In Vitro Analysis of Hrd1p-mediated Retrotranslocation of Its Multispanning Membrane Substrate 3-Hydroxy-3-methylglutaryl (HMG)-CoA Reductase

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Endoplasmic reticulum (ER)-associated degradation (ERAD) is responsible for the ubiquitin-mediated destruction of both misfolded and normal ER-resident proteins. ERAD substrates must be moved from the ER to the cytoplasm for ubiquitination and proteasomal destruction by a process called retrotranslocation. Many aspects of retrotranslocation are poorly understood, including its generality, the cellular components required, the energetics, and the mechanism of transfer through the ER membrane. To address these questions, we have developed an in vitro assay, using the 8-transmembrane span ER-resident Hmg2p isozyme of HMG-CoA reductase fused to GFP, which undergoes regulated ERAD mediated by the Hrd1p ubiquitin ligase. We have now directly demonstrated in vitro retrotranslocation of full-length, ubiquitinated Hmg2p-GFP to the aqueous phase. Hrd1p was rate-limiting for Hmg2p-GFP retrotranslocation, which required ATP, the AAA-ATPase Cdc48p, and its receptor Ubx2p. In addition, the adaptors Dsk2p and Rad23p, normally implicated in later parts of the pathway, were required. Hmg2p-GFP retrotranslocation did not depend on any of the proposed ER channel candidates. To examine the role of the Hrd1p transmembrane domain as a retrotranslocon, we devised a self-ubiquitinating polytopic substrate (Hmg1-Hrd1p) that undergoes ERAD in the absence of Hrd1p. In vitro retrotranslocation of full-length Hmg1-Hrd1p occurred in the absence of the Hrd1p transmembrane domain, indicating that it did not serve a required channel function. These studies directly demonstrate polytopic membrane protein retrotranslocation during ERAD and delineate avenues for mechanistic understanding of this general process.

The endoplasmic reticulum (ER)-associated degradation (ERAD) pathway mediates the destruction of numerous integra...
complex presumably by promoting retrotranslocation of ER-embedded Hmg2p. Due to the requirement for retrotranslocation in all ERAD pathways we have adapted our in vitro assay of Hrd1p-mediated ubiquitination of the normally degraded fusion Hmg2p-GFP to study this ER removal step in ERAD. We have reconstituted Hrd1p-mediated ubiquitination and retrotranslocation of Hmg2p-GFP in vitro (21, 22). We have now directly demonstrated that the entire 8-spanning Hmg2p-GFP protein is removed from the membrane by this process, remaining intact yet soluble after retrotranslocation. The dislocation of intact Hmg2p-GFP required both Cdc48p and hydrolysis of the β-γ bond of ATP. The Ubx2p adaptor protein functioned in a manner consistent with its proposed role in Cdc48p anchoring to the ER. Surprisingly, the Dsk2p/Rad23p proteasomal coupling factors were also required for retrotranslocation. Neither derivatives nor Sec61p were implicated in Hmg2p-GFP retrotranslocation by our assay. Furthermore, an engineered substrate based on HMG-CoA reductase underwent ERAD in the complete absence of Hrd1p or Doa10p and in vitro, full-length retrotranslocation, both indicating that the large transmembrane domains of either of these ERAD E3 ligases were not required for membrane extraction. Taken together, these studies define a core set of proteins that can mediate recognition and retrotranslocation of the HRD substrate Hmg2p-GFP and will allow mechanistic analysis along all points of the ERAD pathway.

**EXPERIMENTAL PROCEDURES**

*Strains and Media—* All strains were derived from the S288C derivative genetic background and grown at 30 °C with aeration, as described previously (23). Yeast strains are listed in Table S1. Cultures used for assessing degradation by cycloheximide chase and microsome donor strains were logarithmically grown in minimum medium with 2% glucose and appropriate amino acid supplements (A_{600} < 0.35). Cytosol donor strains were grown in YPD medium to an A_{600} between 0.8 and 1.2. Standard yeast techniques used to integrate plasmids, prepare gene deletions, and incorporate mutant alleles are detailed in the supplemental material.

*In Vitro Ubiquitination—* In vitro reactions were prepared and analyzed as described previously (21, 22), and are delineated in detail in the supplemental material. Briefly, cytosols from strains overexpressing Ubc7p or an otherwise identical ubc7Δ null strain were prepared similarly to those from the Schekman laboratory (24). Cytosol strains were lysed by grinding under liquid nitrogen and ultracentrifuged for membrane removal. Typically, 1 mM ATP was added to cytosol; however, ATP was not added in AMP-PNP (Sigma) experiments, to allow choice of nucleotide during the two reaction phases. Protein concentration was measured using Bradford reagent. Cytosol concentrations were adjusted to 20–25 mg/ml for ubiquitination and retrotranslocation assays. Microsomes were prepared from ubc7Δ null yeast strain expressing 3HA-Hrd1p and Hmg2p-GFP from the TDH3 promoter by bead lysis followed by membrane fractionation. The microsomal pellets were resuspended in the same buffer used to prepare cytosol. MG-132 (Sigma) was added to microsome and cytosol preparations except where indicated.

One in vitro ubiquitination reaction typically consisted of 10 μl of microsome suspension and 12 μl of cytosol. ATP (500 mM stock) was added to each reaction to a final concentration of 30 mM to initiate reaction, and the reaction was then incubated for 1 h at 30 °C. The assay was stopped by solubilization in 200 μl of SUME (1% SDS, 8 μm urea, 10 mM MOPS, pH 6.8, 10 mM EDTA) with protease inhibitors and 5 mM N-ethylmaleimide. Detergent immunoprecipitation buffer was added prior to incubation of rabbit polyclonal antiserum (anti-GFP, anti-loop and anti-Hrd1p antiseras, prepared in collaboration with Scantibodies, Inc. (Santee, CA)) for immunoprecipitation (IP) of Hmg2p-GFP, Hrd1p, or Hmg1 Hrd1p. Overnight incubation with antiserum was followed with Protein A-Sepharose (GE Healthcare) incubation for 2 h at 4 °C. Protein A beads were washed twice, aspirated to dryness, resuspended in 2 × urea sample buffer (2 × USB: 8 μm urea, 4% SDS, 10% β-mercaptoethanol, 125 mM Tris, pH 6.8), and incubated at 50 °C for 10 min. IPs were resolved by SDS-PAGE using 8% Tris-glycine gels, transferred to nitrocellulose, and immunoblotted with monoclonal antibodies anti-ubiquitin (Zymed Laboratories Inc., South San Francisco, CA), anti-GFP (BD Biosciences), or anti-HA (Covance, Princeton, NJ). Goat anti-mouse conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) recognized primary antibodies. Western Lightning chemiluminescence reagents (PerkinElmer Life Sciences) were used for immunodetection.

