supernatant and pellet fractions. Aliquots of total lysates (T), supernatant fraction (S), and membrane fraction (M) were loaded onto an 8% SDS-PAGE gel, transferred, and immunoblotted with the 9E10 anti-myc antibody.

variant was immunoprecipitated with polyclonal antibodies to the catalytic domain, and the precipitated protein was then immunoblotted for coprecipitated HA-Ub–Hmg1p conjugates. As expected, the added Deg1 sequence caused strong ubiquitination of Deg1-Hmg1p (Figure 3B), whereas normal, stable Hmg1p showed no detectable ubiquitination. Thus, addition of the Deg1 sequence to the stable Hmg1p protein programmed its ubiquitin-dependent degradation.

We then examined the *HRD* gene dependence of Deg1-Hmg1p degradation. Otherwise isogenic strains with the mutations of interest were constructed so that all expressed Deg1-Hmg1p from the same integrated, single genomic copy. Similar to Deg1-Hmg2p, Deg1-Hmg1p was significantly stabilized in the presence of the *ubc7*Δ allele, with little or no degradation observed during the cycloheximide treatment (Figure 3C). Furthermore, degradation of Deg1-Hmg1p was only partially affected by either the *hrd1*Δ or the *hrd3*Δ alleles, whereas the presence of the *ubc7*Δ allele had strong stabilizing effect (Figure 3C). Last, the presence of the *ubc6*Δ allele had a significant, partially stabilizing effect on Deg1-Hmg1p (Figure 3D), but this effect was much less than that observed for the *ubc7*Δ allele, similar to Deg1-Hmg2p.

The *hrd2-1* allele also stabilized Deg1-Hmg1p, and as with the Deg1-Hmg2p protein, caused the appearance of a 60-kDa fragment with the epitope tag and catalytic region (our unpublished results), which was stable over the course of the assay (Figure 3C, arrowhead). Why an impaired protease resulted in the appearance of C-terminal fragments of the Deg1-tagged proteins, or the previously reported Deg1-s62p (Mayer *et al.*, 1998), is unclear. So far, this appears to be a unique proteasomal phenotype for Deg1-containing membrane proteins because we have never seen an intermediate in any other ER degradation substrate examined in any *hrd2-1*-containing strain.

Interestingly, the molecular weight of Deg1-Hmg1p increased during the chase period, and this increase was due entirely to the glycosylation of Deg1-Hmg1p (our unpublished results). Deg1-Hmg1p was not glycosylated under normal cellular growth conditions (Figure 3C, 0 time points), indicating that glycosylation was the result of the cycloheximide treatment. The reason for this is not clear.

We further analyzed Deg1-Hmg1p degradation by flow cytometric analysis of strains expressing a GFP-reporter version of this protein, Deg1-Hmg1p–GFP. This analysis showed that the effects of the *hrd1*Δ and *hrd3*Δ alleles had an approximately twofold stabilizing effect on the degradation of Deg1-Hmg1p (Figure 3E), whereas the *ubc7*Δ allele was completely stabilizing. Thus, alterations in Deg1-Hmg1p stability by the presence of the null alleles, as determined quantitatively by flow cytometric analysis, were nearly identical to those of Deg1-Hmg2p, as determined by pulse–chase or flow cytometric analyses (compare with Figure 2, B and D).

One explanation for the significant independence from *HRD1* and *HRD3* of Deg1-mediated degradation was that

most of the Deg1-modified protein was not membrane bound and, as a result, was degraded in a manner similar to the Deg1-mediated degradation of soluble proteins that is *UBC7* dependent but completely *HRD1* independent (Bordallo *et al.* 1998). To address this, the membrane localization of Deg1-Hmg1p was determined. Almost all of the Deg1-Hmg1p immunoreactivity in whole-cell lysates fractionated with microsomal fractions (Figure 3F), and this membrane association was disrupted only when detergents were added (our unpublished results). Furthermore, cellular localization studies with strains expressing Deg1-Hmg1p–GFP showed typical ER membrane fluorescence that was increased, but not qualitatively changed, by the *ubc7*Δ allele (our unpublished results). Thus, the *HRD*-independent component of Deg1-Hmg1p degradation apparently occurred with membrane-associated protein.

