HERP REGULATES HRD1-MEDIATED UBIQUITYLATION IN A UBIQUITIN-LIKE-DOMAIN-DEPENDENT MANNER

Melanie Kny§*, Sybille Standera§*, Rasmus Hartmann-Petersen¶, Peter-Michael Kloetzel§ and Michael Seeger§

From the §Institut für Biochemie, Charité - Universitätsmedizin Berlin, Institut für Biochemie, D-13347 Berlin, Germany; ¶Department of Biology, Protein Science Section, University of Copenhagen, DK-2200 Copenhagen N, Denmark

Running Head: Herp UBL-domain is required for Hrd1-mediated ubiquitylation

Address correspondence to: Dr. Michael Seeger, Institut für Biochemie, Charité – Universitätsmedizin Berlin, CC 2 Grundlagenmedizin, Oudenarder Str. 16, 13347 Berlin, Germany, Phone: +49 30 450 528 186; Fax: +49 30 450 528 921; Email: michael.seeger@charite.de

Maturation of newly synthesized proteins in the endoplasmic reticulum (ER) is monitored by a number of complex quality control mechanisms (1). One such pathway is known as the unfolded protein response (UPR) that helps the cell to survive under these stress conditions. Herp is a mammalian ubiquitin domain protein, which is strongly induced by the UPR. It is involved in ER-associated protein degradation (ERAD) and interacts directly with the ubiquitin ligase Hrd1, which is found in high molecular mass complexes of the ER-membrane. Here we present first evidence that Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like (UBL)-domain dependent manner. We found that upon exposure of cells to ER-stress, elevation of Herp steady state levels is accompanied by an enhanced association of Herp with pre-existing Hrd1. Hrd1-associated Herp is rapidly degraded and substituted by de novo synthesized Herp, suggesting a continuous turnover of the protein at Hrd1 complexes. Further analysis revealed the presence of multiple Hrd1 copies in a single complex enabling binding of a variable number of Herp molecules. Efficient ubiquitylation of the Hrd1-specific ERAD substrate alpha1-antitrypsin Null Hong-Kong (NHK) required the presence of the Herp UBL domain, which was also necessary for NHK degradation. In summary, we propose that Herp associates with Hrd1-containing ERAD complexes positively regulating the ubiquitylation activity of these complexes, thus permitting survival of the cell during ER stress.

Maturation of newly synthesized proteins in the ER is monitored by a number of complex quality control mechanisms (1). One such pathway is known as the unfolded protein response (UPR) that helps the cell to survive under these stress conditions. Herp is a mammalian ubiquitin domain protein, which is strongly induced by the UPR. It is involved in ER-associated protein degradation (ERAD) and interacts directly with the ubiquitin ligase Hrd1, which is found in high molecular mass complexes of the ER-membrane. Here we present first evidence that Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like (UBL)-domain dependent manner. We found that upon exposure of cells to ER-stress, elevation of Herp steady state levels is accompanied by an enhanced association of Herp with pre-existing Hrd1. Hrd1-associated Herp is rapidly degraded and substituted by de novo synthesized Herp, suggesting a continuous turnover of the protein at Hrd1 complexes. Further analysis revealed the presence of multiple Hrd1 copies in a single complex enabling binding of a variable number of Herp molecules. Efficient ubiquitylation of the Hrd1-specific ERAD substrate alpha1-antitrypsin Null Hong-Kong (NHK) required the presence of the Herp UBL domain, which was also necessary for NHK degradation. In summary, we propose that binding of Herp to Hrd1-containing ERAD complexes positively regulates the ubiquitylation activity of these complexes, thus permitting survival of the cell during ER stress.

Maturation of newly synthesized proteins in the endoplasmic reticulum (ER) is monitored by a number of complex quality control mechanisms (1). One such pathway is known as the unfolded protein response (UPR) that helps the cell to survive under these stress conditions. Herp is a mammalian ubiquitin domain protein, which is strongly induced by the UPR. It is involved in ER-associated protein degradation (ERAD) and interacts directly with the ubiquitin ligase Hrd1, which is found in high molecular mass complexes of the ER-membrane. Here we present first evidence that Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like (UBL)-domain dependent manner. We found that upon exposure of cells to ER-stress, elevation of Herp steady state levels is accompanied by an enhanced association of Herp with pre-existing Hrd1. Hrd1-associated Herp is rapidly degraded and substituted by de novo synthesized Herp, suggesting a continuous turnover of the protein at Hrd1 complexes. Further analysis revealed the presence of multiple Hrd1 copies in a single complex enabling binding of a variable number of Herp molecules. Efficient ubiquitylation of the Hrd1-specific ERAD substrate alpha1-antitrypsin Null Hong-Kong (NHK) required the presence of the Herp UBL domain, which was also necessary for NHK degradation. In summary, we propose that binding of Herp to Hrd1-containing ERAD complexes positively regulates the ubiquitylation activity of these complexes, thus permitting survival of the cell during ER stress.