*In Vitro Retrotranslocation—* Each in vitro retrotranslocation set of total, supernatant, and pellet fractions was typically derived from one 3 × in vitro ubiquitination reaction. The reaction was run at 30 °C for 1 h and terminated by the addition of 1.5 μl of 50 × N-ethylmaleimide to a final concentration of 5 mM. One reaction equivalent (typically 24 μl) was transferred to one tube designated as total (T in Figs. 1–8), and another reaction equivalent was transferred to a tube for centrifugation for 1 h at 25,000 × g at 4 °C. The resulting supernatant (S) was carefully removed, and the resulting pellet (P) was resuspended in the same volume as the supernatant and total fractions. Each fraction was solubilized with SUME and immunoprecipitated and detected as described. The use of identical volumes allowed direct visual comparison of the immunoblotted proteins present in each fraction.

*Nondetergent Immunoprecipitation—* In vitro ubiquitination and retrotranslocation was carried out as described but using non-detergent IP buffer (15 mM Na2HPO4, 150 mM NaCl, 10 mM EDTA) in the immunoprecipitation of the supernatant fraction. Either preimmune, anti-GFP, or anti-loop antiserum was added and incubated in IPs. After the addition and incubation of Protein A-Sepharose, protein-bound beads were washed extensively with non-detergent IP buffer. Immunoprecipitated Hmg2p-GFP was removed from beads by the addition of sample buffer, resolved by 8% SDS-PAGE gels, and immunoblotted as described.

*Ubiquitin Stripping—* Recombinant Usp2-cc, the catalytic core of the deubiquitinating enzyme Usp2p, was a generous gift from the Kopito laboratory (Stanford University) and prepared from a plasmid derived from Rohan Baker (Australian National University). In vitro ubiquitination and retrotranslocation was carried out as described except that leupeptin was not added to any of the buffers used in the preparation of cytosol and micro-
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somates. Seven in vitro ubiquitination reactions were incubated for 1 h at 30 °C and then centrifuged at 25,000 × g. The supernatant fractions were pooled. Two reaction equivalents (50 μl) of supernatant received 5 μl of Usp2-cc (0.8–1.6 mg/ml) or buffer. The Usp2-cc reactions were incubated for 1 h in a 37 °C incubator. The reactions were stopped with SUME containing PIs and N-ethylmaleimide, and retrotranslocated substrate was immunoprecipitated in the same way as described. Half of each IP was used for detection of anti-ubiquitin, and the other half was used to detect Hmg2p-GFP with anti-GFP or Hmg1-Hrd1p with anti-HA antibodies.

Use of GST–Ubiquitin—In vitro reactions were modified by the addition of either GST-ubiquitin (Boston Biochem, Cambridge, MA) or ubiquitin (Sigma) to a final concentration of 20 μM in the reaction mix. Retrotranslocation and detection were carried out as in other reactions, using anti-ubiquitin or anti-GFP immunoblotting.

Cycloheximide Chase of Hmg1-Hrd1p—Preparation of whole cell lysates was previously described (25). Logarithmically growing cells were incubated with the protein synthesis inhibitor cycloheximide (50 μg/ml) for the indicated times and broken by bead lysis. Cell lysates were separated by SDS-PAGE, and the degradation substrate was detected with anti-HA antibodies.

Protease Protection Assay—Microsomes were prepared from the strain expressing the Hmg1-Hrd1p fusion used in the in vitro retrotranslocation assay for trypsin digestion, as previously described (26). The microsomes were resuspended in reaction buffer and incubated with 150 μg/ml trypsin (Sigma) for 0, 2, 8, and 32 min. An equal volume of 2X USB buffer was added to stop the reactions. The samples were then separated by 14% SDS-PAGE, and immunoblots were detected with anti-Myc 9E-10 antibody (hybridoma from ATCC (Manassas, VA)).

Flow Cytometry—Flow cytometry was carried out as previously described (27). Yeasts grown in minimum medium with 2% glucose and appropriate amino acids into log phase (A600 < 0.2) were incubated with cycloheximide (50 μg/ml) for the times indicated. The BD Biosciences FACSCalibur flow cytometer measured the individual fluorescence of 10,000 cells. CellQuest software was used to analyze the data and plotted fluorescence versus cell count histograms.

RESULTS

In these studies, we employed Hmg2p-GFP, in which the catalytic domain was replaced with GFP. This substrate underwent entirely normal, HRD pathway-dependent regulated degradation like its parent protein Hmg2p (27). Hrd1p-mediated ubiquitination of Hmg2p-GFP involves both membrane-bound and soluble proteins. The E2 Ubc7p is soluble, as are ubiquitin, the proteasome, the Cdc48p complex, and other coupling factors, such as Rad23p. Conversely, the E3 ligase, the substrate, and Cue1p, the required anchor for Ubc7p, are integral membrane proteins. Our in vitro assay uses two distinct strains as sources of ER membranes and cytosol that are mixed to initiate the in vitro reaction (21, 22).

The microsome strain expresses epitope-tagged ligase 3HAG-Hrd1p, substrate Hmg2p-GFP, Cue1p, and any other membrane-bound proteins required for the process. The microsome strain harbors a null mutation in UBC7, rendering Hmg2p-GFP in a nonubiquitinated state prior to assay initiation (28). The cytosol strain is devoid of Hmg2p-GFP and expresses Ubc7p from the strong TDH3 promoter to provide a pool of soluble E2, and other cytosolic proteins required for ERAD. These microsome and cytosol strains were modified to produce mutant and null strains presented in every subsequent in vitro experiment.