The results above indicated that *HRD* gene dependence of ER degradation could vary between substrates from complete to very minor, even when *UBC7* dependence remained very strong. It was possible that the minimal role of *HRD1/HRD3* in the degradation of the Deg1-targeted proteins was a particular feature of that degron. Thus, we extended our analyses to other substrates of ER degradation, with particular interest in cases in which degradation is brought about by features of the substrates that are posited to be recognized in the normal functioning of the ER quality control apparatus. Specifically, we assessed the role of the *HRD* genes in two other substrates of ER degradation, unassembled Vph1p and UP*, a misfolded protein.

Vph1p: An Unassembled Subunit of a Protein Complex

Vph1p is a multispanning membrane protein that is a subunit of the multimeric, membrane-bound V_O complex of the vacuolar membrane ATPase (Manolson *et al.*, 1992). Vma21p is a non-V_O protein required for correct assembly of the V_O complex in the ER. In *vma21*Δ strains, which do not express Vma21p, the V_O complex fails to assemble, and the "orphaned" Vph1p protein is retained in the ER where it is degraded (Hill and Stevens, 1994, 1995). Vph1p is analogous to Hmg2p in that it is a normal, multispanning membrane protein that is degraded in the absence of any introduced mutations to the protein itself that might cause misfolding or misassembly. To study the degradation of Vph1p, we introduced a *vma21* null allele into our wild-type strain background. Although we prefer to assay protein stability with multiple degradation assays, the available reagents did not reproducibly give a strong immunoblotting signal in a cycloheximide–chase assay (details available from authors by request). Therefore, we only used pulse–chase experiments with a polyclonal antibody to determine the stability of radiolabeled Vph1p. As reported (Hill and Stevens, 1994), Vph1p was degraded in *vma21*Δ cells but remained stable in wild-type cells (Figure 4A).

We used the *vma21*Δ mutant to test the *HRD* gene dependency of Vph1p degradation. A series of otherwise isogenic strains with various relevant mutations in the *vma21*Δ background were constructed by crossing and isolation of haploid progeny. Vph1p degradation was then compared in this collection of isogenic strains. In contrast to Hmg2p, another natural protein, the presence of the *hrd1*Δ allele caused only partial stabilization of Vph1p (Figure 4, B and C). Similarly, the *ubc7*Δ allele caused similar partial stabilization. Although complete stabilization did not occur, the comparable stabilization caused by either single mutation suggested that the *UBC7*-dependent component of Vph1p degradation was equally dependent on *HRD1*; however, the combined *hrd1*Δ*ubc7*Δ alleles demonstrated a dramatic additive effect on Vph1p stabilization, indicating that Hrd1p and Ubc7p did not necessarily function together in Vph1p degradation (Figure 4, B and C). Similar partial stabilization of Vph1p was also seen in the other *hrd* mutants, suggesting that Hrd2p and Hrd3p were also involved in Vph1p degradation (Figure 4, B and C). The presence of the *ubc6*Δ allele showed no effect on Vph1p degradation, but the combined *ubc6*Δ/*ubc7*Δ alleles showed a similar additive effect as the *hrd1*Δ/*ubc7*Δ allele (Figure 4, B and C).

The role of *HRD1* in Vph1p degradation was complex. Nevertheless, it was clear that *HRD1* was not absolutely required for degradation of this natural protein, because loss of the *HRD1* gene in the presence of *UBC7* caused only a small effect on Vph1p stability; however, the loss of Hrd1p in a *ubc7*Δ background caused a significant increase in stability above that caused by the loss of *UBC7* alone. This implied that in some circumstances *HRD1* could contribute to Vph1p degradation, and in a manner independent of *UBC7*, unlike the equally important, codependent role that theses two genes played in Hmg2p degradation. Because loss of Hrd1p had a much larger effect on Vph1p stability in the *ubc7*Δ null than in the normal strain, it would appear that the dependency of Vph1p degradation on Hrd1p can vary in different genetic circumstances. Finally, there was also a significant component of degradation that was preserved in the *ubc6*Δ/*ubc7*Δ strains.