Maturation of newly synthesized proteins in the endoplasmic reticulum (ER) is monitored by a number of complex quality control mechanisms (1). One such pathway is known as the unfolded protein response (UPR) that helps the cell to survive under these stress conditions. Herp is a mammalian ubiquitin domain protein, which is strongly induced by the UPR. It is involved in ER-associated protein degradation (ERAD) and interacts directly with the ubiquitin ligase Hrd1, which is found in high molecular mass complexes of the ER-membrane. Here we present first evidence that Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like (UBL)-domain dependent manner. We found that upon exposure of cells to ER-stress, elevation of Herp steady state levels is accompanied by an enhanced association of Herp with pre-existing Hrd1. Hrd1-associated Herp is rapidly degraded and substituted by de novo synthesized Herp, suggesting a continuous turnover of the protein at Hrd1 complexes. Further analysis revealed the presence of multiple Hrd1 copies in a single complex enabling binding of a variable number of Herp molecules. Efficient ubiquitylation of the Hrd1-specific ERAD substrate alpha1-antitrypsin Null Hong-Kong (NHK) required the presence of the Herp UBL domain, which was also necessary for NHK degradation. In summary, we propose that binding of Herp to Hrd1-containing ERAD complexes positively regulates the ubiquitylation activity of these complexes, thus permitting survival of the cell during ER stress.

Maturation of newly synthesized proteins in the endoplasmic reticulum (ER) is monitored by a number of complex quality control mechanisms (1). One such pathway is known as the unfolded protein response (UPR) that helps the cell to survive under these stress conditions. Herp is a mammalian ubiquitin domain protein, which is strongly induced by the UPR. It is involved in ER-associated protein degradation (ERAD) and interacts directly with the ubiquitin ligase Hrd1, which is found in high molecular mass complexes of the ER-membrane. Here we present first evidence that Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like (UBL)-domain dependent manner. We found that upon exposure of cells to ER-stress, elevation of Herp steady state levels is accompanied by an enhanced association of Herp with pre-existing Hrd1. Hrd1-associated Herp is rapidly degraded and substituted by de novo synthesized Herp, suggesting a continuous turnover of the protein at Hrd1 complexes. Further analysis revealed the presence of multiple Hrd1 copies in a single complex enabling binding of a variable number of Herp molecules. Efficient ubiquitylation of the Hrd1-specific ERAD substrate alpha1-antitrypsin Null Hong-Kong (NHK) required the presence of the Herp UBL domain, which was also necessary for NHK degradation. In summary, we propose that binding of Herp to Hrd1-containing ERAD complexes positively regulates the ubiquitylation activity of these complexes, thus permitting survival of the cell during ER stress.

Maturation of newly synthesized proteins in the endoplasmic reticulum (ER) is monitored by a number of complex quality control mechanisms (1). One such pathway is known as the unfolded protein response (UPR) that helps the cell to survive under these stress conditions. Herp is a mammalian ubiquitin domain protein, which is strongly induced by the UPR. It is involved in ER-associated protein degradation (ERAD) and interacts directly with the ubiquitin ligase Hrd1, which is found in high molecular mass complexes of the ER-membrane. Here we present first evidence that Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like (UBL)-domain dependent manner. We found that upon exposure of cells to ER-stress, elevation of Herp steady state levels is accompanied by an enhanced association of Herp with pre-existing Hrd1. Hrd1-associated Herp is rapidly degraded and substituted by de novo synthesized Herp, suggesting a continuous turnover of the protein at Hrd1 complexes. Further analysis revealed the presence of multiple Hrd1 copies in a single complex enabling binding of a variable number of Herp molecules. Efficient ubiquitylation of the Hrd1-specific ERAD substrate alpha1-antitrypsin Null Hong-Kong (NHK) required the presence of the Herp UBL domain, which was also necessary for NHK degradation. In summary, we propose that binding of Herp to Hrd1-containing ERAD complexes positively regulates the ubiquitylation activity of these complexes, thus permitting survival of the cell during ER stress.

