Role of HERP and a HERP-related Protein in HRD1-dependent Protein Degradation at the Endoplasmic Reticulum*

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Background: HERP1 has been implicated in ERAD with unknown mechanism.
Results: HERP1 and newly identified HERP2 are integral components of the HRD1 complex, facilitating retrotranslocation by recruiting DERL2 to HRD1.
Conclusion: HERPs are partially redundant, required for HRD1-mediated retrotranslocation.
Significance: HERP1 and HERP2 are essential adaptors between HRD1 and DERL2 that help organizing a functional retrotranslocation complex in HRD1-mediated ERAD.

Misfolded proteins of the endoplasmic reticulum (ER) are retrotranslocated to the cytosol and degraded by the proteasome via a process termed ER-associated degradation (ERAD). The precise mechanism of retrotranslocation is unclear. Here, we use several lumenal ERAD substrates targeted for degradation by the ubiquitin ligase HRD1 including SHH (sonic hedgehog) and NHK (null Hong Kong α1-antitrypsin) to study the geometry, organization, and regulation of the HRD1-containing ERAD machinery. We report a new HRD1-associated membrane protein named HERP2, which is homologous to the previously identified HRD1 partner HERP1. Despite sequence homology, HERP2 is constitutively expressed in cells, whereas HERP1 is highly induced by ER stress. We find that these proteins are required for efficient degradation of both glycosylated and nonglycosylated SHH proteins as well as NHK. In cells depleted of HERPs, SHH proteins are largely trapped inside the ER with a fraction of the stabilized SHH protein bound to the HRD1-SEL1L ligase complex. Ubiquitination of SHH is significantly attenuated in the absence of HERPs, suggesting a defect in retrotranslocation. Both HERP proteins interact with HRD1 through a region located in the cytosol. However, unlike its homolog in *Saccharomyces cerevisiae*, HERPs do not regulate HRD1 stability or oligomerization status. Instead, they help recruit DERL2 to the HRD1-SEL1L complex. Additionally, the UBL domain of HERP1 also seems to have a function independent of DERL2 recruitment in ERAD. Our studies have revealed a critical scaffolding function for mammalian HERP proteins that is required for forming an active retrotranslocation complex containing HRD1, SEL1L, and DERL2.

Misfolded proteins of the endoplasmic reticulum (ER)[3] are recognized by lectins and chaperones in ER lumen, retrotranslocated across the membrane, extracted by an energy-dependent process that involves AAA (ATPase associated with various cellular activities) ATPase p97 (or its homolog Cdc48p in yeast), and then degraded by the 26 S proteasome (1, 2). This highly conserved process termed ER-associated degradation (ERAD) helps adapt cells to proteotoxic stress in the ER and therefore is critical for the life and death decision in cells bearing a stress burden from either high secretory loads in the ER or some pathological conditions (2, 3).

Retrotranslocation is thought to be mediated by one or more "retrotranslocons" in the ER membrane, but the identity of the retrotranslocon(s) is currently unknown. A key retrotranslocation machinery protein is the ER-associated ubiquitin ligase HRD1 in mammals or Hrd1p in *Saccharomyces cerevisiae*. This ligase is anchored to the ER membrane by a multitransmembrane domain with a catalytic RING (Really Interesting Gene) domain exposed to the cytosol that confers ubiquitin ligase activity (4–6). In yeast, Hrd1p functions along with several membrane proteins including Hrd3p, Usa1p, and Der1p, forming a complex essential for retrotranslocation of misfolded proteins. Under normal conditions, Usa1p interacts directly with Hrd1p to promote its oligomerization as well as its stability, which are required for retrotranslocation (7–9). Usa1p also serves as an adaptor that recruits Der1p to the Hrd1p-Hrd3p subcomplex (8, 10), which is required for ERAD of both misfolded lumenal and membrane substrates (7, 9, 11). Hrd3p is also an essential component of the Hrd1p retrotranslocation complex. It is required to stabilize Hrd1p because in its absence Hrd1p is rapidly degraded via a Usa1p-dependent mechanism (5, 9). In addition, together with Der1p, Hrd3p may facilitate the delivery of substrates to Hrd1p for subsequent retrotranslocation (12–14). Intriguingly, although each of these components is essential for ERAD of misfolded luminal proteins under

[3] The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; NHK, null Hong Kong α1-antitrypsin; UBL, ubiquitin-like; Tm, tunicamycin; Tg, thapsigargin.

normal conditions, Hrd3p, Usa1p, and Der1p become dispensable when Hrd1p is overexpressed (7). This observation establishes a pivotal role for Hrd1p and likely its mammalian homolog HRD1 in retrotranslocation.

Compared with *S. cerevisiae*, the composition and organization of the HRD1 complex in mammalian cells are poorly defined, largely because of the complexity and redundancy of the system. For example, three Der1p homologs named Derlin1, 2, and 3, respectively, are found in mammals, and only recently has it become clear that Derlin2 (DERL2) is a major function partner for HRD1-mediated ERAD (15). Likewise, several SEL1L-like proteins in addition to SEL1L exist in humans, but their functional significance is unknown. For Usa1p, at the first glance, there appears to be no obvious homolog in mammalian cells. However, an ER membrane protein named HERP (HERPUD1 in GenBank™ or HERP1 in this study) was shown to have a similar domain structure as Usa1p, although the two proteins do not share much sequence homology (16). Because HERP1 partially rescues the ERAD defect in Usa1p-deficient yeast cells, HERP1 is considered a functional ortholog of Usa1p (7). Anchored to the ER membrane, both Usa1p and HERP1 have UBL domains at their N termini and transmembrane domains at their C termini (10, 16). In mammalian cells, the roles of HERP1 in ERAD are controversial (17, 18).

We recently identified two new ERAD substrates, the glycosylated human SHH (sonic hedgehog) protein and its nonglycosylated counterpart N278A. The degradation of both these substrates is dependent on HRD1, SEL1L, and DERL2 (15, 19). SHH is autoprocessed in the ER after co-translational translocation, generating a cholesterol-modified N-terminal fragment for signaling and a glycosylated C-terminal fragment (hereafter referred as SHH-C) for disposal by ERAD (19). In this study, we report the discovery of a novel ER membrane protein termed HERP2, which is homologous to HERP1 in human cells. We investigate the roles of these HERP proteins in ERAD of SHHs and NHK. Our study reveals a critical role for these HERP proteins in degrading luminal HRD1 substrates, at least in part via regulating the organization of the HRD1 complex in mammalian cells. We propose that both HERP proteins are integral components of the HRD1-SEL1L complex that functions in degradation of both glycosylated and nonglycosylated HRD1 substrates in a partially redundant manner.

EXPERIMENTAL PROCEDURES

The materials used in this study were described previously (15) except the following. Anti-HERP1 antibody is a generous gift from Dr. K. Kokame (National Cerebral and Cardiovascular Center, Suita, Osaka, Japan). Anti-HERP2 was from Abnova. Tunicamycin and thapsigargin were from Enzo Life Science.

DNA Constructs for Mammalian Cell Transfection—Full-length SHH and N278A with HA tag in pIRES-EGFP (Takara Bio Inc.) were described before (15, 19). Expression plasmids coding for FLAG-HERP1, FLAG-HERP2, FLAG-WASP, and HERP1-HA were generated by PCR from human cDNA libraries with appropriate primers and cloned to pIRES-EGFP or pAC vector. Truncations on FLAG-HERP1 and FLAG-HERP2 were carried out using the QuikChange kit (Strategene).