UP: A Quality Control Substrate*

Yeast uracil permease (UP), encoded by the *FUR4* gene, is a plasma membrane protein required for the uptake of uracil (Chevallier, 1982; Chevallier and Lacroute, 1982). A mutated form of the uracil permease, Fur4–430Np, referred to herein as UP*, contains a 3 amino acid residue insertion in a predicted cytoplasmic loop. UP* is retained in the ER, presumably because of improper folding, where it is degraded via the ubiquitin–proteasome pathway (Galan *et al.*, 1998). To assess the involvement of the *HRD* genes in UP* degradation, strains carrying the appropriate *hrd* null alleles were transformed with a 2 μ plasmid containing UP*. Degradation of UP* was assayed by a cycloheximide–chase assay. Experiments were performed at 37°C for optimal degradation, as reported (Galan *et al.*, 1998). Degradation of UP* was slowed significantly in the presence of the *ubc7*Δ allele (Figure 5A), similar to the stabilization previously reported in the presence of both the *ubc6*Δ and *ubc7*Δ alleles (Galan *et al.*, 1998). An isogenic strain with both the *ubc6*Δ and *ubc7*Δ alleles did not show any greater level of stabilization than a strain with only the *ubc7*Δ allele (our unpublished results); however, there was no effect on degradation in strains with either the *hrd1*Δ or *hrd3*Δ alleles (Figure 5A). This lack of effect by either of the *hrd1*Δ or *hrd3*Δ alleles suggested that the *UBC7*-dependent degradation of UP* occurred in a completely *HRD* gene-independent manner. Furthermore, overexpression of Hrd1p to levels that hasten the degradation of various ER degradation substrates (N. Bays, unpublished