Maturation of newly synthesized proteins in the endoplasmic reticulum (ER) is monitored by a number of complex quality control mechanisms (1). One such pathway is known as the unfolded protein response (UPR) that helps the cell to survive under these stress conditions. Herp is a mammalian ubiquitin domain protein, which is strongly induced by the UPR. It is involved in ER-associated protein degradation (ERAD) and interacts directly with the ubiquitin ligase Hrd1, which is found in high molecular mass complexes of the ER-membrane. Here we present first evidence that Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like (UBL)-domain dependent manner. We found that upon exposure of cells to ER-stress, elevation of Herp steady state levels is accompanied by an enhanced association of Herp with pre-existing Hrd1. Hrd1-associated Herp is rapidly degraded and substituted by de novo synthesized Herp, suggesting a continuous turnover of the protein at Hrd1 complexes. Further analysis revealed the presence of multiple Hrd1 copies in a single complex enabling binding of a variable number of Herp molecules. Efficient ubiquitylation of the Hrd1-specific ERAD substrate alpha1-antitrypsin Null Hong-Kong (NHK) required the presence of the Herp UBL domain, which was also necessary for NHK degradation. In summary, we propose that binding of Herp to Hrd1-containing ERAD complexes positively regulates the ubiquitylation activity of these complexes, thus permitting survival of the cell during ER stress.
EXPERIMENTAL PROCEDURES

Plasmids- Plasmids were constructed by PCR-amplification from HeLa cDNA or from cDNA clones, purchased from RZPD/ImaGenes, followed by insertion into pSG5 (Novagen) (HERP, HERP∆UBL encoding aa 1-10 joined with 88-391, HRD1, HRD1-MYC). HRD1-M-MYC encoding Hrd1C329S was generated from HRD1-MYC by site directed mutagenesis using QuickChange (Stratagene). To generate HTB-tagged versions, the RGS6His-TEV-Bio cassette (HTB) derived from pFA6a-HTB-kanMX6 (kindly provided by P. Kaiser, La Jolla, USA) was inserted into the BamHI site of pSG5-based constructs using BamHI/BglII. His-Ub-GFP was generated by subcloning an insert encoding 10His-Ubiquitin into pEGFP-N3. GenBank accession numbers are AF055001 for HERP and AL834263 for HRD1. NHK was expressed from a pBR322 based Ig-lambda expression vector kindly provided by P. Paganetti (Novartis). The shHERP and shGFP plasmids have been described previously (6).

Cell lines and transfections- HeLa cells were grown in Iscove's medium containing 10% fetal bovine serum, 2mM glutamine, 100µg/ml streptomycin and 100 units/ml penicillin. Transfections were performed using the calcium phosphate method. To generate HTB-tagged versions, the RGS6His-TEV-Bio cassette (HTB) derived from pFA6a-HTB-kanMX6 (kindly provided by P. Kaiser, La Jolla, USA) was inserted into the BamHI site of pSG5-based constructs using BamHI/BglII. His-Ub-GFP was generated by subcloning an insert encoding 10His-Ubiquitin into pEGFP-N3. GenBank accession numbers are AF055001 for HERP and AL834263 for HRD1. NHK was expressed from a pBR322 based Ig-lambda expression vector kindly provided by P. Paganetti (Novartis). The shHERP and shGFP plasmids have been described previously (6).

Antibodies- Polyclonal Herp specific antiserum was generated as described (6). The antiserum against Hrd1 was kindly provided by M. Kikkert and E. Wiertz (Leiden University, Netherlands). The RGS-His-antibody was from Qiagen, the p97 antibody (MA3-004) was from Dianova, the polyclonal ubiquitin-specific antiserum was from Dako, the peroxidase-coupled ubiquitin antibody (FK2) was from Enzo, the GAPDH antiserum was from Santa Cruz Biotechnology, the alphaL-antitrypsin antibody used for immunoprecipitation was from Dako, the peroxidase-coupled alphaL-antitrypsin antibody was from Bethyl Labs and the anti-GFP antibody was from Rockland Immunochemicals.

Preparation of cell extracts, streptavidin-agarose-based precipitation of tagged proteins, metabolic labelling, immunoprecipitations and Western Blotting- To prepare cell extracts for Western blot analysis, cells were lysed in RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1mM EDTA, 1% NP40, 0.1% SDS, 0.5% Sodium-Desoxycholate). For analysis of protein complexes by tag-based precipitation or immunoprecipitation, cells were lysed in buffer A (33mM Hepes pH 7.3, 150mM KOAc, 4mM MgOAc, 1% DeoxyBigCHAP (Calbiochem), 10% glycerol) supplemented with Complete™ (Roche) for 30min on ice. Cell debris was removed by centrifugation in a microfuge for 10min at 14000 rpm. For precipitation of HTB-tagged proteins, supernatants were incubated with streptavidin agarose (Novagen) for 2h and washed in buffer B (buffer A, containing 0.2% DeoxyBigCHAP instead of 1%). Beads were then suspended in SDS sample buffer and analyzed by immunoblotting. For metabolic labelling, cells grown to confluency were incubated in methionine-free RPMI for one hour followed by addition of 35S-methionine (200µCi /1x10⁷ cells) for 30min. Samples were normalized for 35S-methionine incorporation by scintillation counting.