TABLE 1
Sequences of siRNA duplexes used in this study

Gene	Nucleotides		Primers
HERP1	609	Forward	AAUGUCUCAGGGACUUGCUUCCAAA
		Reverse	UUUGGAAGCAAGUCCCUGAGACAUU
HERP2	678	Forward	GAGAUUCCUGUGACCCUCAUCAUUA
		Reverse	UAAUGAUGAGGGUCACAGGAAUCUC
SEL1L	450	Forward	GAAACCAGCUUUGACCGCCAUUGAA
		Reverse	UUCAAUGGCGGUCAAAGCUGGUUUC

Cell Culture, Generation of Stable Cells, and Transfection of Plasmids and siRNAs—Human 293T cells were grown in DMEM supplemented with 10% fetal bovine serum, 0.1% penicillin and streptomycin. U2OS cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin. To generate HRD1-Myc stable cell lines, the plasmids encoding HRD1-Myc in pIRES-EGFP vector were transfected to 293T cells and selected by flow cytometry. The expression of the HRD1-Myc proteins was confirmed by Western blot analysis with both Myc and HRD1 antibodies. Transfection of plasmids was carried out using TransIT-LT1 (Mirus) according to the manufacturer's instructions. siRNAs were carried out as described (15, 19). The siRNA sequences used are shown in Table 1.

Immunofluorescence—U2OS cells were cultured on cover slides for 24 h. The cells were fixed in PBS with 4% paraformaldehyde for 5 min and were permeabilized with PBS containing 0.1% Triton X-100 for 20 min. Immunostaining was carried out with anti-HERP2 antibodies and DyLight™488-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). The immunostained cells were imaged by Leica TCS SP5 confocal microscopy.

Subcellular Fractionation—The experiment was carried out similarly as described (20). Cultured cells were harvested and washed with PBS before centrifugation at $3,000 \times g$ for 2 min. The cell pellet was resuspended with hypotonic buffer (50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM DTT, protease inhibitor) and passed through a 25-gauge needle 10 times using a 1-ml syringe. The mixture was centrifuged at $3,000 \times g$, and the supernatant was subjected to ultracentrifugation at $100,000 \times g$ for 20 min at 4 °C. After centrifugation, soup was collected as cytosolic fraction, and pellet was washed with hypotonic buffer following ultracentrifugation. Pellet was collected as membrane fraction and lysed in lysis buffer before immunoblotting. For topology analysis, the syringe passed mixture was treated with proteinase K before ultracentrifugation.

Cycloheximide Chase Assay and Quantitative RT-PCR Analysis—Cycloheximide chase assay was carried out as described (15, 19). Cells were incubated with 100 μg/ml cycloheximide in culture medium at 37 °C. At the times indicated in the figures, cells were harvested and lysed as described before (15, 19). Quantitative RT-PCR analysis was carried out as described (15, 19). The sequences of the primers used to quantify mRNA knockdown are shown in Table 2.

Immunoprecipitation—Immunoprecipitation was carried out as described previously (15, 19). To detect ubiquitinated SHH-HA, the cells were lysed in denaturing buffer with 0.8% SDS, 4 mM DTT, and 5 mg/ml *N*-ethylmaleimide with protease inhibitors. Afterward, the lysate was diluted 5-fold with dena-

TABLE 2
Primer sequences for quantitative PCR used in this study

Homo sapiens gene	Nucleotides		Primers
HERP1	794–907	Forward	TGGATTGGACCTATTCAGCAGC
		Reverse	GCAGGTACATAACAACGGTGGC
HERP2	437–666	Forward	TTCCACAAGCACAAACTGACCA
		Reverse	CTGGGTTGGGTTGACATTTGAT
SEL1L	1750–1931	Forward	CAGGGCTATGAAGTGGCACAAA
		Reverse	TCGGTGCCAAACCCATAGAAAT

turing buffer without SDS and DTT before centrifugation. The supernatant was used for immunoprecipitation with anti-HA antibodies. For other immunoprecipitation experiments, cells were lysed on ice for 20 min in HKM buffer (20 mM HEPES, pH 7.4, 100 mM KOAc, and 2 mM MgOAc) supplemented with protease inhibitors and 1% deoxy-bigChap. The lysate was centrifuged for 20 min at 4 °C and 20,000 × g to collect the supernatant followed by immunoprecipitation with indicated antibodies. For all immunoprecipitation experiments, roughly one-tenth of the total lysate was loaded as inputs.

Chemical Cross-linking—Cross-linking with dithiobis(succinimidyl propionate) was carried out as described (15) according to the manufacturer's instructions.

RESULTS

Identification of a Novel HERP1 Homolog—By sequence analysis, we discovered a HERP1-related protein in humans, which we termed HERP2 (or HERPUD2). HERP2 shares 38% sequence identity and 51% homology with HERP1. Similar to HERP1, it contains a ubiquitin-like (UBL) domain at the N terminus and a long hydrophobic segment close to the C-terminal region.

To understand the function of HERP2, we first determined its subcellular localization. To this end, we carried out immunostaining experiments with HERP2 antibodies using U2OS cells stably expressing the ER-localized membrane protein Sec61α-RFP as an ER marker. The specificity of HERP2 antibody had been verified by immunoblotting, showing that it did not cross-react with HERP1 (data not shown). The results showed that endogenous HERP2 displayed a perinuclear localization that overlapped with Sec61α-RFP, indicating that it is predominantly localized to the ER (Fig. 1A).

We next tested whether HERP2 is membrane-bound like HERP1. Cells were fractionated into membrane and cytosolic fractions. Immunoblotting showed that HERP2 was exclusively present in the membrane fraction (Fig. 1B, lane 3). To determine whether HERP2 has a membrane topology similar to that of HERP1 and Usa1p, we tagged the N and C termini of HERP2 with FLAG and HA epitopes, respectively (Fig. 1C). Cells expressing tagged HERP2 were fractioned, and the membrane fractions containing HERP2 were treated with proteinase K. HERP2 could hardly be detected by antibodies against either FLAG or HA after the membranes were exposed to proteinase K (Fig. 1, D and E, lanes 5 versus lanes 3), indicating that its N and C termini were both exposed to the cytosol (Fig. 1C). We conclude that HERP2 is a HERP1 homolog located predominantly on the ER, with both N and C termini in the cytosol.

Both HERP Proteins Function in ERAD of SHHs and NHK—To investigate whether HERP proteins are both involved in

ERAD, we first used siRNA to deplete HERP1 and HERP2 either individually or together and examined the degradation of SHH-C by cycloheximide chase. Depletion of HERP2 did not inhibit the degradation of SHH-C, whereas HERP1 depletion had a minor but statistically significant inhibitory effect toward SHH-C degradation (Fig. 2A; see also Fig. 6E). By contrast, knockdown of both HERP1 and HERP2 simultaneously resulted in much stronger stabilization of SHH-C than depletion of either HERP1 or HERP2 (Fig. 2A; see also Fig. 6E). These results suggest that HERP1 and HERP2 may have a partially redundant function in ERAD, but it appears that HERP1 may contribute slightly more to ERAD of SHH-C than HERP2 in unstressed 293T cells.

We also investigated whether HERP1 and HERP2 are involved in degradation of a nonglycosylated SHH variant (N278A), which is also a HRD1 substrate (15). Depletion of either HERP1 or HERP2 individually did not cause significant stabilization of SHH N278A, but simultaneous depletion of both HERP proteins resulted in strong inhibition of N278A degradation (Fig. 2B). Consistent with the cycloheximide shutoff experiments, significant accumulation of wild type SHH-C and the N278A mutant at the steady state level was only detected when both HERP proteins were depleted (Fig. 2, A and B). These results show that HERPs also play an overlapping function in ERAD of a nonglycosylated SHH variant.

We also evaluated whether HERP1 and HERP2 are required for degradation of another HRD1 substrate, NHK (14). We found that similar to SHHs, only when both HERP1 and HERP2 were depleted was the degradation of NHK inhibited most significantly (Fig. 2C). This result again indicates at least a partial redundant function for HERP proteins in ERAD of HRD1 substrates.