The reaction is started by mixing Ubc7p-containing cytosol with microsomes and ATP, followed by incubation at 30 °C. Ubiquitin transfer is measured by immunoprecipitation of Hmg2p-GFP, SDS-PAGE, and immunoblotting for Hmg2p-GFP itself or ubiquitin. The assay reaction as used in this work follows a number of biological criteria of specificity, including strict dependence on Hrd1p, lysine-6 of Hmg2p,3 and Ubc7p, and its membrane anchor Cue1p (22).

Hrd1p is expressed from the TDH3 promoter at levels sufficient to operate in the absence of a number of ERAD factors, including Hrd3p (see below), Usa1p,4 and Yos9p.5 The assay thus defines the minimal components sufficient for successful retrotranslocation, providing the best avenue for complete reconstitution. Hrd1p at this level causes sufficient ubiquitination of Hmg2p-GFP for the recovery and direct detection of the retrotranslocated substrate (see below), allowing analysis of the dislocated Hmg2p-GFP species and the partner molecules needed for solubilization of a multispanning membrane protein. A typical ubiquitination reaction is shown in Fig. 1A, demonstrating the dependence on Hrd1p.

To directly evaluate Hmg2p-GFP retrotranslocation, we fractionated the in vitro reaction mix to assess the amount of ubiquitinated Hmg2p-GFP present in the soluble phase. Identical volumes were withdrawn from a reaction mix after incubation (see “Experimental Procedures”). One was processed without fractionation to evaluate total ubiquitinated Hmg2p-GFP. The other was centrifuged at 25,000 × g. The supernatant was removed, and the membrane pellet was resuspended in reaction buffer to the same volume as the removed supernatant. All three equal volume samples were then analyzed for ubiquitinated Hmg2p-GFP. The results of this retrotranslocation assay are shown in Fig. 1B, as three immunoblotting lanes labeled T (total reaction mix), S (supernatant), and P (pellet). Each lane includes the ubiquitin immunoblot (for ubiquitinated Hmg2p-GFP) and the GFP blot for unmodified Hmg2p-GFP. A significant portion of the ubiquitinated Hmg2p-GFP is in the supernatant, consistent with retrotranslocation. Immunoprecipitation of the supernatant with preimmune serum (see Fig. 2B) resulted in no anti-ubiquitin immunoreactivity, indicating that the ubiquitin immunoreactivity was from Hrd1p-modified Hmg2p-GFP generated in the reaction. The appearance of ubiquitinated Hmg2p-GFP in the supernatant was time-dependent (Fig. S1). We found that the intensity of the signal for retrotranslocated protein was greater (compare S lanes in Fig. 1C) when proteasome inhibitor MG-132 was added, indicating that the 20S core protease activity was contributing to lessened signal, either due to in vitro degradation of a fraction of the substrate or due to proteasome-bound ubiquitin proteases that are sensitive to core protease inhi-
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FIGURE 1. In vitro reconstitution of Hmg2p-GFP ubiquitination and retrotranslocation. A, Hmg2p-GFP in vitro ubiquitination required Ubc7p and Hrd1p. Microsomes (MIC) were prepared from strains expressing TDH3pro-Hmg2p-GFP without Hrd1p (hrd1Δ), with TDH3pro-Hrd1p (WT) or TDH3pro-C399S-Hrd1p (RING mutant). Cytosol (CYT) was prepared from strains expressing TDH3pro-Ubc7p (UBC7) or no Ubc7p (ubc7Δ). Reaction mixtures composed of microsomes and cytosols, as indicated, were incubated for 1 h, immunoprecipitated with anti-GFP, and immunoblotted for ubiquitin (anti-UB), as indicated. The arrows show mobility of unmodified Hmg2p-GFP, B, in vitro retrotranslocation of Hmg2p-GFP. Wild-type in vitro ubiquitination reactions were carried out as described in A but with 3× volume (72 μl). Hmg2p-GFP was immunoprecipitated and immunoblotted from a 1× (24-μl) aliquot of the nonfractionated mixture (T) or from the supernatant (S) and pellet (P) of an identical volume of the same reaction mixture that was centrifuged, as described. C, proteasome inhibitor increased the level of ubiquitinated Hmg2p-GFP. D, in vitro reactions were carried out with microsomes with Hrd3p (HRD3) or with no Hrd3p (hrd3Δ) incubated with cytosols with Ubc7p (UBC7) or with no Ubc7p (Δ).

bition. Thus, our in vitro ubiquitination and retrotranslocation assays included the proteasome inhibitor MG-132.

Membrane-embedded substrates and lumenal substrates can be divided into two subpathways of ERAD, referred to as ERAD-M and ERAD-L, respectively. ERAD-L requires more components, including lectins/chaperones, such as Yos9p (29, 30), and traditional chaperones like Kar2p (31). These lumenal proteins are not required for regulated degradation of Hmg2p. Furthermore, sufficient levels of Hrd1p will allow ERAD of both lumenal and membrane-bound substrates in the absence of Hrd3p (30, 32). Because Hrd3p serves to anchor these lumenal factors to the ER surface (30, 33), we tested if Hrd3p was required for the in vitro reactions described herein. The ubiquitination and retrotranslocation of Hmg2p-GFP in an hrd3Δ null strain was indistinguishable when compared with the normal HRD3 strain (Fig. 1D). Thus, Hrd1p appears to play a central role in recognition and retrotranslocation of its substrate Hmg2p, as anticipated from its central role as defined by earlier genetic studies.

Retrotranslocation of the 8-spanning, ER-resident membrane protein, Hmg2p, is not energetically intuitive. We next performed several tests to evaluate if the appearance of ubiquitinated Hmg2p-GFP in the S fraction was bona fide retrotranslocation. Alternatively, the ubiquitin immunoreactivity that was immunoprecipitated with anti-GFP antibodies could be GFP-containing products cleaved from the transmembrane region in the microsomal membrane. We immunoprecipitated Hmg2p-GFP with antibodies against the first luminal loop (anti-loop) and the fourth luminal loop (data not shown), normally within the ER, and obtained results identical to those obtained with anti-GFP antibodies, indicating that both transmembrane domain and GFP epitopes are present in the soluble ubiquitinated substrate (Fig. 2A).