For immunoprecipitations RIPA-extracts were incubated for 4h with the antibody prior to addition of ProteinG Sepharose for 16h. Beads were then washed with buffer B and eluted in SDS sample buffer. For sequential immunoprecipitation cells were lysed in buffer A. Incubation with the first antibody (anti-Herp or anti-p97) and ProteinG Sepharose for 16h was followed by a first wash with buffer B, followed by 30min incubation in RIPA buffer to dissociate ERAD complexes. Proteins still attached to the beads were analyzed by SDS-PAGE and autoradiography while supernatants were subjected to a second IP (anti-Hrd1) and analyzed accordingly. Western blotting was performed according to standard protocols using secondary antibodies from Seramun and the ECL-Plus detection kit (GE Healthcare). To analyze immunoprecipitations from non-labelled cell extracts by Western blotting, peroxidase-coupled primary antibodies or ProteinA-HRP (BioRad) were used.

In-vivo-ubiquitylation studies- Formation of His-tagged-ubiquitin conjugates was monitored as described (16). Briefly,
HeLa cells (1 10⁷) were transfected with NHK, 10HisUB-GFP, HERP, HERPΔUBL, HRD1-MYC or HRD1-M-MYC as indicated for 48h and treated with 10µM MG132 for 6h. After cell lysis using 6M guanidinium-HCl, ubiquitin conjugates were isolated using Ni-NTA-agarose, washed and analyzed by immunoblotting.

In the alternative approach 1 10⁷ HeLa cells were transfected with NHK, shHERP, shGFP, HERP, HERPΔUBL, HRD1-MYC or HRD1-M-MYC as indicated, cultured for 24h followed by MG132 treatment for 6h. Preparation of cell extracts, immunoprecipitation and Western blotting was performed as described above.

Glycerol gradient centrifugation- Cells (2 10⁷) were lysed in buffer A, loaded on a glycerol gradient (BufferA, but with 0.2% DeoxyBigCHAP, no inhibitors and 15-50% glycerol) and subjected to centrifugation in a Beckman SW40 rotor at 40,000rpm for 15h and 49min at 4°C.

RESULTS

Differential regulation of Herp and Hrd1 steady state levels and their enhanced association during ER stress. Upon treatment of cells with ER-stress-inducing agents such as thapsigargin and tunicamycin, both Herp and Hrd1 are induced by the UPR (4,5,17). As data from previous studies imply a more stringent regulation of Herp in comparison with Hrd1 (4,15,17-21), we set out to compare alterations in their protein levels and elucidate the formation of protein complexes containing both proteins upon exposure of cells to ER stress.

To this end, HeLa cells were treated with either thapsigargin or tunicamycin and cell extracts were subjected to immunoprecipitation and analyzed by Western blotting. As expected, Herp levels were low in untreated cells, but were boosted dramatically within 4h after exposure to thapsigargin or tunicamycin (Fig. 1A). In order to detect steady state levels of Hrd1 we had to perform Hrd1-specific immunoprecipitations, as available Hrd1 antibodies were insufficient to directly detect the protein in immunoblots of HeLa cell extracts. We found that in contrast to Herp the amount of Hrd1 remained virtually constant within 8h of treatment with thapsigargin or tunicamycin. However, the elevation of Herp levels detected upon addition of the reagents was accompanied by an enhanced coprecipitation of Herp with Hrd1 and vice versa.

In summary, exposure of cells to thapsigargin or tunicamycin led to a dramatic increase of Herp steady state levels and an enhanced formation of Herp and Hrd1 containing complexes.

Dynamics of Hrd1-associated Herp. To study the dynamics of the interaction between Herp and Hrd1, pulse chase experiments followed by co-immunoprecipitations were performed. These experiments enabled us to monitor the stability of Herp and its association with Hrd1 over time. As a control we used p97, which is known to be a stable protein that also interacts with Hrd1. HeLa cells were treated with thapsigargin for 6h to induce ER stress and the expression of UPR target proteins such as Herp and Hrd1. Then cells were metabolically labelled with 35S-methionine for 60min and analyzed immediately or after incubation for 3 and 6h with medium lacking radioactively labelled amino acids (Fig. 1B). We found that de novo synthesized Herp and Herp- or p97-associated Hrd1 were increased in cells exposed to thapsigargin, while p97 levels seemed not to be affected. Though Herp is rapidly degraded, p97 as well as Herp- or p97-associated Hrd1 species were stable for at least 6h. As this data indicated that labelled Herp associated with Hrd1 is replaced by de novo synthesized Herp, we used cycloheximide (CHX) to block translation and therefore prevent synthesis of Herp after pulse labelling. Indeed, addition of CHX resulted in a decreased coprecipitation of labelled Hrd1 with Herp, while the amount of Hrd1 coprecipitated with p97 remained constant for at least 6h. The data therefore suggest a continuous process in which de novo synthesized Herp binds pre-existing Hrd1 and is then degraded.