HERPs Are Integral Components of the HRD1-SEL1L-DERL2 Complex—To determine whether HERP2 is part of the HRD1-containing retrotranslocation complex, we carried out immunoprecipitation experiments in 293T cells using HRD1 antibodies. Immunoblotting showed that endogenous SEL1L and DERL2 could be co-precipitated with HRD1, whereas calnexin and BiP were not, indicating that the antibodies specifically pulled down the HRD1 complex (Fig. 3A). Under the same condition, both endogenous HERP1 and HERP2 were co-precipitated with HRD1, indicating that HERP1 and HERP2 are part of the HRD1-SEL1L-DERL2 complex (Fig. 3A).

Next, we investigated whether HERP1 and HERP2 could coexist in the same HRD1-SEL1L complex by expressing FLAG-tagged HERP1 or HERP2 in 293T cells. Immunoprecipitation of either HERP1 or HERP2 was carried out with FLAG antibodies, which also specifically precipitated HRD1, SEL1L, and DERL2, but not calnexin (Fig. 3B, lanes 2 and 3). As a control, immunoprecipitation of an unrelated protein FLAG-WASP (Wiskott-Aldrich syndrome protein) was performed, which did not pull down HRD1 or its associated components (Fig. 3B, lane 1). Interestingly, immunoprecipitation of HERP1 with FLAG antibodies precipitated a significant fraction of endogenous HERP2, and vice versa, suggesting that HERP1 and HERP2 can form a hetero-oligomer either directly or through the HRD1 complex. Together, our co-immunoprecipitation and RNA interference results (Figs. 2 and 3) suggest that both HERPs can
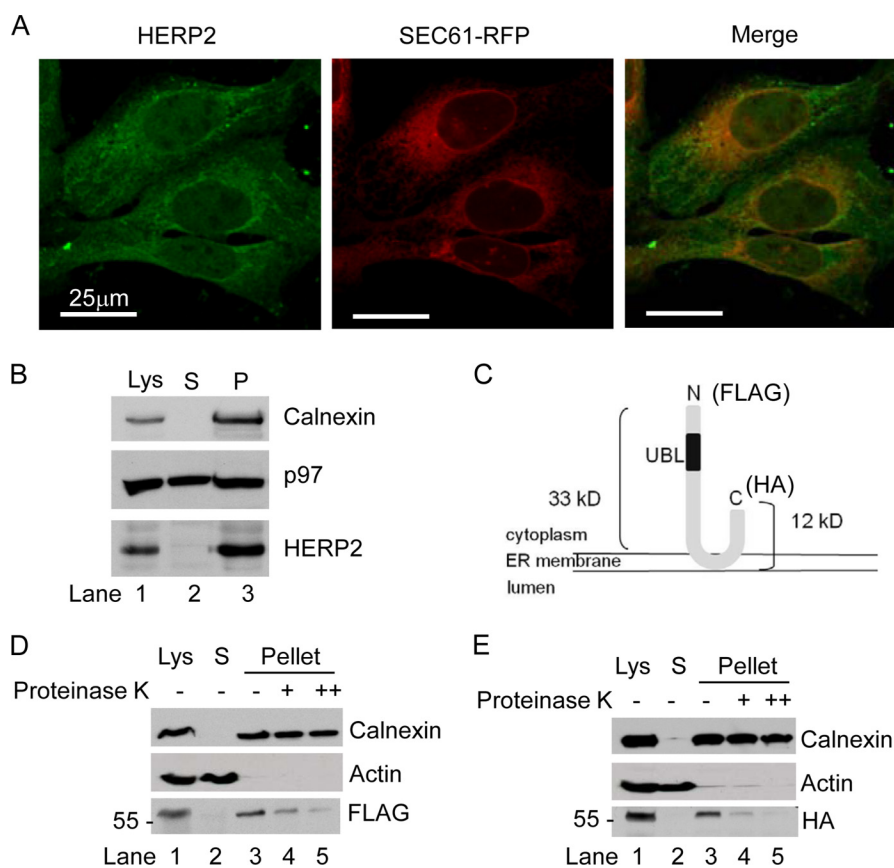
FIGURE 1. **HERP2 locates in endoplasmic reticulum with similar membrane topology as HERP1.** *A*, HERP2 is primarily localized on ER. Immunostaining was carried out with anti-HERP2 antibody using U2OS cells stably expressing Sec61α-RFP proteins. The localization was determined by fluorescence microscopy. The location of ER was revealed by Sec61α-RFP fusion proteins. *B*, 293T cells were fractioned into membrane (P) and cytosolic fractions (S). The fractions were separated on SDS-PAGE and immunoblotting with indicated antibodies. Calnexin was used as a marker for membrane. *C*, the predicted membrane topology of HERP2. HERP2 was tagged with FLAG or HA epitopes at the N or C terminus. *D*, 293T cells were transfected with FLAG-tagged HERP2 and fractioned into cytosolic (S) and membrane fractions (P). The membrane fractions were treated with proteinase K at a final concentration of 0 (−), 0.026 μg/ml (+), and 0.052 μg/ml (++), respectively. The cell lysates and fractions were separated on SDS-PAGE and immunoblotting with calnexin, actin, and FLAG antibodies. Actin was used as a marker for cytosolic fraction. *E*, as in *D*, but with 293T cells transfected with HA-tagged HERP2.

interact with the HRD1-SEL1L complex to function in ERAD, likely in a partially redundant manner.

It has been shown that recombinant HERP1 protein interacts with the RING domain of HRD1 *in vitro* through a region between its UBL domain and the transmembrane domain (amino acids 88–240) (21). To determine whether this region is critical for targeting HERP1 proteins to the HRD1 complex in cells, we generated three different truncation mutants, lacking either the UBL domain (amino acids 2–87) (ΔUBL), a region from amino acids 120 to 200 (Δ120–200), or the transmembrane domain containing C terminus (ΔTM) (Fig. 3C). These HERP1 variants were expressed in 293T cells and subsequently immunoprecipitated from cell extracts under native conditions using FLAG antibodies. As a control, FLAG-tagged WASP was used. Immunoblotting showed that immunoprecipitation of FLAG-WASP did not pull down HRD1 or its associated factors (Fig. 3D, *lane 1*), whereas wild type HERP1 as well as the ΔUBL and ΔTM mutants all interacted with HRD1 and DERL 2 (Fig. 3D, *lanes 2*, *3*, and *5*). The Δ120–200 mutant on the other hand did not interact with HRD1 in cells (Fig. 3D, *lane 4*), confirming that this region of HERP1 binds to HRD1. Similar experiments carried out with HERP2 confirmed the role of the corresponding central region in binding HRD1 (amino acids 136–221) (Fig.

3E). Interestingly, an interaction with DERL2 was observed even for the mutant HERP1 that did not bind HRD1 (Fig. 3D, *lane 4*), suggesting the existence of an interaction between HERP1 and DERL2 that is independent of HRD1.