We next used serial immunoprecipitations with the anti-GFP and anti-luminal loop antisera to discern if the ubiquitinated Hmg2p-GFP in the soluble fraction was intact. If full-length ubiquitinated Hmg2p-GFP was moved to the cytosolic fraction in the assay, then either antibody should completely remove the ubiquitinated material from the soluble fraction. Three pairs (six identical samples total) of reaction supernatants were immunoprecipitated with either anti-GFP (two samples), anti-loop (two samples), or preimmune serum (two samples). The two identical supernatants from each pair were next reprecipitated with either anti-GFP or anti-loop antibodies. The precipitated material from all reactions was immunoblotted with antibodies against the first luminal loop (anti-loop) and the fourth luminal loop (anti-loop) and the fourth luminal loop (data not shown), normally within the ER, and obtained results identical to those obtained with anti-GFP antibodies, indicating that both transmembrane domain and GFP epitopes are present in the soluble ubiquitinated substrate (Fig. 2A).

The serial IP experiment indicated that the retrotranslocated Hmg2p-GFP was intact. Immunoprecipitation of the soluble, ubiquitinated Hmg2p-GFP with either GFP or luminal antibodies completely cleared all of the ubiquitin immunoreactivity, resulting in no further pull-down of ubiquitinated Hmg2p-GFP with either antibody in the second IP. Conversely, reimmunoprecipitation of the supernatants from the preimmune pair with either antibody resulted in a

D. Finley, personal communication.
The above experiments all indicate that intact, ubiquitinated Hmg2p-GFP is moved to the soluble fraction in vitro. To directly test if full-length Hmg2p-GFP was retrotranslocated, we used the recombinant catalytic core of the ubiquitin protease USP2 (Usp2-cc) that efficiently strips ubiquitin from multiubiquitin chains on substrates (34), allowing us to directly examine the retrotranslocated material by GFP immunoblotting. Before immunoprecipitating the retrotranslocated, ubiquitinated protein, we divided the sample into three aliquots. One was incubated with buffer on ice, and the other two were incubated at 37 °C with either buffer or Usp2-cc (Fig. 2D). The upper panel shows an anti-ubiquitin blot of the sample and demonstrates the effect of the Usp2-cc. The lower panel shows the same sample immunoblotted with anti-GFP antibodies. Without ubiquitin stripping, there is little detectable GFP, whereas in the stripped sample, the GFP immunoreactivity appears at the molecular weight of Hmg2p-GFP (arrow). Importantly, only the mobility of full-length Hmg2p-GFP increased after Usp2-cc treatment. This experiment directly demonstrated that intact Hmg2p-GFP was being moved to the cytosol as a multiubiquitinated protein.

We noticed that a band of Hmg2p-GFP immunoreactivity was seen in the “unstripped” S fraction at varying intensities between experiments, which was not dependent on ubiquitination or the factors needed for retrotranslocation, and may be caused by some fragmentation of the ER. This background signal was not present when ubiquitin was used to detect the retrotranslocated material, and thus we used this method of detection. Nevertheless, it was important to directly demonstrate that Hmg2p-GFP is moved in its entirety to the soluble fraction.

A unifying feature of many ERAD pathways is the proposed role of the hexameric AAA-ATPase Cdc48p (p97 in mammals) in retrotranslocation (12, 35), along with its binding partners Ufd1p and
FIGURE 3. Role of Cdc48p in Hmg2p-GFP retrotranslocation. **A**, in vivo Hmg2p-GFP degradation was blocked by the **cdc48-2** mutant. WT (**CDC48**) and **cdc48-2** strains expressing Hmg2p-GFP were subjected to a cycloheximide chase and flow cytometry at the indicated times to evaluate Hmg2p-GFP levels. The experiment was done at 30 °C, a permissive temperature for this allele. Histograms of 10,000 cells are shown, with the number of cells versus GFP fluorescence. **B**, in vitro retrotranslocation of Hmg2p-GFP required Cdc48p. In vitro reactions were carried out in which both components were derived from either WT (**CDC48**) or **cdc48-2** strains (these panels were extracted from the more elaborate panel **D**). **C**, same experiment using reaction mixtures from WT or **cdc48-3** strains. **D**, strong requirement for cytosolic Cdc48p in retrotranslocation. The indicated combinations of WT or **cdc48-2** reaction components (microsomes (**MIC**)) and cytosol (**CYT**)) were mixed, as indicated, to evaluate relative contribution to Hmg2p-GFP retrotranslocation. **E**, in vitro Hmg2p-GFP ubiquitination with GST-ubiquitin does not support Hmg2p-GFP retrotranslocation. Exogenous ubiquitin or GST-ubiquitin was added to the in vitro retrotranslocation assay, and anti-ubiquitin immunoblotting was used for detection of modified Hmg2-GFP. Lanes labeled Δ and **ubc7Δ** indicate reactions carried out with **ubc7Δ** cytosol and specified microsomes in **B–E**. **T**, total reaction mix; **S**, supernatant; **P**, pellet.
Npl4p (35, 36). Hmg2p is strongly stabilized by mutations in the Cdc48p complex, with a block that occurs after ubiquitination (12). We first confirmed that the cdc48-2 allele shows a strong in vivo block in Hmg2p-GFP degradation, even at the permissive temperature of 30 °C (Fig. 3A). Then we tested the effect of this allele in our in vitro assay. When both microsome and cytosol strains were cdc48-2, ubiquitination occurred, but there was a nearly complete block in release of ubiquitinated Hmg2p-GFP into the cytoplasmic S fraction (Fig. 3B). Similar results were obtained using the cdc48-3 allele (Fig. 3C).

The Cdc48p complex is found both in the microsome fraction and in the cytoplasmic pool (37), and some of the complex is bound to Hrd1p (30, 33, 38). Thus, we wondered whether membrane-bound or soluble Cdc48p provided the activity for retrotranslocation. We prepared microsomes and cytosol from strains with either normal CDC48 or the cdc48-2 allele and ran the retrotranslocation assay with the various combinations of mutant or wild-type material, as indicated (Fig. 3D; the first four lanes and the last three lanes of D are the same as shown in B). It was clear that the cytosol alone contributes the majority of the needed Cdc48p activity. This implies that soluble Cdc48p complexes are recruited from the aqueous medium for their role in retrotranslocation.