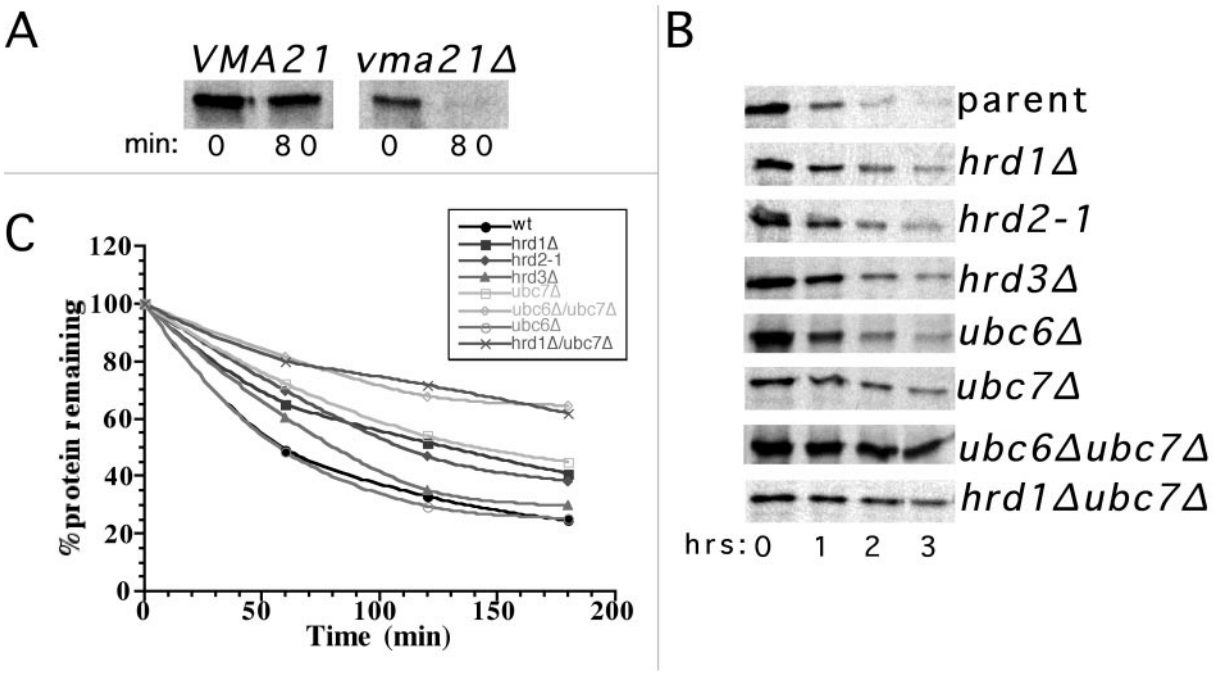

Figure 4. Vph1p degradation was partially dependent on the *HRD* genes and *UBC7*. (A) Degradation of Vph1p in a *vma21*Δ strain. Results of pulse–chase experiment of *VMA21* (RHY566) and *vma21*Δ (RHY918) strains are shown. Cells were pulse-labeled with ^{35}S-Express for 10 min and chased for the indicated times. Vph1p was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. (B) Pulse–chase experiment of strains containing the *vma21*Δ allele (parent strain: RHY918) and the *hrd1*Δ allele (RHY1032), the *hrd2-1* allele (RHY1067), the *hrd3*Δ allele (RHY1034), the *ubc6*Δ allele (RHY1228), the *ubc7*Δ allele (RHY1069), the *ubc6*Δ*ubc7*Δ allele (RHY1488), or the *hrd1*Δ *ubc7*Δ allele (RHY1491). (C) Densitometric analysis of the pulse–chase experiments in B. Each value is the average of at least two independent pulse–chase experiments. SDs were <10%.

results) similarly had no effect on UP* steady-state level or degradation rate (our unpublished results).

To further test this surprising independence of ER degradation from *HRD1/HRD3*, we also evaluated the role of these genes in ubiquitination of UP*, because both are required for ubiquitination of Hmg2p (N. Bays and R. Hampton, unpublished results). Otherwise isogenic strains carrying the appropriate null alleles and expressing a single integrated copy of the UP* coding region from the strong GAPDH promoter were compared in a direct ubiquitination assay. UP* was immunoprecipitated with an N-terminal anti-Fur4p antibody, and the precipitates were immunoblotted for coprecipitated, covalently linked ubiquitin with an anti-ubiquitin monoclonal antibody. In the wild-type strain, ubiquitinated UP* ran as a distribution of high molecular weights (Figure 5B). UP* ubiquitination was strongly dependent on *UBC7*, indicated by the attenuation of the signal caused by the *ubc7*Δ allele. A deficiency in proteasomal function caused by the presence of the *hrd2-1* allele resulted in the expected increase in UP* ubiquitination; however, in agreement with the degradation experiments, UP* ubiquitination was completely unaffected by the presence of either the *hrd1*Δ or the *hrd3*Δ alleles. Thus, in two different assays of degradation, UP* degradation was dependent on *UBC7* but completely independent of *HRD1* and *HRD3*.

DISCUSSION

The *HRD* gene-encoded proteins are responsible for the degradation of a wide variety of ER-associated proteins, including Hmg2p, CPY*, and Sec61–2p (Hampton *et al.*, 1996a; Bordallo *et al.*, 1998). The diversity of these substrates has led to the reasonable proposal that the *HRD* gene-encoded proteins function in a general ER degradation pathway, which targets proteins for ubiquitination mediated by the ER-associated, ubiquitin-conjugating enzymes Ubc7p and Ubc6p. Other studies on various substrates have indicated that Ubc7p and Ubc6p are the main, and perhaps only, ubiquitin-conjugating enzymes that participate in ER degradation (Hiller *et al.*, 1996; Hampton and Bhakta, 1997; Sommer and Wolf, 1997). Thus, the simplest model for ER-associated degradation is that Hrd1p and Hrd3p work together with Ubc7p, and to a lesser and variable extent Ubc6p, in a single pathway for ER degradation, and that all ER degradation substrates are equally dependent on this mechanism; however, this hypothesis has never been systematically tested by direct comparison of various substrates in isogenic strains. Accordingly, we analyzed various ER membrane proteins for *HRD* gene and *UBC7* dependence, with the expectation that all ER-associated degradation substrates would show a strong and equivalent dependence on the *HRD* gene-encoded proteins and the ER ubiquitin-con-