Herp binds to Hrd1 oligomers. In a previous study we have shown that in vitro, a cytosolic C-terminal fragment of Hrd1 is able to oligomerize and bind directly to an N-terminal fragment of Herp (6). As the presence of oligomeric Hrd1 would enable binding of multiple Herp molecules to a single Hrd1-complex, we asked whether Hrd1 forms oligomeric structures in the cell. To test this hypothesis we generated HeLa cell lines expressing Hrd1 with a C-terminal tandem tag containing RGS6His and an in vivo...
biotinylation site (Hrd1-HTB). Extracts from two Hrd1-HTB expressing cell clones (6 and 36) were subjected to immunoprecipitation with Hrd1-specific antiserum and streptavidin-agarose precipitation (Fig. 2A). As evident from the Western blots of the Hrd1 immunoprecipitates stained by the RGS-His antibody, levels of Hrd1-HTB in clone 6 were lower when compared to clone 36. Thus, the amount of the fusion protein in clone 6 was below the detection limit of the Hrd1 antiserum. In both clones levels of wt-Hrd1 were similar to the HeLa control and exceeded the level of Hrd1-HTB by far. Streptavidin-agarose precipitation of Hrd1-HTB resulted in coprecipitation of wt-Hrd1 indicating that both Hrd1 species are present in a common complex. Notably, in both clones the amount of wt-Hrd1 coprecipitated with Hrd1-HTB exceeded the amount of Hrd1-HTB considerably. Thus, we concluded that the number of Hrd1 molecules in a single complex is at least equal to the sum of the wt-Hrd1 and Hrd1-HTB amounts divided by the amount of Hrd1-HTB.

While similar amounts of Herp were co-precipitated with the Hrd1 antiserum from the HeLa control as well as from the transfectants, it was as expected absent from streptavidin-agarose-precipitates of the control cells. Due to higher amounts of Hrd1-HTB present in clone 36 as compared to clone 6, there was also an increased coprecipitation of wt-Hrd1 and Herp. To ensure that Hrd1-HTB is present only in high molecular mass complexes, we subjected extracts of Hrd1-HTB cells (clone 36) to glycerol gradient centrifugation followed by streptavidin-agarose precipitation (Fig. 2B). Hrd1-HTB was detected exclusively in high density fractions of the gradient, in which it was found to coprecipitate with endogenous Herp and p97. Therefore, Hrd1-HTB is completely integrated into high molecular mass complexes. As coprecipitation of p97 peaks between fractions 8 and 9, while the Hrd1 and Herp peaks are found in fractions 7 and 8, it is likely that there are different Hrd1-containing multimers, which vary with respect to their association with p97. In addition, we observed that Hrd1 proteins in complexes containing Hrd1-HTB were stable for at least 6h indicating that the tag did not interfere with their stability (Fig. 2C).

The data therefore suggest the existence of oligomeric complexes comprising multiple Hrd1 molecules that each contain a Herp-binding site.

**Herp promotes Hrd1-mediated ubiquitylation in a UBL-domain-dependent manner.** Considering the association of Herp with Hrd1 we reasoned that Herp might be required for the Hrd1-mediated ubiquitylation of substrate proteins. To test this hypothesis, we employed an in-vivo-ubiquitylation assay to analyze the effect of Herp on the ubiquitylation of alpha1-antitrypsin Hong-Kong (NHK), which has been identified as a substrate of Hrd1 (22). NHK was coexpressed with His-tagged ubiquitin and the accumulation of specific ubiquitin conjugates was monitored upon inhibition of the proteasome. Analysis of Ni-NTA-associated ubiquitin-conjugates using an alpha1-antitrypsin-specific antibody revealed that expression of Herp lacking the UBL domain (HerpΔUBL) led to an inhibition of NHK ubiquitylation when compared to wtHerp (Fig. 3A, compare lanes 4 and 5). The observed reduction of NHK ubiquitylation was similar to the effect observed upon expression of Hrd1C329S, a RING-domain-mutant of the E3 (17) (Fig. 3A, compare lanes 6 and 7).

An increase of endogenous Herp indicated that expression of NHK causes ER stress (Fig. 3A, compare lanes 1 and 2), which is reduced upon coexpression of the ubiquitin construct (Fig. 3A, lane 3). In the presence of Hrd1C329S further enhancement of endogenous Herp levels is most likely due to impaired Hrd1 function.