The Cdc48p complex has binding sites for ubiquitin, located both on Ufd1p and Cdc48p itself (36, 39). The importance of these sites has been demonstrated for the case of US11-mediated major histocompatibility complex I retrotranslocation, where p97-mediated retrotranslocation does not occur when a molecule in the retrotranslocation process. Interestingly, a similar role in retrotranslocation. We capitalized on the differing use of ATP requirement in retrotranslocation. We prepared microsomes and cytosol from strains with either normal CDC48 or the cdc48-2 allele and ran the retrotranslocation assay with the various combinations of mutant or wild-type material, as indicated (Fig. 3D; the first four lanes and the last three lanes of D are the same as shown in B). It was clear that the cytosol alone contributes the majority of the needed Cdc48p activity. This implies that soluble Cdc48p complexes are recruited from the aqueous medium for their role in retrotranslocation.

The Cdc48p complex has binding sites for ubiquitin, located both on Ufd1p and Cdc48p itself (36, 39). The importance of these sites has been demonstrated for the case of US11-mediated major histocompatibility complex I retrotranslocation, since p97-mediated retrotranslocation does not occur when a GST-ubiquitin fusion is used instead of native ubiquitin. Similarly, Hmg2p-GFP retrotranslocation was dependent on native ubiquitin. Use of the N-terminal GST-ubiquitin fusion allowed in vitro ubiquitination to proceed, producing large conjugates in the reaction mix (Fig. 3E). However, there was little or no movement of the GST-ubiquitin-derivatized Hmg2p-GFP to the cytosol, underscoring the importance of the ubiquitin molecule in the retrotranslocation process. Interestingly, a similar experiment with K6W ubiquitin, which blocks proteasomal degradation (40), did not inhibit the retrotranslocation assay (data not shown).

Dislocation of an 8-spanning integral membrane protein would require significant energy. Hexameric Cdc48p is a member of the large AAA-ATPase family, and this activity is thought to drive retrotranslocation. We wanted to directly examine this question with our assay. To specifically test the role of ATP in retrotranslocation, we capitalized on the differing use of ATP by the ubiquitin E1 or the AAA-ATPase domains of Cdc48p. Ubiquitin addition to the E1 is driven by hydrolysis of the α–β phosphodiester bond, whereas the AAA-ATPases hydrolyze the β–γ bond (41). In numerous cases, the β–γ imido analog of ATP, called AMP-PNP, with a normal α–β bond but a non-hydrolyzable phosphodiimido linkage to the γ-phosphate position, will drive ubiquitination (42) but not reactions that depend on β–γ hydrolysis. We employed AMP-PNP to test for a role of ATP in retrotranslocation separate from its known involvement in ubiquitination. The direct approach of simply adding AMP-PNP to the retrotranslocation assay rather than normal ATP was not an effective way to ask this question. In our in vitro assay, although AMP-PNP did support Hmg2p-GFP ubiquitination, the reaction did not proceed to nearly the same extent as those run with ATP, prohibiting direct comparison with the two nucleotides (Fig. 4A, left). This appeared to have some specificity for the HRD pathway, since AMP-PNP did support total lysate in vitro ubiquitination, evaluated by ubiquitin immunoblotting of the nonimmunoprecipitated proteins in the total reaction mix (Fig. 4A, total in vitro ubiquitination; right).

This requirement for the β–γ ATP bond in Hmg2p-GFP ubiquitination required a more complex procedure to test an ATP requirement in retrotranslocation. We first ran a ubiquitination phase of the assay with ATP to ensure sufficient Hmg2p-GFP ubiquitination and then a retrotranslocation phase with either ATP or AMP-PNP to test for ATP dependence of retrotranslocation of the ubiquitinated Hmg2p-GFP. The major contribution of soluble Cdc48p to Hmg2p-GFP retrotranslo-
tion allowed us to accomplish this separation. This was done by running the “ubiquitination phase” with cdc48-2 microsomes and cdc48-2 cytosol with ATP present. After a reaction period, we recovered the cdc48-2 ubc7/H9004 microsomes from the reaction by centrifugation and initiated the second “retrotranslocation phase” by adding CDC48 cytosol from a ubc7/H9004 null to those microsomes. This CDC48 ubc7/H9004 cytosol was supplemented with either ATP or AMP-PNP, along with a control in which buffer was added to the microsomes. The reaction mixes were then fractionated to evaluate soluble and membrane-bound ubiquitinated Hmg2p-GFP (Fig. 4B). The reaction with AMP-PNP did not support retrotranslocation, but did allow continued ubiquitination. Clearly, a β-γ ATP bond is needed for Cdc48p-dependent retrotranslocation.

How does the Cdc48p complex engage the HRD complex at the ER surface? It has been proposed that the ER membrane protein Ubx2p serves as an ER-localizing “receptor” or docking site for the Cdc48p complex (43–45). However, in vivo, loss of Ubx2p only partially blocks ERAD. This is clear in previous reports (43–45), and is demonstrated in Fig. 5A, with Hmg2p-GFP cycloheximide chase. Thus, there may be other ways for Cdc48p to get to the ERAD process in vivo, since a complete null of the Ubx2p “receptor” has a less strong effect on Hmg2p-GFP ERAD than the partially functioning hypomorph of cdc48-2 (Fig. 3A). Furthermore, ubx2Δ null strains have numerous physiological alterations, such as a highly elevated unfolded protein response7 that may obscure studies of the molecular

7 B. K. Sato and R. Y. Hampton, unpublished observations.
actions of Ubx2p in vivo. Thus, we used the in vitro assay to directly test the role of this adaptor in Cdc48p-dependent retrotranslocation.

When the assay was run with ubx2Δ cells, retrotranslocation was completely abrogated, as shown by the lack of immunoreactivity in the soluble fraction (Fig. 5B). Furthermore, cytosol from a wild-type UBX2 strain did not allow retrotranslocation from membranes prepared from a ubx2Δ null strain (data not shown). However, ubx2Δ microsomes also showed unexpected ubiquitination of Hmg2p-GFP in the absence of added Ubc7p. This Hrd1p-dependent ubiquitination of Hmg2p-GFP is present in the microsome fraction before the reaction is initiated and was observed when Hmg2p-GFP was directly immunoprecipitated from lysates of the ubx2Δ ubc7Δ double null microsome strain (data not shown). This ubx2Δ-caused "preubiquitination" was highly reproducible and implies that Ubx2p may have roles at other positions in the ERAD pathway, at least of Hmg2p-GFP. At present, we do not know how this process occurs.