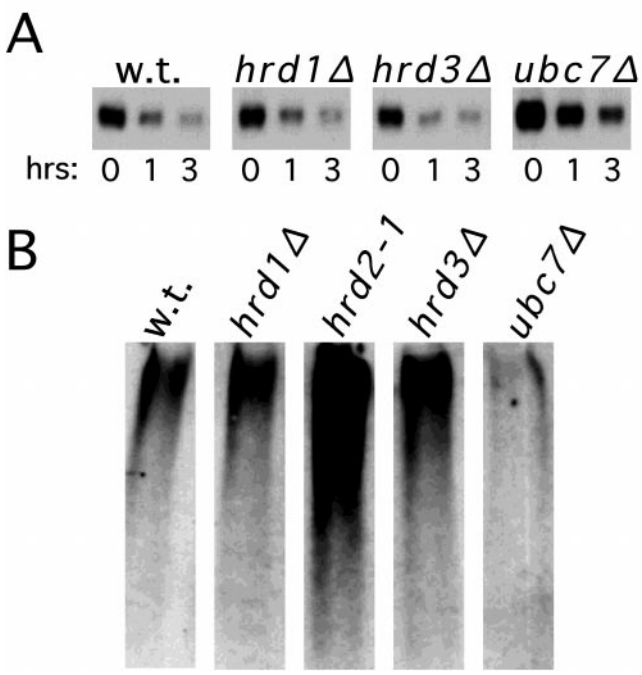

Figure 5. UP* degradation was independent of *HRD1* and *HRD3* but dependent on *UBC7*. (A) Cycloheximide–chase assay of strains expressing UP* in a wild-type (RHY1951), *hrd1*Δ (RHY2094), *hrd3*Δ (RHY1904), and *ubc7*Δ (RHY1900) genetic background. After addition of cycloheximide, lysates were prepared at the indicated times and immunoblotted with antiserum generated against the last 10 residues of uracil permease. (B) Levels of UP* ubiquitination correlated with its *HRD*-independent degradation. Cultures of strains expressing UP* in a wild-type (RHY1216), *hrd1*Δ (RHY1222), *hrd2-1* (RHY1218), *hrd3*Δ (RHY1223), or *ubc7*Δ (RHY1221) genetic background were lysed, and UP* was immunoprecipitated with antibodies generated against the N-terminus of uracil permease. Ubiquitination of UP* was assayed by immunoblotting with an anti-ubiquitin antibody.

jugating enzyme Ubc7p for degradation. In contrast to this simple model, we discovered that even when the analysis was restricted to only membrane proteins, the *HRD* gene dependence of ER degradation varied widely.

As expected from our previous results, Hmg2p degradation was strongly dependent on *HRD1*, *HRD3*, and *UBC7*, such that Hmg2p was completely stable in stains that carried null alleles of these genes. Furthermore, *HRD1* and *UBC7* appeared to work together because the presence of both null alleles had a stabilizing effect on Hmg2p degradation identical to that of either of the single null alleles. Hmg2p degradation had very little dependence on *UBC6*. Whether this reflects unique features within Hmg2p that allow it to be a regulated substrate of ER degradation or is simply an extreme example of the often-observed predominance of *UBC7* in ER degradation is not yet clear. Nevertheless, the roles of Hrd1p, Hrd3p, and Ubc7p in Hmg2p degradation indicated the existence of a single degradation mechanism codependent on each of these proteins.

In contrast to Hmg2p, our studies with several other substrates revealed that *UBC7*-dependent degradation of an ER-associated protein could proceed independently of the *HRD* gene-encoded proteins. Either Hmg1p or Hmg2p with an appended Deg1 sequence was subject to degradation that was almost completely dependent on *UBC7* but showed little requirement for *HRD1* or *HRD3*, providing an example of *UBC7*-dependent degradation that was uncoupled from the *HRD* gene-encoded proteins. This possibility was demonstrated even more strikingly with the misfolded UP* protein, which was subject to *UBC7*-dependent degradation and ubiquitination that was completely independent of either *HRD1* or *HRD3*. Thus, it is clear that the *HRD* gene-encoded proteins, although important for various quite distinct degradation substrates, are not globally involved in the degradation of all ER-associated proteins

HRD1 and *HRD3* are required for the degradation of a diverse collection of proteins that appear to have in common only the presence of misfolding mutations. Thus, it would seem reasonable to imagine that the *HRD* gene-encoded proteins are involved in the recognition of common features of quality control substrates, as well as some natural proteins, such as Hmg2p, that may also have these features as part of their native structure; however, the results with UP* indicated that the action of the *HRD* gene-encoded proteins cannot be this general. UP* is an example of a typical ER quality control substrate in which a mutation results in aberrant ER retention and degradation. Yet, the degradation and ubiquitination of UP* showed no detectable requirement for either *HRD1* or *HRD3*. In contrast, another "classic" quality control substrate, Pdr5p*, also a mutant membrane transporter that is retained and degraded in the ER, shows significant and equal dependence on *HRD1* and *UBC7* (Plemper *et al.*, 1998). The reason why diverse substrates such as Hmg2p, CPY*, and Pdr5p* share comparable *HRD* gene dependence, but similar substrates such as UP* and Pdr5p* have distinctly different *HRD* gene requirements, is not yet clear.

Taken together, our results indicated that the role of the *HRD* gene-encoded proteins in ER degradation vary widely from complete, to partial, to no involvement at all. The varying degrees of *HRD* gene dependence that we observed might suggest that there are multiple mechanisms to present substrates to the ER-associated ubiquitin-conjugating enzymes, such as Ubc7p. One simple model is that Hrd1p and Hrd3p form part of an ER-specific E3 ubiquitin ligase that helps target a subset of ER degradation substrates for Ubc7p/Ubc6p-dependent degradation. This is quite reasonable considering that Hrd1p is homologous to a family of known ubiquitin ligases that all share a functionally required motif known as an H2-RING finger (Joazeiro *et al.*, 1999; Lorick *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999). Furthermore, we have recently demonstrated that Hrd3p physically interacts with a specific region of Hrd1p (R. Gardner, G. Foss, and R. Hampton, unpublished results). Thus, it is reasonable to imagine that Hrd1p and Hrd3p are part of an ER-associated ubiquitin ligase complex that promotes transfer of ubiquitin from specific E2s such as Ubc7p to specific degradation substrates. Substrates that completely require *HRD1* and *HRD3* for degradation, such as Hmg2p, would interact only with the Hrd1p/Hrd3p-containing ubiquitin ligase. Conversely, ER substrates that undergo ubiquitin-mediated degradation in a manner independent of the *HRD* gene-encoded proteins, such as UP*, may be recognized by different ubiquitin ligases or alternatively may

not require the action of an E3. Whatever the mechanism of Hrd1p/Hrd3p, it is not yet clear what determines whether a substrate will be *HRD1/HRD3* dependent or independent.

In addition to varying *HRD1/HRD3* dependence, our panel of substrates exhibited varying degrees of *UBC7* dependence as well. Degradation of either UP* or Vph1p was only partially dependent on *UBC7/UBC6*, indicating the possibility that alternative mechanisms of ER-associated degradation using different ubiquitin-conjugating enzymes, or perhaps even distinct mechanisms, may be at play. The discovery and analysis of more ER degradation substrates will help reveal the rules that determine cellular targeting of ER degradation substrates. This endeavor combined with ongoing analysis of molecular mechanisms of degradation in well-studied substrates will clarify the cellular strategies used to recognize and destroy ER-associated proteins.

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