To validate the data from this experiment, we performed an inverse approach using an alpha1-antitrypsin-specific antibody to immunoprecipitate NHK from cells that were cotransfected with NHK and a HERP-specific shRNA construct (shHERP)(Fig. 3B). As observed in the previous experiment (Fig. 3A), there is a modest increase in endogenous Herp in the presence of NHK, which is further enhanced upon expression of Hrd1C329S. We found that the shRNA-mediated reduction of Herp levels is accompanied by a decrease in ubiquitylated NHK. Although transfection with HERP-specific shRNA resulted in a reduction of endogenous Herp already at 24h after transfection (Fig. 3B, compare lanes 2 and 3), it was not sufficient to efficiently inhibit expression of Herp from a plasmid. Therefore, we were able to rescue the shRNA effect by cotransfection of wtHERP,
while HERPΔUBL led to a further reduction of ubiquitylated NHK.

Differences between ubiquitylation patterns obtained by the different experimental setups are most likely due to the presence of the His-tag in the ubiquitin-construct used in the approach presented in Figure 3A.

Taken together, the data demonstrate that the UBL-domain of Herp is essential for the ubiquitylation of NHK detected upon inhibition of the 26S proteasome. Furthermore, our data confirm previous reports on the requirement of the Hrd1-activity for the ubiquitylation of NHK (22).

Herp is required for the Hrd1-mediated degradation of glycosylated NHK. As ubiquitylation of substrate proteins is necessary for their degradation by the proteasome, requirement of the Herp UBL domain for the ubiquitylation of NHK suggested that it is also important for the degradation of the substrate protein. To prove this hypothesis we monitored the degradation of NHK upon addition of tunicamycin, an inhibitor of N-glycosylation. As NHK is N-glycosylated and tunicamycin prevents this modification, addition of tunicamycin allowed us to monitor the fate of the glycosylated substrate pool.

Treatment of cells with tunicamycin led to a reduction of glycosylated NHK, which was accompanied by an increase of its non-glycosylated form (Fig. 4). To ensure that the observed non-glycosylated NHK species are synthesized de novo after the exposure of cells to tunicamycin, we performed a control experiment in which cycloheximide (CHX) was used to block translation. Indeed, when protein synthesis was inhibited by CHX, the non-glycosylated form of NHK was not detected. As expected, Herp levels were increased after 4h of treatment with tunicamycin, while inhibition of translation by CHX led to the opposite effect.

Inhibition of Herp synthesis with specific shRNA resulted in a stabilization of glycosylated NHK. Cotransfection with wtHERP restored substrate degradation, while cotransfection of HERPΔUBL failed to rescue the shRNA phenotype. In summary, the data confirmed our hypothesis that degradation of glycosylated NHK is dependent on the Herp-UBL-domain.

**DISCUSSION**

Components of the ERAD machinery such as Hrd1 are induced by the UPR to facilitate the disposal of aberrant proteins from the ER (4,5,17). Hrd1 is a central component of membrane-resident ERAD complexes connecting the ubiquitylation of ERAD substrates and their extraction to the cytosol (6,10-12). It therefore seems likely that rapid adaptation of substrate turnover to cellular requirements is achieved predominantly by varying the activity of ERAD complexes that are available at the time, rather than by regulating their abundance. As Herp binds Hrd1 and promotes the ubiquitylation activity of the E3, it is able to act as a regulator of Hrd1-dependent protein degradation. This hypothesis is further supported by our data on the dynamics of the association between Herp and Hrd1 upon exposure of cells to ER stress.

While the increase of Herp levels in cells exposed to ER stress is accompanied by an elevation of Herp-associated Hrd1, the steady state level of total Hrd1 appears to remain unchanged for at least 8h. In contrast, we observed an increase of Hrd1 synthesis within that period of time. However, it has been reported that HRD1 mRNA expression peaks at 6h of exposure to ER stress, while steady state levels of the Hrd1 protein are elevated only modestly with a peak between 12h and 24h (20). As Hrd1 is a stable protein displaying a half life of 15h (19), it is likely that the amount of Hrd1 synthesized de novo within 8h after induction by the UPR is considerably lower than the Hrd1 amount already present in the cell. The consequence is only a minor increase of the Hrd1 steady state level that is not detected in our experiments. As the half life of Herp is only 2h (15), its rapid induction by the UPR also leads to the dramatic increase of its steady state levels already 4h after exposure to ER stress. The enhanced association of Herp with Hrd1 then permits an increase of Hrd1-mediated ubiquitylation. Once the cell has overcome the stress situation, UPR signalling is disabled, which leads to a decrease in Herp-associated Hrd1 allowing a re-adjustment of Hrd1-dependent ubiquitylation to standard conditions.