We confirmed the generality of the requirement for Ubx2p in retrotranslocation by examining Hrd1p itself as a substrate. Hrd1p levels were sufficiently elevated to allow in vitro Hrd1p self-ubiquitination, mediated by the RING domain of Hrd1p (28). In vitro Hrd1p retrotranslocation occurred and was also dependent on Ubx2p (Fig. 5C). The block to retrotranslocation caused by ubx2Δ was strong with either Hmg2p-GFP or Hrd1p.

A number of studies have implicated the set of adaptor proteins Dsk2p and Rad23p that facilitate transfer of ubiquitinated ERAD substrates to the proteasome. These adaptors have both ubiquitin-binding Uba motifs and proteasome-binding Ubl motifs (46, 47). Although these adaptors are proposed to function in proteasome delivery, they in fact had a strong role in Hmg2p-GFP retrotranslocation (Fig. 6A).

Both Rad23p and Dsk2p are soluble proteins (48) and might be expected to be supplied from the cytosolic fraction. However, the actions of the pair were more complex and indicated that they may have multiple roles in the ERAD pathway. It appeared that Rad23p/Dsk2p functioned both in the extent of Hmg2p-GFP ubiquitination and retrotranslocation (Fig. 6B, compare first and third sets). Furthermore, these two actions were to a large extent separable between the cytosolic and membrane fractions used in the assay. When the rad23Δ dsk2Δ double null was present in both the microsomes and cytosol, retrotranslocation was severely curtailed, and the extent of Hmg2p-GFP ubiquitination (compare total reaction lanes from all-WT and all-null sets) was lessened. Use of individual fractions with or without Rad23p and Dsk2p showed that the retrotranslocation was significantly restored (but not completely) by the presence of these factors solely in the microsome strains (Fig. 6B, second set, MIC: RAD23 DSK2 with CYT: ΔΔ Δ Δ UBC7), without alleviation of the lessened extent of ubiquitination. Conversely, the presence of Rad23p and Dsk2p in only the cytosol fraction allowed for ubiquitination of the higher molecular weight conjugates, but retrotranslocation was still poor (Fig. 6B, fourth set, MIC: rad23A dsk2Δ with CYT: RD UBC7). Thus, it is likely that Rad23p and Dsk2p have functions at a number of points along the ERAD pathway and distinct functions at the ER surface and in the cytosolic fractions.

It is widely thought that a pore or channel is required for the removal of ERAD substrates. The most often proposed candidates for such a pore include members of the derlin family (49, 50), or the anterograde pore Sec61p. Yeast expresses two derlins, the original Der1p protein and its homologue Dfm1p that has demonstrable Cdc48p-binding activity (51), but the double null has no in vivo defect on Hmg2p-GFP ERAD. In vivo analysis of the role of Sec61p in ERAD or retrotranslocation has been challenging, because hypomorphs of this essential gene have a variety of effects on ER processes. Nevertheless, a sec61-2 mutant has small but reproducible deficiencies in ERAD of Hmg2p-GFP. We wondered if the highly sensitive retrotranslocation assay would reveal any roles for these factors that are harder to observe in vivo, as appears to be the case for the ubx2Δ null above. We tested membranes from a der1Δ dfm1Δ double null strain in the retrotranslocation assay (Fig. 7A) and membranes from the sec61-2 strain (Fig. 7B). As a control, we included the ubx2Δ null that had blocked Hmg2p-GFP retrotranslocation. In no case was there any effect of these mutants on in vitro retrotranslocation of Hmg2p-GFP. Furthermore, assay of sec61-2 function in microsomes at the nonpermissive temperature (37 °C) had no effect on in vitro retrotranslocation (data not shown).

These results led us to wonder if Hrd1p itself was providing channel function in addition to its role as the E3 ligase. Hrd1p...
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FIGURE 7. Direct evaluation of channel candidates derlins Der1p/Dfm1p or Sec61p in Hmg2p-GFP retrotranslocation. In vitro Hmg2p-GFP retrotranslocation reactions consisting of WT derlins (DER1 DFMI) or der1Δ dfm1Δ microsomes in A and the anterograde channel SEC61 or sec61-2 microsomes in B were reacted with ubc7Δ (Δ) or UBC7 cytosol. T, total reaction mix; S, supernatant; P, pellet; MIC, microsomes; CYT, cytosol.

has a multspanning membrane domain N-terminal to its RING ligase domain, which would allow simultaneous coupling of protein dislocation and ubiquitination.

The simple experiment of removing Hrd1p to test its role in retrotranslocation was not feasible, because it is also necessary for Hmg2p ubiquitination, which is a prerequisite for retrotranslocation. To separate the Hrd1p ligase function from other possible activities, we devised a “self-destructive” substrate that employs the Hrd1p RING domain in the absence of its transmembrane region. We prepared a coding region that expresses the fusion being correctly inserted in the ER membrane. Microsomes prepared from strain expressing Hmg1-Hrd1p were digested with trypsin for the indicated times and immunoblotted with anti-Myc. C and D, degradation behavior of Hmg1-Hrd1p was assayed by cycloheximide chase. C, degradation of Hmg1-Hrd1p was dependent on Ubc7p. D, degradation of Hmg1-Hrd1p was dependent on cdc48-2 and hrd2-1. The ubc7Δ and hrd2-1 lanes include lower intensity exposures to facilitate comparison with the wild-type lanes. E, in vitro retrotranslocation of Hmg1-Hrd1p fusion was dependent on Cdc48p. Reactions with Ubc7p from WT (CDC48 UBC7) or cdc48-2 (cdc48-2 UBC7) and without Ubc7p (Δ) were carried out with hrd1Δ microsomal strains CDC48 (WT) or cdc48-2 expressing Hmg1-Hrd1p from the TDH3 promoter. Fusion protein was immunoprecipitated with anti-Hrd1p antiserum and immunoblotted with anti-ubiquitin and anti-HA antibodies. Anti-ubiquitin (anti-UB) panels were derived from the same gel, as were anti-HA panels. Extraneous lanes were removed. M, mutant cdc48-2. F, Usp2-cc ubiquitin protease treatment revealed that full-length Hmg1-Hrd1p was retrotranslocated in vitro. Supernatant fractions from in vitro reactions were treated either with buffer or with ubiquitin-specific protease Usp2-cc. The arrows point to mobility of unmodified Hmg1-Hrd1p. Total reaction mix; S, supernatant; P, pellet; MIC, microsomes; CYT, cytosol.

lhrd1Δ null mutant (Fig. 8C) and in the hrd1Δ doa10Δ double mutant (data not shown). Hmg1-Hrd1p degradation was also slowed by mutations cdc48-2 or the proteasomal RPN1 hypomorph hrd2-1 (Fig. 8D). Again, a lighter exposure of the hrd2-1 mutant at a similar initial intensity to the wild-type is included to facilitate comparison. The rapid, Cdc48p-dependent degradation of Hmg1-Hrd1p implied that the autonomously degrading fusion protein undergoes retrotranslocation. We tested this directly in vitro. Hmg1-Hrd1p underwent Ubc7p-dependent in vitro ubiquitination and showed the expected movement to the soluble supernatant fraction (Fig. 8E). Furthermore, Hmg1-Hrd1p retrotranslocated from microsomes derived from a hrd1Δ null strain. Hmg1-Hrd1p retrotranslocation was partially blocked in an assay run with both cytosol and supernatant.
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from the cdc48-2 mutant (Fig. 8E). Finally, use of the Usp2-cc ubiquitin protease demonstrated that full-length Hmg1-Hrd1p was being retrotranslocated (Fig. 8F). Thus, Cdc48p-mediated retrotranslocation of this HRD pathway substrate can occur in the complete absence of the Hrd1p transmembrane domain.

DISCUSSION

In this study, we have successfully reconstituted Hrd1p-dependent retrotranslocation of a multispanning membrane substrate of the HRD pathway, Hmg2p-GFP. By multiple criteria, we have shown the ubiquitinated, full-length Hmg2p-GFP is moved to the supernatant fraction. The ability to observe this process with Hmg2p-GFP both defines the capacity of this transfer mechanism and allows its intimate mechanistic study in ways that are not possible using intact cells.

In our approach, we have used sufficient Hrd1p levels to drive ubiquitination of Hmg2p-GFP without need for Hrd3p or Usa1p. Thus, we are examining the minimal requirements for in vitro ubiquitination and retrotranslocation. Importantly, in vitro ERAD of Hmg2p-GFP by this method has all of the features of in vivo Hmg2p-GFP degradation. Ubc7p and its anchor Cue1p are required, as is the critical lysine 6 of Hmg2p-GFP (52, 53). Furthermore, Hrd1p-dependent ubiquitination of Hmg2p-GFP is specifically blocked by chemical chaperones as is in vivo degradation of this substrate (52). These studies also reinforce the idea that Hrd1p is a central organizer of ERAD, consistent with multiple observations that Hrd1p alone can drive ERAD of membrane-anchored substrates (32, 54).

A recent, elegant study of ERAD using radioiodinated ubiquitin provides a distinct approach for the biochemical study of these processes (55). The radiochemical analysis therein is more sensitive and somewhat more flexible in analysis of input strains. However, the ubiquitination state of the substrates at the start of the assay is available in our approach and cryptic in the radiochemical method. Furthermore, the higher amount of retrotranslocated material in our studies allows for direct detection of the retrotranslocated substrate in the soluble fraction, thus providing direct evidence of full dislocation of a polyanionic ERAD substrate. In addition, the elevated levels of Hrd1p in our protocol provide a useful internal control, since Hrd1p undergoes Ubc7p-dependent self-ubiquitination in addition to transfer to substrates under study. Thus, both approaches have distinct advantages, and the successful analysis of ERAD mechanisms will benefit from the use of each.

In both mammals and yeast, the AAA-ATPase Cdc48p/p97 is a key component of the postubiquitination step in ERAD of multiple substrates. Cdc48p was similarly required for the complete removal of Hmg2p-GFP from the ER membrane. Despite there being an ER-bound pool of Cdc48p/p97 (37), the cytosolic Cdc48p was the most important source of this activity. Perhaps Cdc48p plays a role in stabilizing the retrotranslocated Hmg2p-GFP, so it must be replenished from the soluble pool as the reaction proceeds, as has been proposed from structural studies of the mammalian p97 protein’s role in ERAD (56).

Cdc48p AAA-ATPase is thought to power retrotranslocation. By capitalizing on the cell biology of the retrotranslocation reaction and the differing requirements for ATP by E1 and Cdc48p, we directly demonstrated an ATP requirement for Hmg2p-GFP retrotranslocation. This separation depended on using AMP-PNP during the retrotranslocation phase of an experiment to show the specific need for a β–γ bond in this step. Interestingly, the simple method of separating ubiquitination from retrotranslocation by use of AMP-PNP instead of ATP in the complete reaction was not feasible, because AMP-PNP only poorly supported Hmg2p-GFP ubiquitination in the reconstituted HRD reactions.

This unexpected, HRD-specific requirement for the β–γ bond of ATP may be due to a requirement for Cdc48p in initiating Hmg2p-GFP ubiquitination. Consistent with this idea, in vitro Hmg2p-GFP ubiquitination was lower in reactions with cdc48-2 preparations. It has been posited that Cdc48p may play a role in major histocompatibility complex I recognition as well as retrotranslocation in the US2- and US11-dependent pathway (35), and other AAA-ATPases have been implicated in the recognition of misfolded proteins for destruction (57, 58). This additional role for Cdc48p will be investigated in the future. It is also possible that Cdc48p-dependent retrotranslocation allows the movement of luminal lysines to the cytoplasmic face during ubiquitination, thus increasing the extent of ubiquitination observed when Cdc48p is fully functional. The in vitro approach makes the separation of such intertwined functions feasible.