HERPs Are Required for Retrotranslocation of SHH-C out of the ER—SHH-C protein is deglycosylated upon retrotranslocation into the cytosol, and the deglycosylated SHH-C can accumulate in cells when the proteasome is inhibited (19). Thus, the amount of deglycosylated SHH-C in proteasome-inhibited cells reflects the retrotranslocation activity (15). To determine whether HERPs function before, during, or after SHH-C was retrotranslocated, we treated cells with a proteasome inhibitor, which led to accumulation of the fast migrating deglycosylated SHH-C (Fig. 4A, *lane 1*). As expected, when HRD1 was depleted, little deglycosylated SHH-C was found, indicating that SHH-C was not retrotranslocated into the cytosol (Fig. 4A, *lane 3*) (15). This observation is consistent with the proposed role of HRD1 in retrotranslocation of ERAD substrates. When both HERP1 and HERP2 were depleted, we reproducibly observed much fewer deglycosylated SHH-C molecules, as shown by three independent experimental repeats (Fig. 4A, *lanes 2*, *5*, and *8 versus lanes 1*, *4*, and *7*). These results indicate that HERPs are required for moving SHH-C into the cytosol. In
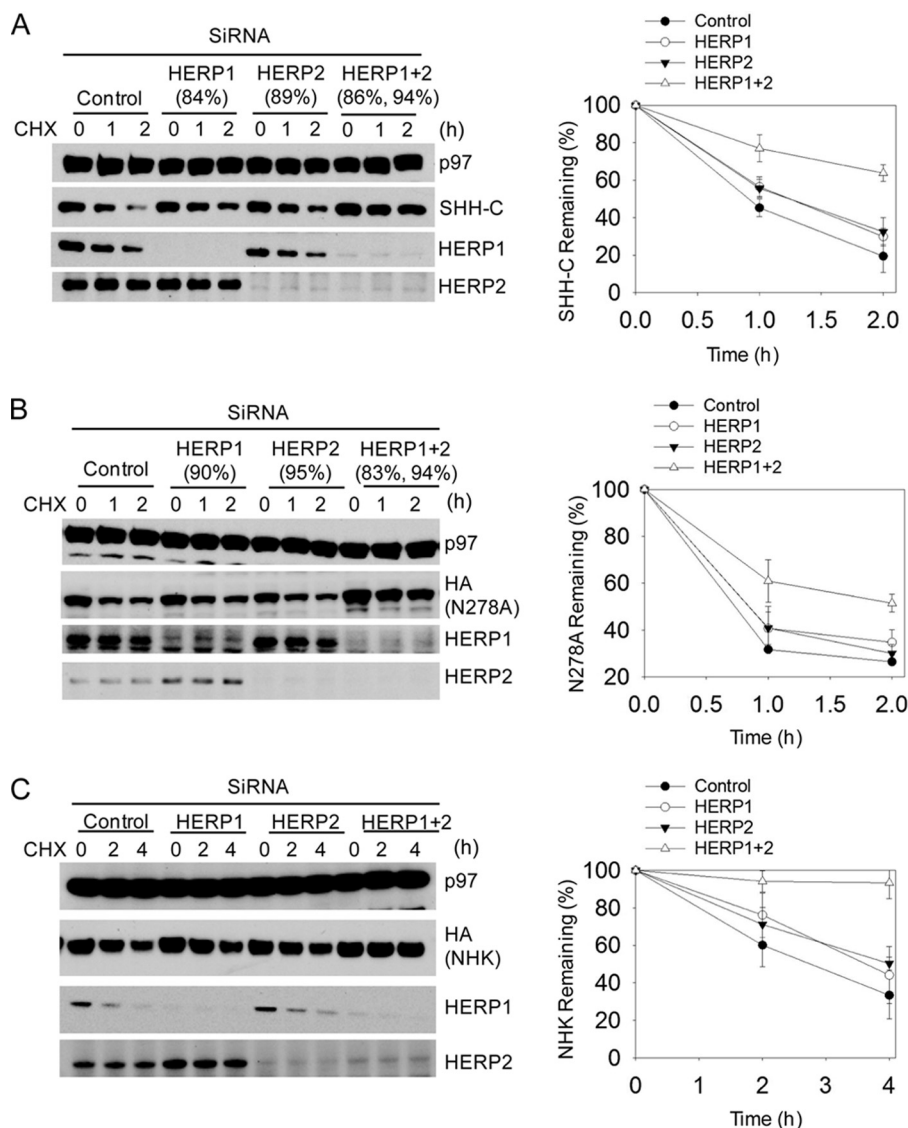
FIGURE 2. **HERP1 and HERP2 are required for the degradation of SHHs and NHK.** The extent of the depletion (in *parentheses*) was determined by quantitative RT-PCR (shown in *parentheses* on top of the panels) and immunoblotting. Immunoblotting with p97 antibodies was used as loading control. The quantification was carried out with three independent experiments. *A*, 293T cells stably expressing SHH-HA were depleted of HERP1 or/and HERP2 by siRNA, and the fate of glycosylated SHH-C proteins was followed after cycloheximide addition. Controls were treated with a negative control siRNA duplex (Invitrogen). All samples were analyzed by SDS-PAGE and immunoblotting with HA, HERP1, and HERP2 antibodies. The *right graph* shows quantification of SHH-C in the experiment. *B*, as in *A*, but with 293T cells stably expressing N278A-HA. *C*, as in *A*, but with 293T cells stably expressing NHK-HA. *CHX*, cycloheximide.

further support of this notion, we found that ubiquitination of SHH-C was reduced in cells depleted of HERPs (Fig. 4*B*). Thus, similar to DERL2 knockdown (15), in HERP-depleted cells, SHH-C appeared to be trapped inside the ER without being retrotranslocated.

To determine whether the substrates were delivered to the HRD1-SEL1L complex in the absence of HERPs, immunoprecipitation was carried out after knocking down HERP proteins in cells. Similar to DERL2 depletion, a small fraction of the substrates was co-precipitated with SEL1L (Fig. 4*C*). Putting these results together, we concluded that HERPs along with DERL2 are essential for the retrotranslocation of SHH-C protein in mammalian cells. Without DERL2 or HERP proteins, SHH-C is trapped inside the ER with a fraction bound to the HRD1 complex.

HERPs Do Not Regulate the Stability or Oligomerization of HRD1—In *S. cerevisiae*, Hrd1p is rapidly degraded in cells lacking Hrd3p (5). Interestingly, when Usa1p is further deleted, Hrd1p becomes stable again (10), indicating that Hrd1p degradation requires Usa1p. We therefore wondered whether HERP proteins play a similar role in mammalian ERAD. We first checked whether the stability of HRD1 and its associated components was affected by depletion of HERPs or SEL1L. Cycloheximide chase experiments showed that depletion of both HERP proteins did not significantly affect the half-life nor the protein levels of HRD1 and its associated components including SEL1L and DERL2 (Fig. 5*A*, *lanes 10–12*). By contrast, knockdown of SEL1L proteins reduced HRD1 levels significantly as observed previously (Fig. 5*B*, *lanes 4–6 versus lanes 1–3*) (15). The remaining HRD1 in SEL1L knockdown cells was
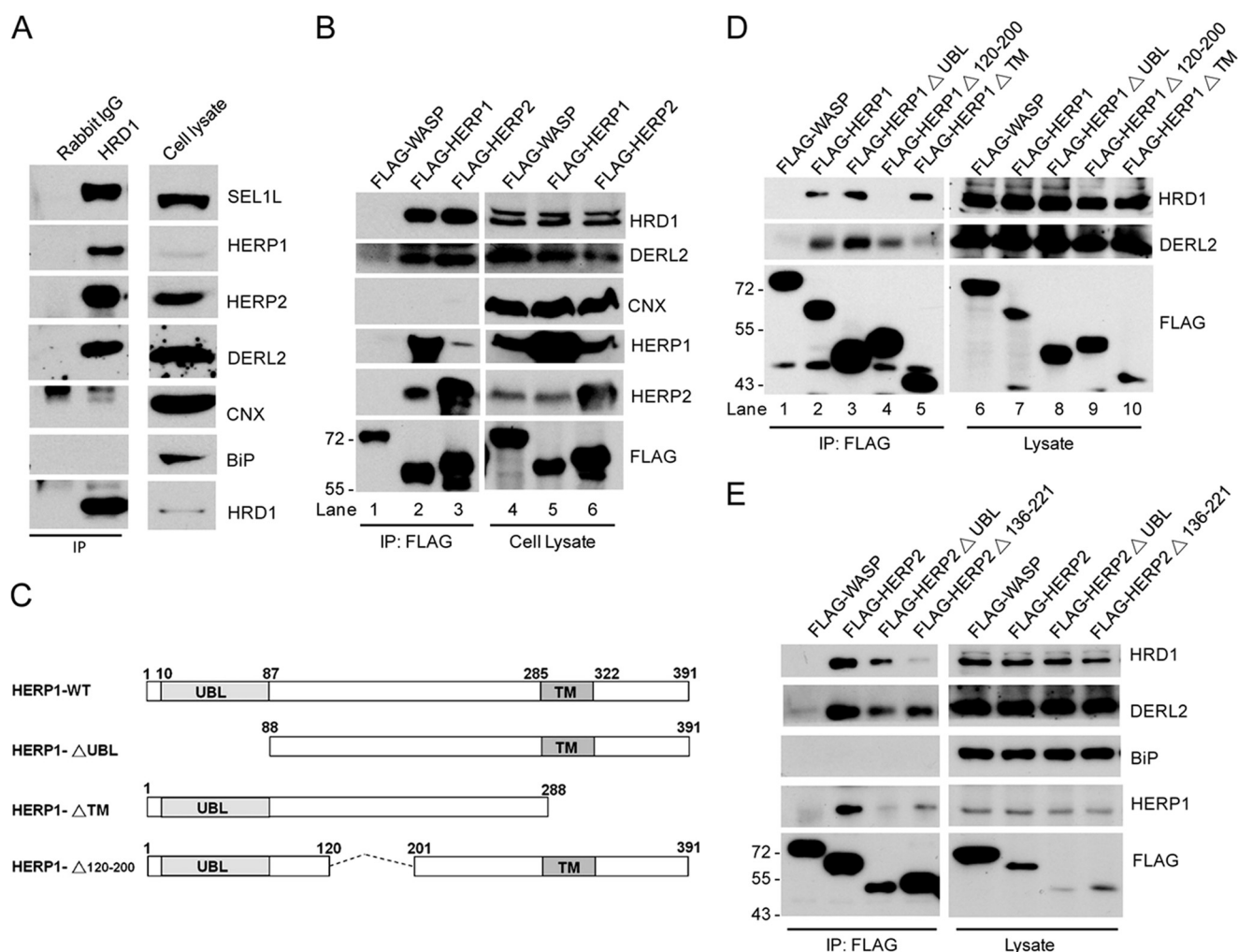
FIGURE 3. **HERP1 and HERP2 are integral components of HRD1-SEL1L complex.** *A*, 293T cells were lysed in HKM buffer with 1% deoxy-bigChap and protease inhibitors, and immunoprecipitated (*IP*) with HRD1 antibodies. The precipitates were separated in SDS-PAGE and immunoblotting with the indicated antibodies. *B*, 293T cells were transfected with FLAG-tagged HERP1, HERP2, or WASP plasmids as indicated. Immunoprecipitation was carried out with FLAG-M2 beads. The proteins were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. *C*, schematic representation of full-length HERP1 and its deletion mutants. *D*, 293T cells were transfected with various FLAG-tagged HERP1 plasmids as indicated. Cells were lysed, and immunoprecipitation was carried out with FLAG-M2 beads. Precipitates were separated on SDS-PAGE and immunoblotting with indicated antibodies. *E*, as in *D*, but with different FLAG-tagged HERP2 plasmids as indicated.

stable likely because it was associated with residual SEL1L as the consequence of incomplete depletion (Fig. 5*B*, *lanes 4–6 versus lanes 1–3*). When HERPs proteins were co-depleted together with SEL1L, it did not restore the protein level of HRD1 (*lanes 7–9*). We concluded that the mechanism of HRD1 degradation in mammalian cells is distinct from that of budding yeast; the regulation of HRD1 stability in mammalian cells does not involve HERP proteins.

Interestingly, despite significant sequence similarity and functional redundancy between the two HERP proteins, endogenous HERP2 appeared to be a stable protein, whereas HERP1 was rapidly turned over with a half-life of less than 1 h (Figs. 2 and 5*A*). The degradation of HERP1 was almost completely inhibited when SEL1L was depleted from cells (Fig. 5*B*, *lanes 4–6*), suggesting that HERP1 itself is turned over by the ERAD system. This is consistent with a recent report demonstrating that an ER-associated E3 ligase is responsible for HERP1 turnover (22).

In *S. cerevisiae*, Usa1p was shown to regulate the oligomerization of Hrd1p (7, 8). Because HRD1 can also oligomerize in 293T cells (Fig. 5*C*) (15, 18, 23), we asked whether HERP proteins were required for HRD1 oligomerization in mammalian cells. We treated control or HERPs-depleted cells with the chemical crosslinker dithiobis(succinimidyl propionate). Immunoblotting showed that depletion of HERP proteins did not affect HRD1 oligomerization (Fig. 5*D*). We concluded that the maintenance of HRD1 oligomers does not require stoichiometric amount of HERP proteins.

HERPs Link DERL2 to HRD1-SEL1L Complex—In *S. cerevisiae*, HERP homolog Usa1p links Der1p to Hrd1p (7), and Der1p is essential for retrotranslocation of substrates with luminal lesion (11, 24, 25). Because we showed that HERPs and DERL2 are both integral part of the HRD1-SEL1L complex in mammalian ERAD (Figs. 3 and 4) (15), we wondered whether HERPs also link DERL2 to the HRD1-SEL1L complex in mammalian cells. To investigate this possibility, HERP proteins were
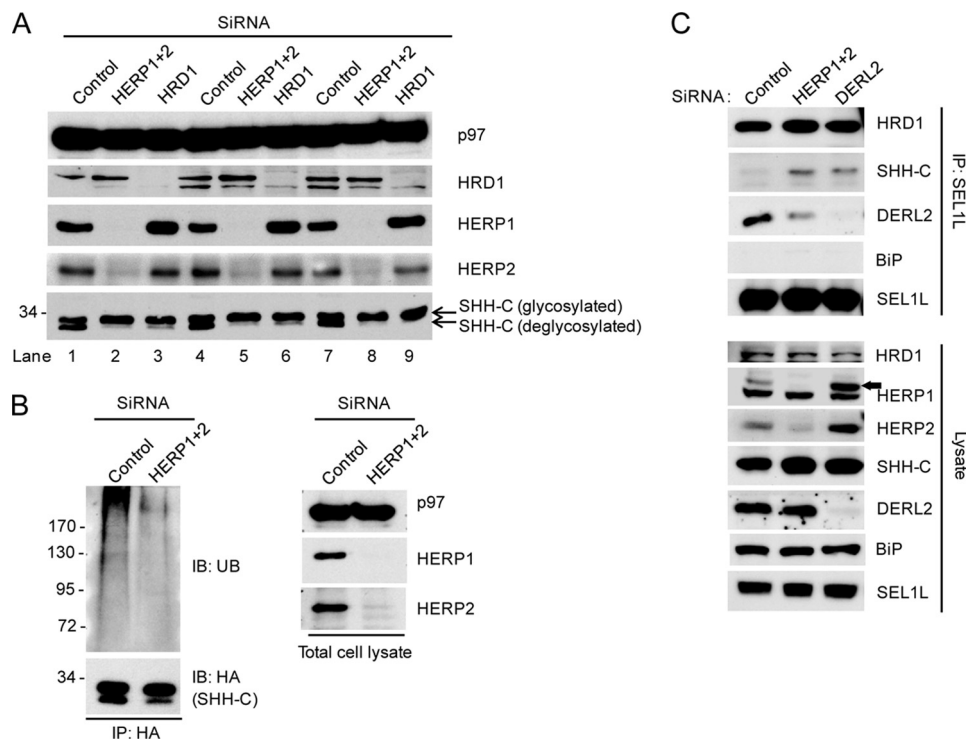
FIGURE 4. **Depletion of HERP1 and HERP2 traps the substrates inside ER.** *A*, cells stably expressing SHH-HA were depleted of both HERP1 and HERP2, or HRD1 by siRNA oligonucleotides. Cells were then treated with MG132 (150 mM) for 4 h before lysed in lysis buffer containing 1% Triton X-100 and protease inhibitors. Cell lysates were separated by SDS-PAGE and immunoblotting (*IB*) with indicated antibodies. Immunoblotting with p97 was used as loading control. SHH-C, glycosylated or not, was as indicated. Three independent repeats are shown. *B*, as in *A*, but depleted of both HERP1 and HERP2. Cells were lysed in denaturing buffer containing SDS followed by immunoprecipitation (*IP*) with HA antibodies as described under "Experimental Procedures." The precipitates were separated on Bis-Tris gradient gel and immunoblotting with ubiquitin antibodies. Total cell lysates were also separated on SDS-PAGE and immunoblotting with HERP1, HERP2, and p97 antibodies. *C*, as in *A*, but depleted with both HERP1 and HERP2 or DERL2 siRNA oligonucleotides. Cells were lysed in HKM buffer containing 1% deoxy-bigChap and immunoprecipitated with SEL1L antibodies. The precipitates and the total cell lysates were separated on SDS-PAGE and immunoblotting with the indicated antibodies.

depleted by siRNA from cells, and immunoprecipitation was carried out with HRD1 antibodies. From control knockdown cells, SEL1L, HERP1, HERP2, and DERL2, but not calnexin, were co-precipitated with HRD1 (Fig. 5*E*, *lane 1*), indicating that HRD1 interacts specifically with these ERAD factors. When individual HERP was depleted, the interaction of HRD1 with SEL1L and DERL2 was largely maintained (Fig. 5*E*, *lanes 2* and *3*). However, when both HERP proteins were knocked down, we consistently observed reduced amount of DERL2 in association with HRD1 (Fig. 5*E*, *lane 4*). After normalizing the DERL2 levels by the amount of HRD1 in the precipitated samples from three independent experiments, the levels of DERL2 associated with HRD1 were reduced by ~4-fold after depletion of both HERP proteins. The results indicated that HERP proteins play a key role in recruiting DERL2 to the HRD1-SEL1L complex.

HERP1 Is Induced by ER Stress, Whereas HERP2 Is Constitutively Expressed—The expression of HERP1 is highly induced by ER stress, as reported previously (16). We therefore tested whether HERP2 was also regulated by ER stress. Whole cell extracts from cells treated with different ER stress inducers such as DTT, tunicamycin (Tm) or thapsigargin (Tg) were analyzed by immunoblotting. The result showed that HERP1 was highly up-regulated by these treatments, but HERP2 expression remained unaffected (Fig. 6, *A* and *B*). We conclude that unlike HERP1, HERP2 is a constitutively expressed ERAD factor.

Given its up-regulation during ER stress, we asked whether HERP1 would become more indispensable for ERAD of SHH-C

in cells undergoing ER stress induced by the pharmacological stress inducers such as Tm and Tg. Cycloheximide chase experiments done with both stressed and nonstressed cells revealed no additional inhibition of SHH-C degradation by either HERP1 or HERP2 knockdown in Tm-treated cells compared with control unstressed cells (Fig. 6*C*, *lanes 3*, *4*, *9*, and *10*). Interestingly, in Tm-treated cells, a large fraction of SHH-C cannot be glycosylated because of inhibition of glycosylation (*lanes 7–12*). This nonglycosylated SHH species was degraded rapidly in both control and HERP1- or HERP2-depleted cells (Fig. 6*C*, *lanes 9* to *12*, respectively). Likewise, the effect of either HERP1 or HERP2 knockdown on the degradation of SHH-C was not significantly altered by exposing the cells to Tg (Fig. 6*D*), as revealed by quantitative analysis of three independent experiments (Fig. 6*E*). Similar results were also obtained with another HRD1 substrate NHK (data not shown). Together, these results imply that although HERP1 induction under ER stress condition had been presumed to increase ERAD capacity, it does not significantly affect the degradation rate for a couple of HRD1 substrates tested here.

The UBL Domains in HERP1 and HERP2 Are Functionally Different—Expression of a UBL-deleted HERP1 mutant was shown to have a dominant negative effect on degradation of an ERAD substrate (21). We investigated whether the UBL domains in HERP1 and HERP2 are equally important for ERAD of SHH-C. As shown in Fig. 7, expression of the UBL-deleted HERP1 mutant, but not a similar HERP2 mutant, resulted in
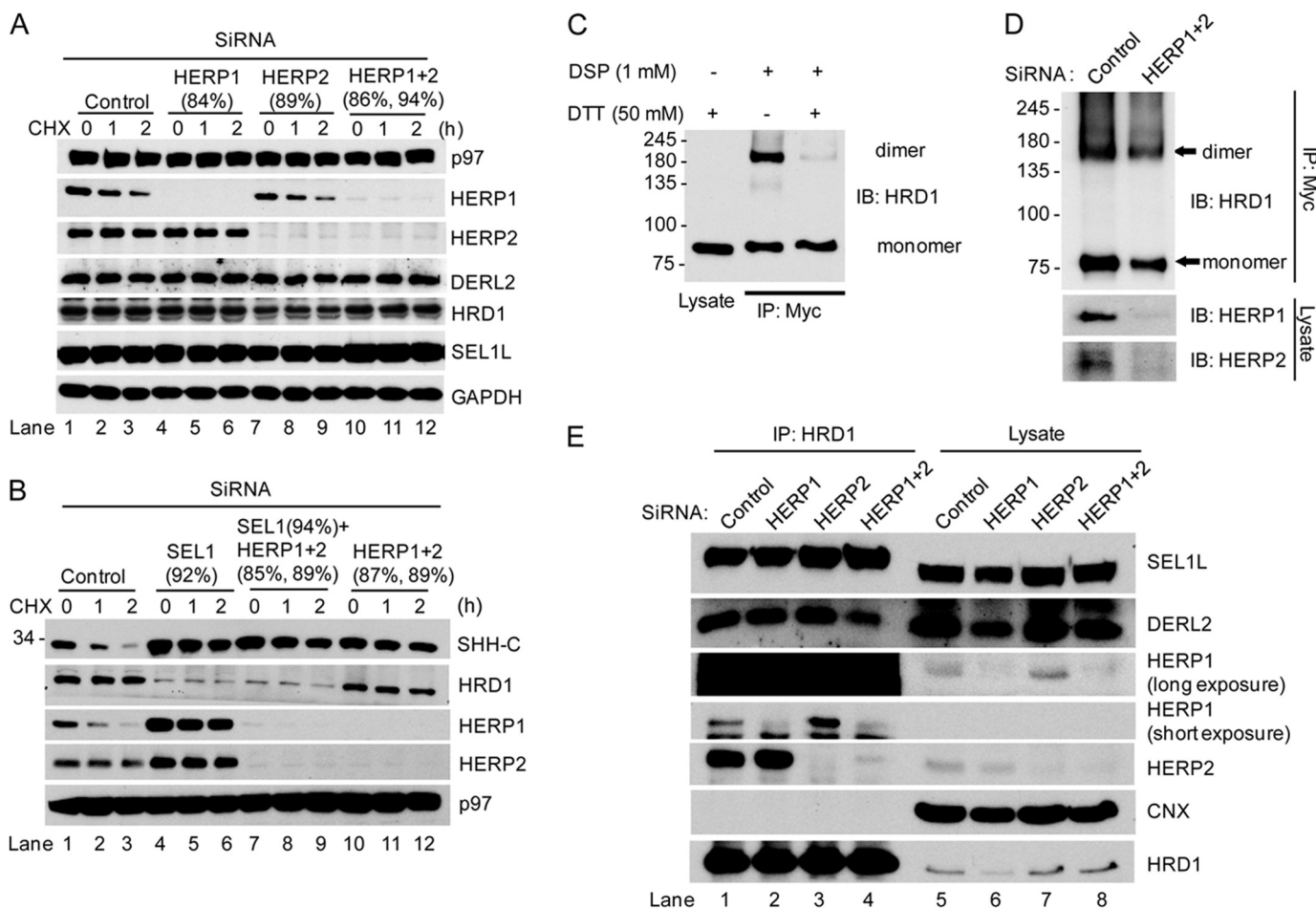
FIGURE 5. **HERPs link DERL2 to HRD1-SEL1L complex.** *A*, 293T cells were depleted of HERP1 or/and HERP2 by siRNA. Cells were treated with cycloheximide and lysed in lysis buffer containing 1% Triton X-100 and protease inhibitors. Cell lysates were analyzed by SDS-PAGE and immunoblotting (*IB*) with indicated antibodies. Immunoblotting with GAPDH was used as loading control. The depletion was also quantified by quantitative RT-PCR (in *parentheses*). *B*, as in *A*, but with cells stably expressing SHH-HA depleted of SEL1L, HERP1, and HERP2 as indicated. Immunoblotting with p97 was used as loading control. *C*, cells stably expressing HRD1-Myc were treated with chemical cross-linkers dithiobis(succinimidyl propionate) (*DSP*) as described under "Experimental Procedures." Cell lysates were then immunoprecipitated (*IP*) with Myc antibodies. Immunoblotting with HRD1 antibodies was carried out. *D*, as in *C*, but with cells depleted of both HERP1 and HERP2. Immunoblotting was carried out with the indicated antibodies. *E*, 293T cells were depleted of HERP1, HERP2, or both HERPs. Cells were lysed in HKM buffer with 1% deoxy-bigChap and protease inhibitors and immunoprecipitated with HRD1 antibodies. The precipitates and lysates were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. *CHX*, cycloheximide.

significant inhibition of SHH-C degradation (Fig. 7). Consistent with our conclusion that increased HERP1 expression does not significantly increase ERAD efficiency, we found that overexpression of wild type HERP1 did not increase SHH-C turnover (Fig. 7). These results indicated that the UBL domains of the HERP proteins may have an additional function in ERAD.

DISCUSSION

HERP1 contains an N-terminal UBL domain that is essential for its function in ERAD (18). Because HERP1 was previously reported as an ER stress-induced factor that associates with many ER-bound E3 ubiquitin ligases including HRD1 and gp78 (21, 26), it was proposed to serve as an essential regulator of these E3 ligases (21). However, despite extensive studies, how HERP1 facilitates ERAD has been unclear.

In this study, we report a novel HERP1-related protein named HERP2 that shares similar amino acid sequence, membrane topology, and subcellular localization with HERP1. Interestingly, whereas HERP1 is an unstable protein whose expression is highly up-regulated by ER stress, HERP2 is a stable

protein that is constitutively expressed in cells. Our results suggest that both HERP1 and HERP2 function in ERAD as an integral component of the HRD1 ubiquitin ligase complex and that they regulate the degradation of glycosylated SHH-C, NHK, and nonglycosylated ERAD substrate SHH N278A (Fig. 2). Combined with our previous study (15), we conclude that the HRD1-SEL1L complex consisting of DERL2 and HERPs proteins is responsible for ERAD independent of its glycosylation status. This conclusion is in line with a previous study showing that HERP1 depletion attenuates the degradation of a nonglycosylated ERAD substrate (17). However, it is noteworthy that the study by Okuda-Shimizu and Hendershot (17) concluded that HERP1 is not required for degradation of glycosylated NHK. However, a more recent study showed that depletion of HERP1 does partially stabilize NHK (18). In addition, HERP1 is also involved in the degradation of another glycosylated substrate, a mutant CD3 (21), suggesting that HERP1 may not be specific for nonglycosylated misfolded proteins. In our study, much more dramatic inhibition on the degradation of both glycosylated and nonglycosylated SHH and NHK is observed when
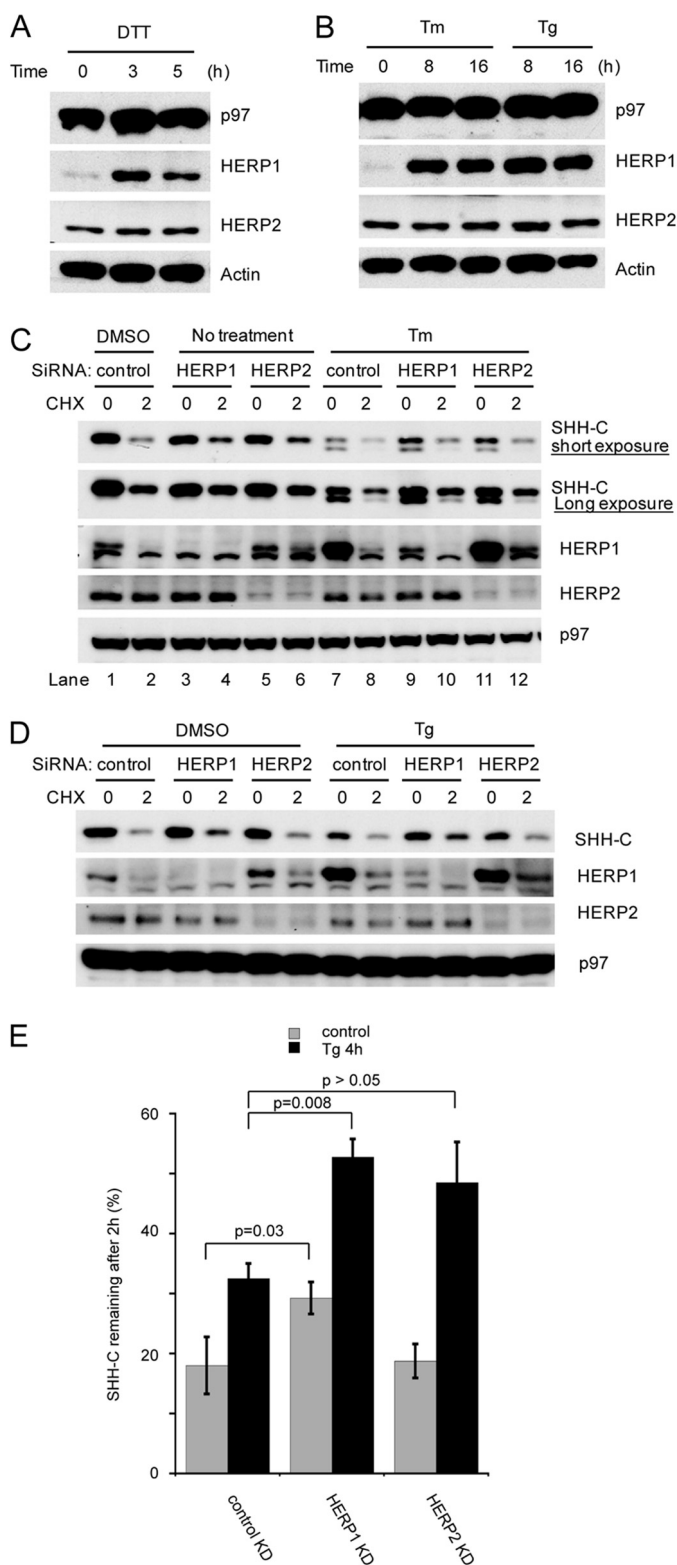
FIGURE 6. **Expression of HERP1 and HERP2 is differentially regulated by ER stress.** Immunoblotting with p97 is used as loading control. *A*, 293T cells were treated with 1 mM DTT for indicated hours. Cells were lysed in lysis buffer containing 1% Triton X-100 and protease inhibitors. Cell lysates were separated on SDS-PAGE and immunoblotting with HERP1, HERP2, and actin. Immunoblotting with actin was used as loading control. *B*, as in *A*, but treated with Tm (2 μg/ml) or Tg (0.1 μM) for the indicated duration. *C*, cells stably expressing SHH-HA were depleted of HERP1 or HERP2. Cells were treated with/without tunicamycin (2 μg/ml) for 8 h in the presence or absence of 100 μg/ml cycloheximide for 2 h. Cells were then lysed and separated on SDS-

both HERP proteins are depleted (Fig. 2). The discovery of HERP2 may explain some discrepancy reported in the literature, because HERP2 may compensate for the loss of HERP1 under certain experimental settings. Our results clearly highlight an essential role for both HERP proteins in mammalian ERAD.

In *S. cerevisiae*, Usa1p (potential ortholog of HERP1) appeared to serve a scaffolding function that links Der1p to Hrd1p (7), the homologs of Derlin and HRD1 in mammalian cells. Our study suggests that the mammalian HRD1-containing ERAD complex appears to be organized in a similar way (Fig. 5*E*). However, our study also reveals several important distinctions between these two ERAD systems. First, in mammals, oligomerization of HRD1 does not require stoichiometric amount of HERP proteins (Fig. 5*D*). By contrast, Usa1p is required for oligomerization of Hrd1p (7, 8). In mammalian cells, although HRD1 still forms oligomer in the absence of HERP proteins, it is no longer active in retrotranslocation, and ERAD substrates are largely trapped inside ER without being ubiquitinated (Figs. 2 and 4). This is also different from that in yeast, in which oligomerization of Hrd1p appears to be essential for the retrotranslocation (7). One more distinction between the yeast and human ERAD systems is the regulation of the HRD1 stability. In yeast, Hrd1p stability is regulated by both Hrd3p and Usa1p (5, 9). In mammalian cells, the stability of HRD1 is regulated by SEL1L, but not by HERPs (Fig. 5, *A* and *B*) (15).

Our study demonstrates that HERP proteins provide an important scaffolding function that links DERL2 to the HRD1-SEL1L subcomplex, which results in the formation of a functional complex consisting of HRD1, SEL1L, and DERL2 (15). In this complex, both HRD1 and DERL2 are homo-oligomeric (15, 18). How HERPs is organized into this HRD1-containing retrotranslocon remains to be elucidated. In the absence of both HERP1 and HERP2, significantly less DERL2 is found in association with HRD1 (Fig. 5*D*). However, when only one HERP protein is depleted, we observed no difference in the amount of DERL2 that is in complex with HRD1, although under the HERP1 depletion condition, a small ERAD inhibition phenotype could be observed for a given HRD1 substrate (Figs. 2 and 6*E*). It is possible that in cells lacking one HERP protein, the complex of HRD1-DERL2 is altered, but not to the extent that significant dissociation of DERL2 from HRD1 has occurred. Alternatively, it is also possible that other than linking DERL2 to the HRD1 complex, HERP proteins may have additional function(s) in ERAD, as suggested by our observation that the UBL domain of HERP1 is functionally important in ERAD of SHH-C, although this domain is not involved in interaction with DERL2 (Fig. 7). The precise function of the HERP1 UBL domain remains to be elucidated.

Each component of the HRD1-SEL1L-HERP-DERL2 complex plays a unique and important role in retrotranslocation of ERAD substrates across the ER membrane because depletion of each of them abrogates retrotranslocation. Within this com-

PAGE and immunoblotting with the indicated antibodies. *D*, as in *C*, but treated with Tg for 4 h. *E*, statistical analysis of *D* from three independent experiments. *CHX*, cycloheximide.
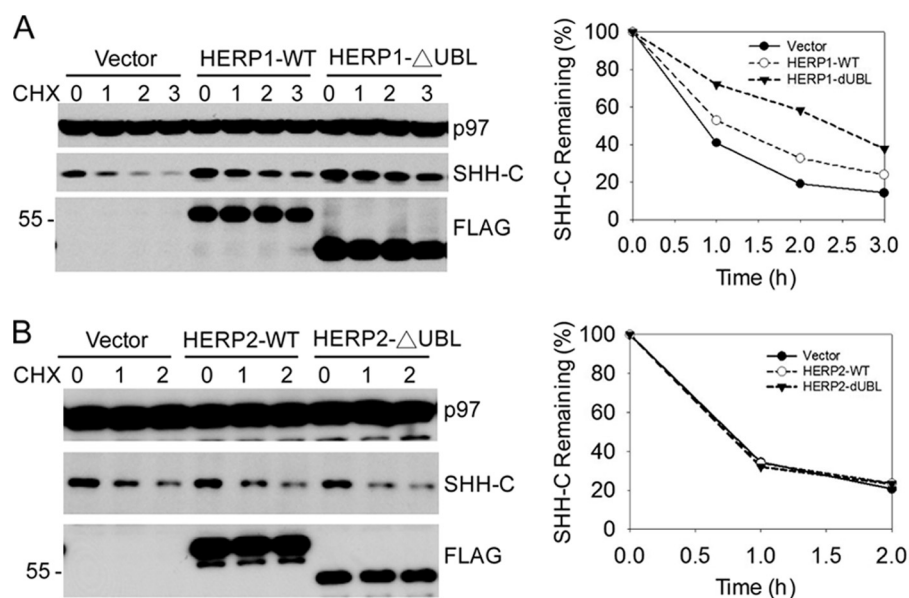
FIGURE 7. **UBL domains of HERP1 and HERP2 affect differentially the degradation of SHH-C.** *A*, 293T cells stably expressing SHH-C were transfected with FLAG-tagged full-length HERP1 and its UBL-deleted mutant as indicated. Cells were treated with cycloheximide and lysed in lysis buffer. The cell lysate were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies. *B*, as in *A* but with HERP2 and its UBL-deleted mutant. What is shown is one representative experiment from two independent repeats. *CHX*, cycloheximide.

plex, yeast Hrd1p may oligomerize to form a large assembly capable of mediating retrotranslocation of luminal substrates all by itself at least when overexpressed (7, 12). This observation places Hrd1p and by extrapolation the mammalian homolog HRD1 at the central place in the retrotranslocation system, perhaps as a major candidate for the putative retrotranslocation channel. SEL1L/Hrd3p likely serves as a major substrate recruiting adaptor that targets ERAD substrates to HRD1 (14). In addition, our gene knockdown experiments clearly demonstrates that SEL1L is required for stabilizing endogenous HRD1 in mammalian cells (Fig. 5*B*) (15), similar to that in yeast (5, 9). Because DERL2 is an essential component of the HRD1 complex that facilitates retrotranslocation (15), it is tempting to speculate that by recruiting DERL2, the scaffolding function of HERPs helps to mold HRD1 into an active state competent for retrotranslocation. Whether HERP proteins can have any additional function in regulating HRD1 activity is currently unclear.

HERP1 and HERP2 appear to play partially redundant roles in ERAD, at least for a subset of ERAD substrates such as SHH-C, N278A, and NHK. Depletion of both HERP1 and HERP2 is required to strongly inhibit their degradation (Fig. 2). Interestingly, despite similarity at the protein sequence level, the UBL domain of these two proteins appear to act differentially in ERAD because deletion of the UBL domain from HERP1, but not that of HERP2, produces a dominant negative effect on SHH-C degradation (Fig. 7). The two HERP proteins are also differentially regulated in expression and stability. HERP1 is highly up-regulated by ER stress (16), but its rapid turnover is not affected by the presence of ER stress (Fig. 6). On the other hand, HERP2 is a stable protein that is constitutively expressed (Figs. 2 and 6). In 293T cells exposed to an ER stress inducer, knockdown of HERP1 does not cause more inhibition in SHH-C and NHK degradation compared with unstressed cells (Fig. 6, *C–E*, and data not shown). This seems to suggest that HERP1 up-regulation upon ER stress induction is not suf-

ficient to alter the degradation kinetics for individual ERAD substrate. The degradation rate of individual substrate may be primarily determined by a key rate-limiting step regulated by the interplay between substrates and certain ERAD machinery factors. Alternatively, the fraction of HERP1 involved in ERAD may not be significantly altered during ER stress induction, even though the total level of HERP1 is induced. ER stress-induced HERP1 may be primarily involved in other cellular processes that adapt cells to ER stress such as calcium homeostasis or apoptosis regulation (27, 28). Future research will be required to determine whether there may exist a clear division of labor for the two HERP proteins under certain physiologically relevant stress conditions or in cells specializing in secretion function.

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