Considering that Herp is able to bind Hrd1 directly (6) the presence of multiple Hrd1 molecules in a single complex may permit also binding of multiple Herp molecules to such a
complex. Thus, it seems possible that either occupation of all Herp binding sites on a Hrd1 ERAD complex is required for substrate ubiquitylation or the ubiquitylation activity of the complex is dependent on the number of Herp molecules bound, introducing a further level of regulation. As oligomerization of gp78, a homologue of Hrd1, as well as its yeast counterpart Hrd1p have recently been demonstrated to be crucial for the ubiquitylation of substrate proteins (23-25), it is likely that also Hrd1 is active only in an oligomeric state.

Previously, it has been shown that, when expressed in budding yeast, Herp is able to partially rescue the phenotype caused by the deletion of USA1, although no significant sequence similarity between both proteins was detected (26). Similar to Herp, also Usa1p contains a UBL domain and was found to associate with Hrd1p. The partial rescue of Usa1p deficient cells by Herp can be explained by the requirement of both proteins for ERAD of transmembrane substrates (6,7,24-28). However, the UBL-domain of Usa1p seems to be dispensable for the ubiquitylation and degradation of soluble ER-proteins (24,25,26,27), while our data demonstrate that the Herp UBL domain is crucial for the ubiquitylation and degradation of the soluble NHK. In addition, Usa1p is a stable protein that functions as a scaffold, crucial for the integrity of Hrd1p-based complexes (24,25,26), while the association of Herp with Hrd1 is highly dynamic. Thus, Herp and Usa1p differ with respect to these important functions and display therefore only limited functional similarities.

Taking into account that the Herp UBL domain is not required for Hrd1-binding (6), it is free to interact with other factors, which may be required for the ubiquitylation of specific Hrd1-substrates. Thus, it is conceivable that Herp acts as an adapter protein enabling Hrd1-mediated ubiquitylation. However, further studies will be required to identify interaction partners of the Herp UBL domain.

In conclusion, the current data favour a model in which ER stress induces increased binding of Herp to Hrd1 molecules present in ERAD complexes. This results in enhanced Hrd1-dependent ubiquitylation and rapid disposal of aberrant proteins to prevent the formation of toxic aggregates and apoptotic cell death.

REFERENCES


FOOTNOTES

*These authors contributed equally to this work.

We thank M. Kikkert, E. Wiertz and K. Hendil for providing antibodies, P. Kaiser and P. Paganetti for plasmids as well as F. Kriegenburg and W. Dubiel for critical reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SE 938/3).

The abbreviations used are: CHX, cycloheximide; E3, ubiquitin-protein ligase; ERAD, endoplasmic reticulum associated protein degradation; GFP, green-fluorescent protein; Herp/Herpud1, homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein; HTB, RGS6His TEV-protease-site biotinylation-sequence; NHK, alpha1-antitrypsin Null Hong Kong; shRNA, small hairpin RNA; UBL, ubiquitin domain protein

FIGURE LEGENDS

Fig. 1. Dynamics of Hrd1-associated Herp. A. Extracts of HeLa cells exposed to either 150nM thapsigargin (tg) or 10µg/ml tunicamycin (tu) for the indicated time were subjected to immunoprecipitation with preimmune serum (ctrl IP) or anti-serum specific for Hrd1 (Hrd1 IP) or Herp (Herp IP). Cell extracts and immunoprecipitated proteins were analyzed by Western blotting as indicated. GAPDH was used as a control to compare protein amounts of the extracts used for immunoprecipitation. An unspecific band detected with the Herp antiserum in the cell extracts is marked with an asterisk.

B. HeLa cells exposed to 150nM thapsigargin (tg) for 6h or untreated were pulse labelled with 35S-methionine for 60min. The chase was performed in the absence or presence of CHX for the indicated time. Cell extracts were subjected to immunoprecipitation (IP 1) as indicated (ctrl IP, Herp IP, p97 IP). Precipitates were washed with RIPA buffer to dissociate coprecipitated Hrd1 that was captured in a second immunoprecipitation using Hrd1 specific antiserum (IP 2 Hrd1). Samples were analyzed by SDS PAGE followed by autoradiography. Unspecific bands are marked with an asterisk.