A number of studies have implicated the ER-localized Ubx2p as an ER receptor for soluble Cdc48p. We directly tested this idea and found that microsomal Ubx2p is required for retrotranslocation of both Hmg2p-GFP and Hrd1p itself. Although the effects of Ubx2p are quite clear in vitro, in vivo Hmg2p-GFP degradation was slowed more by the partial loss of function cdc48-2 mutant than by a complete ubx2Δ null (Fig. 3A compared with Fig. 5A). This implies that other ways are available for Cdc48p to engage the ERAD machinery, consistent with the observation that loss of Ubx2p does not completely remove Cdc48p association with the ER membrane (44). Nevertheless, our results directly implicate Ubx2p as a Cdc48p receptor (43, 45). Perhaps our assay is more sensitive due to the dilution of the cytosol rendering the system more dependent on the ability of the Ubx2p microsomes to attract Cdc48p. Ubx2p functions are probably broader than Cdc48p anchoring, since we have observed that a ubx2Δ null has a profound up-regulation of the unfolded protein response that is much greater than that caused by strong ERAD-inhibiting mutants of Cdc48p. The in vitro assay is particularly useful when this is considered, since in intact cells, a ubx2Δ null mutant is causing ongoing strong regulatory responses that may cloud observation of its direct ERAD functions.

We examined the involvement of the ubiquitin-binding, proteasome delivery adaptors Rad23p and Dsk2p in vitro. Although current models predict that these soluble adaptors would have a role only after retrotranslocation, we observed that their absence caused a strong block to retrotranslocation. The Rad23p and Dsk2p adaptors are predicted soluble proteins, but we saw significant restoration of retrotranslocation when the microsomal fraction was the sole source of Rad23p and

8 R. M. Garza, B. K. Sato, and R. Y. Hampton, unpublished observation.
Hrd1p degradation was unaffected in both a required Cdc48p and the proteasome (Fig. 8). However, Hmg1- by formation of the “self-destructive” Hmg1-Hrd1p protein of Hrd1p functions as a channel. We tested this idea for Hrd1p doa10 mutant even the MAMT 29, 2009• VOLUME 284• NUMBER 22 JOURNAL OF BIOLOGICAL CHEMISTRY, 284, 14721–14729 Hrd1p-dependent Retrotranslocation of HMG-CoA Reductase

Dsk2p. Furthermore, cytosol devoid of Rad23p and Dsk2p supported a lesser extent of Hmg2p-GFP ubiquitination whether the microsome fraction was wild-type or double null. This could be due to a role in recruiting the “E4” Ufd2p, which has been posited to enhance ubiquitination of ERAD substrates, including Hmg2p-GFP (39), or altered recruitment of the ERAD-implicated ubiquitin protease Ufd3p (59). Thus, it appeared that these adaptors have added roles in ERAD that impact both the extent of ubiquitination and retrotranslocation, in addition to their proposed role as proteasomal adaptors. One possibility is that these upstream effects are all caused by proteasome recruitment to the ER. The 26S proteasome has both associated E3 ligases and multiple AAA-ATPase activities that could affect the degree of substrate ubiquitination and assist in Cdc48p-dependent extraction of ERAD substrates, respectively. Whatever the mechanism, it is clear that these adaptors have more complex functions than previously appreciated from genetic analysis of intact cells.

One of the challenging open questions concerning ERAD is the mechanism of exit from the ER membrane, and a number of candidate channels have been proposed (60, 61). We directly examined both the pair of derlins, Der1p and Dfm1p, and Sec61p in our assay and confirmed that neither the double mutant der1Δ dfm1Δ nor the temperature-sensitive sec61-2 mutant had any detectable effects on in vitro retrotranslocation of Hmg2p-GFP. This is consistent with our in vivo studies that similarly showed a lack of effect of the der1Δ dfm1Δ mutant or even the der1Δ dfm1Δ sec61-2 triple mutant on in vivo degradation of Hmg2p-GFP (51).

One appealing idea is that the large transmembrane domain of Hrd1p functions as a channel. We tested this idea for Hrd1p by formation of the “self-destructive” Hmg1-Hrd1p protein with the ubiquitination activity of Hrd1p but lacking the Hrd1p transmembrane domain. Hmg1-Hrd1p underwent ERAD that required Cdc48p and the proteasome (Fig. 8). However, Hmg1-Hrd1p degradation was unaffected in both a hrd1Δ null and a doa10Δ hrd1Δ double null, indicating that delivery to the proteasome could occur without either of these ER ligase transmembrane regions being present in the cell. Consistently, full-length Hmg1-Hrd1p underwent Cdc48p-dependent in vitro retrotranslocation in an hrd1Δ null.

These studies indicate that none of the channel candidates show a role in Hmg2p-GFP retrotranslocation. Perhaps there is a yet undiscovered protein required for retrotranslocation that is essential to cells or a poor genetic target, making its isolation by screening difficult. It may further be that there are redundant routes of extraction, thus masking the effects of the loss of any one. Finally, it is possible that retrotranslocation of Hmg2p-GFP occurs in a way that does not require a channel but instead involves the recruitment of lipids to form a soluble intermediate. One version of this idea has been suggested in a recent essay (62). Although we find this possibility unlikely, it cannot be ruled out until the route or exit is understood by either discovery of the still cryptic channel or the complete reconstitution of the process in a pure system devoid of channel candidates.

Our studies directly demonstrated retrotranslocation of full-length Hmg2p-GFP. Recent work on the Ste6-166p transmembrane Doa10p substrate implied that this multispanning membrane protein was similarly moved to the soluble fraction (55). Combined, these results indicate that movement of entire transmembrane substrates is commonly occurring in ERAD, as has been suggested from in vivo studies with CFTR-Δ508 in mammalian cells (63). It is not clear how the 8-spanning integral membrane protein Hmg2p-GFP remains soluble in the cytosol. It will be important to analyze the physical state and binding partners of this molecular species to gain insight into what processes and proteins are responsible for this heroic thermodynamic event.

This and other in vitro approaches will allow detailed mechanistic analysis of the ERAD pathway. Our future directions will include the analysis of the nature and composition of the retrotranslocated Hmg2p-GFP and the role of the sterol regulatory signals that control Hmg2p and Hmg2p-GFP degradation in coordinating this key step with the earlier events that are required for movement of substrates from the ER to their proteasomal destruction.

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