Fig. 2. Oligomerization of Hrd1. A. HeLa cells (ctrl) and Hrd1-HTB cells (clone6, clone36) were lysed and subjected either to immunoprecipitation using Hrd1 antiserum (Hrd1 IP) or streptavidin agarose precipitation (strept. PD Hrd1-HTB). GAPDH was used as a control,
demonstrating similar protein concentrations in the cell extracts ( extr.). Precipitates were analyzed by Western blotting as indicated. A dash indicates the position of a 72kDa protein marker ( - ), one asterisk the position of wt-Herp ( * ) and two asterisks the position of Herp-HTB ( ** ). B. Hrd1-HTB cell extracts (clone 36) were subjected to glycerol gradient centrifugation. Fractions were incubated with streptavidin agarose to precipitate Hrd1-HTB containing complexes, which were analyzed by immunoblotting as indicated. For comparison sedimentation of PA28 (200kDa approx.) and proteasomes (700 to 3000 kDa approx.) is indicated by bars labelled accordingly C. Hrd1-HTB cells (clone 36) were treated with 50µg/ml CHX for the indicated periods of time. Cell extracts were subjected to streptavidin agarose precipitation. Precipitates were analyzed by immunoblotting as indicated. GAPDH was used as a loading control.

Fig. 3. The Herp UBL domain is required for the ubiquitylation of NHK. A. HeLa cells transfected with indicated constructs were cultured for 48h and treated with MG132 for 6h prior to harvesting. HRD1-M-MYC encodes the RING-domain mutant Hrd1C329S. Cell extracts were analyzed by immunoblotting with antibodies specific for α-tubulin, Herp, myc, alpha1-antitrypsin ( α1-AT ) or GFP as indicated. As the His-ubiquitin-GFP fusion protein (His-Ub-GFP) is cleaved at the ubiquitin N-terminus by endogenous ubiquitin specific hydrolases, GFP serves as transfection control. Extracts were subjected to Ni-NTA precipitation to capture His-tagged ubiquitin conjugates, which were analyzed by Western blotting using antibodies specific for ubiquitin and alpha1-antitrypsin ( α1-AT ). Bands marked by asterisks ( * ) may reflect unspecific His-Ub-independent binding of NHK to the beads, as they are not detected in cells that do not express the substrate (lane 1). Although also observed in other lanes, these bands appear to be pronounced in lane 2 and 5 due to higher steady state levels of NHK. B. Upon transfection with indicated constructs, HeLa cells were grown for 24h followed by treatment with MG132 for 6h. Cell extracts were then subjected to immunoprecipitation with an alpha1-antitrypsin-specific antibody ( α1-AT-IP ). Extracts and immunoprecipitated proteins were analysed by Western blotting using antibodies for GAPDH, peroxidase-coupled alpha1-antitrypsin ( α1-AT ), Herp, myc and peroxidase-coupled FK2 (ubiquitin). Apart from staining ubiquitylated NHK (ub-NHK), the ubiquitin antibody FK2 appears to crossreact with immunoglobulin heavy chains ( Ig ) from the alpha1-antitrypsin antibody used for the immunoprecipitation.

Fig. 4. Efficient degradation of NHK requires the UBL-domain of Herp. Hela cells were cotransfected with NHK and either GFP-specific shRNA (shGFP), HERP-specific shRNA (shHERP), HERP-specific shRNA and a plasmid encoding wt-Herp (shHERP + HERP) or HERPshRNA and a plasmid encoding HerpΔUBL (shHERP + HERPΔUBL). Upon 48h after transfection, the cells were exposed to 10µg/ml tunicamycin or 10µg/ml tunicamycin and 50µg/ml CHX as indicated. To identify glycosylated NHK species, cell extracts were treated with 1000units of PNGaseF (PNGase). GAPDH, Herp and HerpΔUBL as well as glycosylated (NHK+CHO) and non-glycosylated (NHK-CHO) NHK were detected by immunoblotting. Bands of glycosylated NHK were further analyzed by densitometry, normalized to GAPDH and plotted. Error bars represent the standard deviation ( n=3 ).
Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>tg</th>
<th>tu</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Western Blot

<table>
<thead>
<tr>
<th></th>
<th>ctrl IP</th>
<th>Herp IP</th>
<th>Hrd1 IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hrd1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>IP 1</th>
<th>ctrl IP</th>
<th>Herp IP</th>
<th>p97 IP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hrd1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hrd1

- p97
- Herp
- Hrd1

H chase

- + + + + + + + + - + + + + + + + + - + + + + + + + + + + CHX
Figure 3

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-Ub-GFP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NHK</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HERP</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HERPΔUBL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HRD1-MYC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HRD1-M-MYC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Condition</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHK</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>shGFP</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>shHERP</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HERP</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HERPΔUBL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HRD1-MYC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HRD1-M-MYC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Herp regulates HRD1-mediated ubiquitylation in a ubiquitin-like-domain-dependent manner
Melanie Kny, Sybille Standera, Rasmus Hartmann-Petersen, Peter-Michael Kloetzel and Michael Seeger

J. Biol. Chem. published online December 13, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.134551

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts