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A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1☆

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Abstract

The yeast hHrd1 is a ubiquitin-protein ligase (E3) involved in ER-associated degradation. It was originally identified by genetic methods as an E3 of the yeast cholesterol biosynthetic enzyme HMG-CoA reductase (HMGR). We report the identification and cloning of a human homologue of Hrd1 (hHrd1). Immunofluorescence imaging confirms that the endogenous hHrd1 resides in the ER and in vitro assay demonstrates that it has a ubiquitin-ligase activity. However, the homology between the human and yeast Hrd1 is limited to the N-terminal domain of the proteins, and hHrd1 does not appear to be involved in the degradation of mammalian HMGR.

Keywords: Ubiquitin-protein ligase; Endoplasmic reticulum-associated degradation; HMG-CoA reductase; Cystic fibrosis transmembrane conductance regulator; hHrd1

The ubiquitin system plays a central role in regulated degradation of cellular proteins under different physiological conditions. It also eliminates misfolded or incompletely assembled proteins of the secretory pathway, as part of quality control mechanism [1–3].

Proteins are marked to degradation by covalent binding of multiple moieties of ubiquitin molecules, to generate a polyubiquitin chain. The protein-ubiquitin conjugate is recognized as a substrate by the 26S proteasome complex that cleaves the protein into peptides and releases free ubiquitin [2].

Three enzymes, E1, E2, and E3, operate in concert with conjugate ubiquitin to the target substrate. While a single E1 participates in all ubiquitination processes, several E2s and multiple E3s mediate ubiquitination of the different cellular targets [2,4].

E2s, also known as ubiquitin-conjugating enzymes (UBCs), serve as carriers of the ubiquitin molecules from E1 to the target proteins with the mediation of E3s. The E2s transfer the ubiquitin molecule by covalently binding it through a conserved internal Cys residue [4].

E3s, also known as ubiquitin-protein ligases, play the most crucial role in determining the specificity of the ubiquitination process. They may operate in complex with other proteins and recognize specific motives in their targets. Regulation of the ubiquitination process is usually achieved by modification of the E3 complex and/or the target protein. Recently it has been recognized that many of the familiar E3s contain a structural motif known as RING finger. This motif consists of a consensus sequence CX2CX(9-39)CX(1-3)HX(2-3)C/HX2CX(4-48)CX 2C that binds two zinc ions through the Cys or His residues and forms a structure that interacts with other

☆ *Abbreviations:* ER, endoplasmic reticulum; ERAD, ER-associated degradation; UBC, ubiquitin-carrier proteins; HRD, hydroxymethylglutaryl reductase degradation; HMGR, hydroxymethylglutaryl-CoA reductase; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; WT, wild-type; Ub, ubiquitin; DOX, doxycycline; CH, cycloheximide.

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proteins. The role of the RING finger in E3s is not clear yet and it is believed that it interacts and recruits the E2 [5–7].

Much effort is being dedicated lately to elucidate the mechanisms that underlie the degradation of ER-proteins, as it became evident that they are substrates to the ubiquitin–proteasome machinery. ER-associated degradation (ERAD) refers to degradation of ER resident proteins, feedback-regulated degradation, and quality control processes [1].

HMG-CoA reductase (HMGR) represents a well-studied example of feedback-regulated ERAD. HMGR is anchored in the ER-membrane and functions as the key enzyme in the biosynthetic pathway of cholesterol and other derivatives of mevalonate. The level of HMGR is regulated by a feedback loop that responds to the level of the end products. When cells are deprived of cholesterol, HMGR is stabilized and its level is high, but when cholesterol is abundant HMGR is rapidly degraded and its level decreases [8]. The regulated degradation requires the N-terminus half of the protein and it is carried out by the ubiquitin–proteasome system [9,10].

The regulated degradation of HMGR has been investigated mainly in yeast. Hampton and colleagues have shown that the yeast ubiquitin ligase Hrd1 [11] is required for ubiquitination and degradation of HMGR, as well as in quality control degradation of other misfolded ER proteins [12]. Hrd1 is anchored in the ER membrane by six transmembrane domains and it contains a RING finger motif at the C-terminal domain which faces the cytosol. The RING is essential for Hrd1 activity, as substitution of a conserved Cys residue in the RING abrogates ubiquitination [13].

The cystic fibrosis transmembrane conductance regulator (CFTR) is a clinically important ERAD substrate. Only 25–50% of the wild-type protein matures to the cell surface, whereas most of the molecules do not fold properly and are subjected to ERAD. A single mutation in CFTR, ΔF508, is the underlying cause of most cases of cystic fibrosis (CF). CFTRΔF508 protein is inefficiently folded and fails to be glycosylated and delivered to the plasma membrane [14]. The immature CFTR molecules in the ER membrane are rapidly degraded by the ubiquitin–proteasome system [15].

In the course of studying the regulated degradation of HMGR by the ubiquitin system, we aimed to identify its specific ubiquitin-protein ligase (E3). We identified and cloned the human homologue of the yeast HRD1. While our study was in its final stages of writing, Kaneko and colleagues [16] have reported the cloning of the human Hrd1 (hHrd1) and the exploring of its involvement in unfolded protein response and stress-induced apoptosis. In this paper we further characterize hHrd1 and examine its involvement in the regulated degradation of mammalian HMGR and CFTR.

Materials and methods

Cloning of hHRD1. The amino acid sequence of the yeast Hrd1 protein (Z74755, GI:1419784) was subjected to homology search by the bioinformatic tool Gencarta (Compugen). The search yielded a predicted sequence of 941 nucleotides, which is composed of two ESTs (expressed sequence tags). The predicted sequence has 54% similarity and 41% identity to a segment of the yeast Hrd1p that includes the RING finger motif. The full-length cDNA was isolated in a Rapid Amplification of cDNA Ends (RACE) process from a cDNA library of human placenta (Clontech Laboratories). Subcloning during the process was performed with Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The full-length mRNA was ~2.5 kb long with ORF of 1851 bases. The coding region of hHRD1 was amplified by PCR as a single DNA transcript using primers that contain the following ends of the mRNA: 5′-ATGTTCCGCACGGCAGTGATG-3′ at the N terminus and 5′-GTGGGCAACAGGAGACTC-3′ at the C terminus. Using the identified cDNA sequence, the full genomic sequence was found in the Human Genome Database, located on chromosome 11 (GI:8118762 and GI:7770682). The coding sequence of hHrd1 was cloned into pcDNA3 vector, fused to a 6× His tag at the C-terminal end of the protein.

In vitro ubiquitination system. Soluble hHrd1 (S-hHrd1) lacks the first 233 amino acid residues that span the transmembrane domains. It contains the RING finger domain and the C-terminal catalytic site, and is fused to a 6× His tag. S-hHrd1 was cloned into pET28α vector, expressed in bacteria (BL21), and purified to homogeneity by Ni–NTA His Bind Resin (Novagen) according to the manufacturer's instructions. S-hHrd1(C291S) was generated using QuickChange site directed mutagenesis kit (Stratagene). UbcH5c and E2-25K were cloned in pT7-7 vectors, fused to a 6× His tag that was expressed in bacteria, and purified to homogeneity by Ni–NTA His Bind Resin [17]. Human E1 [18] was subcloned into baculovirus expressing system and purified with ubiquitin affinity column [19].

Assays for in vitro ubiquitination were performed in 24 μl reaction mixture that contained 40 mM Tris–HCl, pH 7.6, 2.5 mM $MgCl_2$, 0.5 mM DTT, and 2 mM ATP. The following purified components were added: 0.2 μg E1, 0.5 μg UbcH5c, 4 μg ubiquitin, and 1 μg S-hHrd1. The reactions were incubated for 30 min at 37 °C.

Cells, media, and transfections. HeLa and Tet-On CHO (Clontech) cells were cultured in DMEM with 10% FBS (Biological Industries, Israel). Cholesterol-deprived conditions were obtained by substitution of FBS with lipoprotein-deficient serum (LPDS) and addition of 1 μM lovastatin (kindly given by Merck). LPDS was prepared by increasing the serum density to 1.25 g/ml (by the addition of 0.398 g KBr/ml serum) followed by ultracentrifugation at 100,000*g* for 48 h. The upper layer that contains the lipoproteins was discarded and the serum was extensively dialyzed to remove KBr. High-cholesterol medium contains 2.5 μM hydroxy-cholesterol and 2 mM sodium mevalonate. pCMV-CHA-CFTR WT and pCMVCHA-CFTRΔF508 plasmids were kindly provided by Dr. J.M. Rommens. (University of Toronto, Canada) and are described in [20]. Transfections were carried out using FuGENE 6 (Roche) according to manufacturer's instructions.

Immunofluorescence. HeLa cells grown on glass slides were rinsed three times with PBS and fixed for 10 min in PBS containing 3.7% paraformaldehyde and 0.18% Triton X, followed by extensive rinsing with PBS. Cells were blocked for 30 min with PBS containing 5% goat serum and 0.1% gelatin, and incubated with primary and secondary antibodies.

Preparation of cell lysates and Western blotting. Samples and solutions were maintained in 4 °C throughout the procedure. Cells were thoroughly rinsed with PBS, collected by scraping, and centrifuged at 400*g* for 10 min. Cells were resuspended in lysis buffer (PBS, 1%NP-40, 1%NaDOC, 5 mM EDTA, 5 mM EGTA, and 2.5 μg/ul calpain inhibitor I, and protease inhibitor cocktail of Roche), and sonicated for 10 s in a bath sonicator. Samples were clarified by 10 min centrifugation at 12,000*g* and transferred to clean vials. Protein concentration was

determined according to Bardford [21]. Samples were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and incubated with the appropriate antibodies.

Antibodies. Polyclonal anti-hHrd1 antibody was raised against the peptide sequence spanning residues 598–617 (C)PDAAELRRRRLQ KLESPVAH (Alpha Diagnostics International, Texas). Anti-ubiquitin antibody (P4G7) was purchased from Covance Research Products. Anti-6× His peroxidase and anti-HA peroxidase antibodies were purchased from Roche. Monoclonal antibody against HMGR (IgG-A9 ATCC# CRL1811) was kindly provided by Dr. J. Goldstein (University of Texas, Dallas). Anti-calnexin and anti-BiP monoclonal antibodies were purchased from Transduction Laboratory and FITC antibodies were purchased from Jackson ImmunoResearch Laboratories.

Results and discussion

Characterization of hHRD1 protein

hHrd1 is 617 amino acid long protein, predicted to be anchored in the ER by six transmembrane regions with a type 3a topology. The RING finger motif is located between residues 291 and 329 (Fig. 1A). hHrd1 contains proline-rich clusters which are not found in the yeast Hrd1 but characterize the Cbl E3 family [22,23] (Fig. 1A). Proline clusters are known to be important in

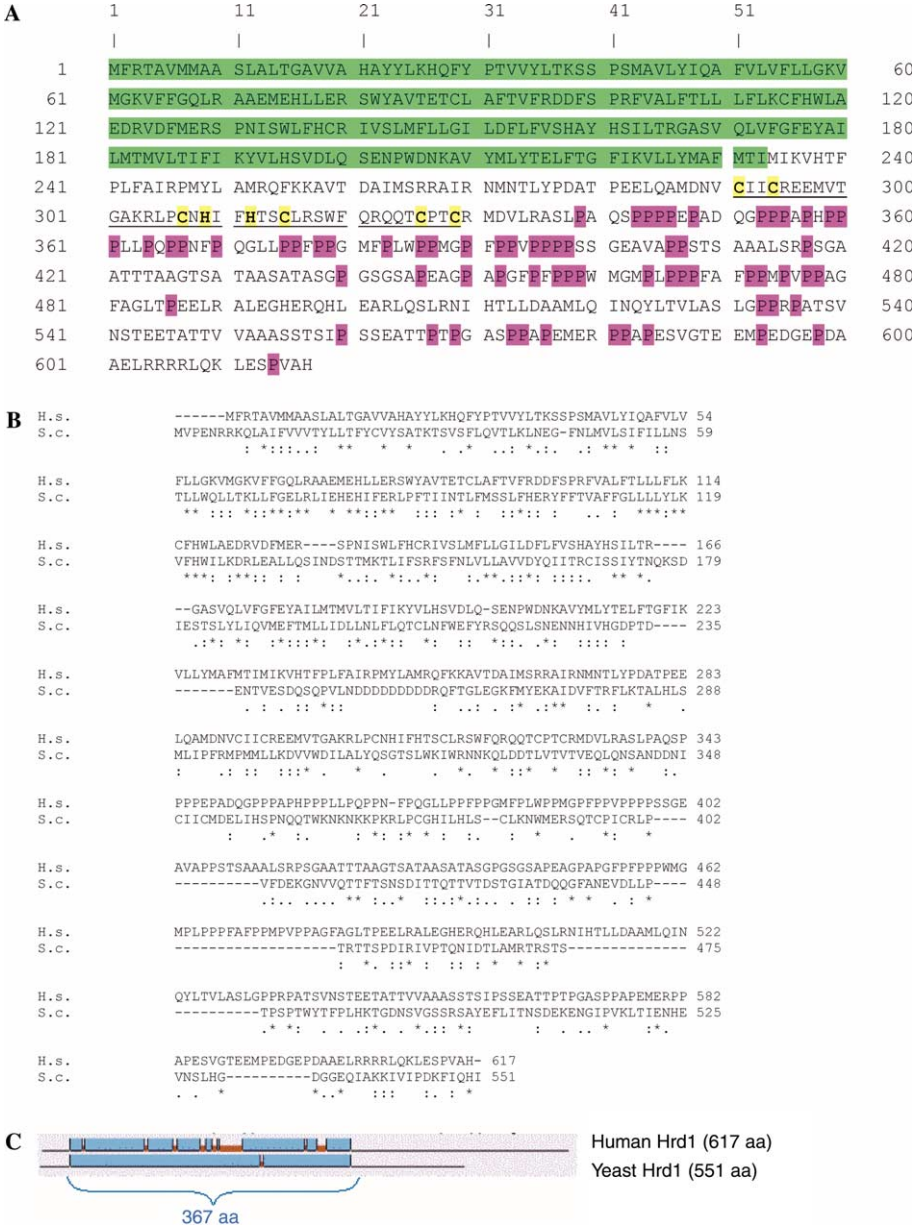

Fig. 1. Amino acid sequence and motives of hHrd1. (A) The predicted membrane-anchored segment of the protein is marked with green. The RING finger is underlined and its active C/H are marked with yellow. All the Pro residues downstream to the RING finger are marked with purple. (B) Amino acid alignment of *Homo sapiens* (H.s.) and *Saccharomyces cerevisiae* (S.c.) Hrd1. "*" Denotes identical residues, ":" denotes conserved substitution, and denotes semi-conserved substitution. (C) Schematic view of the comparison between hHrd1 and yeast Hrd1. Blue boxes represent identical or similar amino acids and red lines represent gaps.

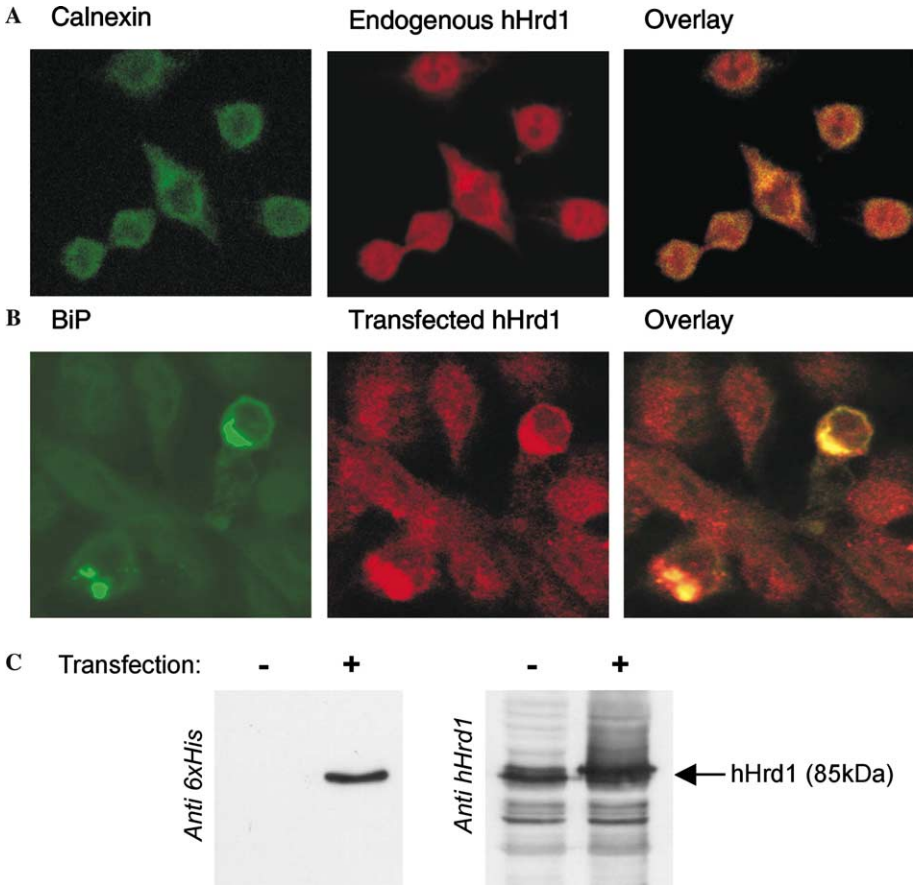

Fig. 2. Endogenous and transfected hHrd1. (A) Immunofluorescence of endogenous hHrd1 and Calnexin. (B) Immunofluorescence of transfected hHrd1 with anti-6× His antibody and BiP. (C) HeLa cells were transfected with either hHrd1 (+) or empty vector (−). Endogenous (anti-hHrd1) and transfected (anti-6× His) hHrd1 were detected by Western blot.

protein–protein recognition via interaction with various domains, including Src homology 3 (SH3) and WW. Moreover, the proline clusters of the E3 Cbl-b were shown to be necessary for the binding and ubiquitination of the p85 regulatory subunit of PI3-K [22,24]. Comparison of the homology between the hHrd1 and the yeast Hrd1 (Z74755, GI:1419784) reveals that it is confined to the N-terminal fragment (amino acids 1–370 in hHrd1), which includes most of the transmembrane region and the RING finger domain. The overall identity of the two proteins is 24% and similarity is 43% (Figs. 2B and C). The limited homology suggests that the two proteins may be involved in recognition of distinct substrates. Computational prediction indicates that hHrd1 has a cleavable signal peptide (1–17) and the theoretical p*I* is 6.5.

Intracellular localization and tissue distribution of hHrd1

Anti-peptide polyclonal antibody was prepared based on the sequence of the cloned hHrd1. This antibody was used for immunofluorescence imaging to identify endogenous hHrd. The intracellular distribution of endogenous hHrd1 has an ER-pattern and it is co-localized with Calnexin which serves as a marker (Fig. 2A). Similarly, the transfected hHrd1 is also localized to the ER, as indicated by the marker BiP (Fig. 2B).

Endogenous hHrd1 migrates as a 85 kDa protein in SDS–PAGE. Transfection of the 6× His tagged hHrd1 yields a protein that migrates in an identical manner to the endogenous protein (Fig. 2C).

hHrd1 was screened in several human tissue preparations (liver, brain, lung, kidney, spleen, testis, ovary, heart, and pancreas) by immunoblotting and was found to reside in all of them (Fig. 3). Not surprisingly, it is extremely abundant in the liver, an organ highly involved in the synthesis of secreted proteins. The kidney is also rich in Hrd1, which may be due to its extensive turnover of membrane pump subunits and ion channels, including ENaC. While Hrd1 is clearly not the E3 of ENaC, which is targeted by NEDD4 [25], the ER of the kidney must be involved in quality control of ensuring tight regulation of these proteins.

hHrd1 was also searched in mice liver and spleen preparations and in several mammalian cell lines. In all cases, it was detected as a 85 kDa protein (data not shown).

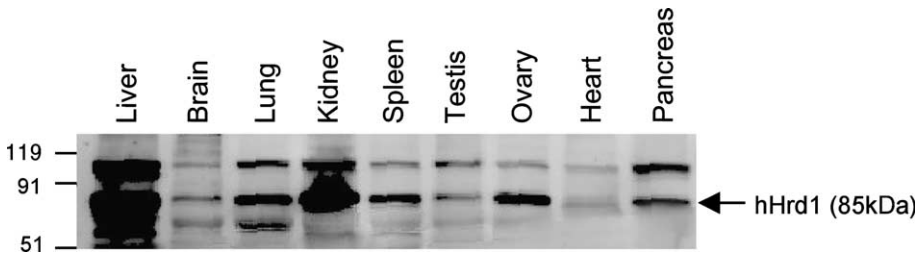

Fig. 3. Western blot analysis of multiple human tissues with anti-hHrd1 antibody. A pre-made nitrocellulose blot of protein extracts from the indicated human tissues (Geno Technology) was subjected to Western blot as described in "Materials and methods."

hHrd1 catalyzes self-ubiquitination in vitro

RING finger ubiquitin-protein ligases have the ability to catalyze in vitro the transfer of ubiquitin from E2s to target proteins or to themselves (self-ubiquitination) [26]. In order to directly assess the activity of hHrd1, S-hHrd1 was incubated in an in vitro ubiquitination system as described in "Materials and methods." The reaction products were identified by Western blot with anti-ubiquitin antibody (Fig. 4A, upper panel) or anti-6× His antibody (Fig. 4A, lower panel). hHrd1 was found to be considerably active in self-ubiquitination. The appearance of ubiquitin conjugates corresponded to the decrease in free hHrd1. Exclusion of any reaction component completely abrogated the in vitro reaction (-E1, -E2, -Ub, -ATP, and -hHrd1).

To demonstrate that the ubiquitination depends on an active RING finger, a RING-mutated S-hHrd1, S-hHrd1(C291S), was tested. When S-hHrd1(C291S) was incubated in the in vitro ubiquitination system, no ubiquitination activity could be detected (Fig. 4B).

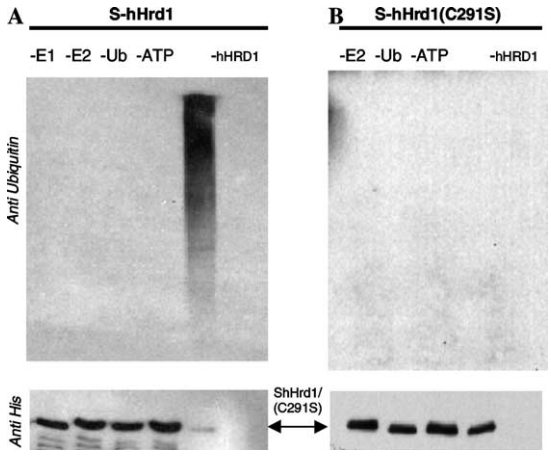

Fig. 4. Self-ubiquitination of hHrd1 in vitro. Purified recombinant E1 and E2 (UbcH5c), purified ubiquitin,and reaction buffer containing ATP were incubated with S-hHrd1 (A) or with S-hHrd1(C291S) (B) for 30 min at 37 °C as described in "Materials and methods." As indicated in the figure, one of the components was excluded each of the control reactions. Following incubation, equal aliquots from each reaction were subjected to Western blot analysis with anti-ubiquitin (upper panel) and anti-6× His tag (lower panel) antibodies.

A similar in vitro ubiquitination assay was performed with purified E2-25K instead of UbcH5c. In this experiment, the level of ubiquitination was significantly lower (data not shown).

hHrd1 is not involved in the degradation of HMGR or CFTR

As described above, our initial identification of hHrd1 was based on homology analysis with the yeast Hrd1. In yeast, Hrd1 was shown to be the specific ubiquitin-protein ligase of HMGR [12]. Thus, we investigated the hypothesis that hHrd1 serves as the specific ubiquitin-protein ligase of HMGR in mammals.

The degradation of HMGR is regulated by the cellular level of cholesterol. When the level of cholesterol is low, HMGR is stabilized and its cellular level is high. Supplementation of cholesterol results in raid degradation of HMGR by the ubiquitin–proteasome system [8]. This regulated degradation was tested in the presence of wild-type or RING-mutated C291S hHrd1 (Fig. 5). C291S hHrd1 cannot recruit the E2 component of the ubiquitination machinery, but can bind the substrate and is therefore expected to stabilize it (dominant negative effect). Overexpression of the wild-type hHrd1 did not accelerate cholesterol-induced degradation of HMGR, and the mutant enzyme did not stabilize it (Fig. 5).

A different experimental approach was to test the effect of hHrd1 on the steady-state level of HMGR. For that purpose, cell lines expressing inducible wild-type or C291S hHrd1 (Tet-On system, Clontech) were prepared. Wild-type hHrd1 was tested for its ability to reduce HMGR level in cholesterol-deprived conditions, in which the level of HMGR is high. In contrast, C291S hHrd1 was tested for its ability to increase HMGR level in high-cholesterol medium, conditions under which the level of HMGR is low ('dominant negative' effect). As shown in Fig. 6, induction of hHrd1 expression did not affect the steady-state level of HMGR.

We tested the possibility that hHrd1 serves as ubiquitin-protein ligase of CFTR. We monitored the degradation of CFTRΔF508 in Tet-On cells, expressing inducible wild-type or C291S hHrd1 (Fig. 7). The presence of either wild-type or C291S hHrd1 did not affect

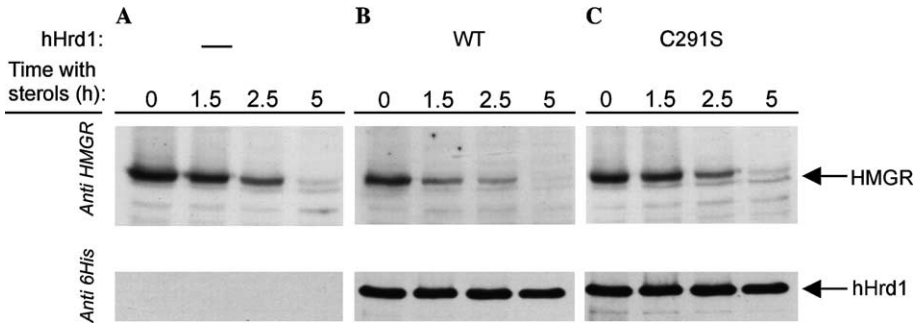

Fig. 5. Cholesterol-induced degradation of HMGR in the presence of hHrd1. HeLa cells were transfected with either empty vector (A), 6× His tagged wild-type hHrd1 (B) or C291S mutated hHrd1 (C). After 24 h, cells were cultured in a cholesterol-deprived medium for additional 24 h. Degradation of HMGR was induced by the addition of hydroxy-cholesterol (2.5 μM) and sodium mevalonate (2 mM) 1.5, 2.5 or 5 h prior to harvesting the cells. Equal protein amounts of cell lysates were subjected to Western blot analysis with anti-HMGR (upper panel) and anti-6× His (lower panel) antibodies.

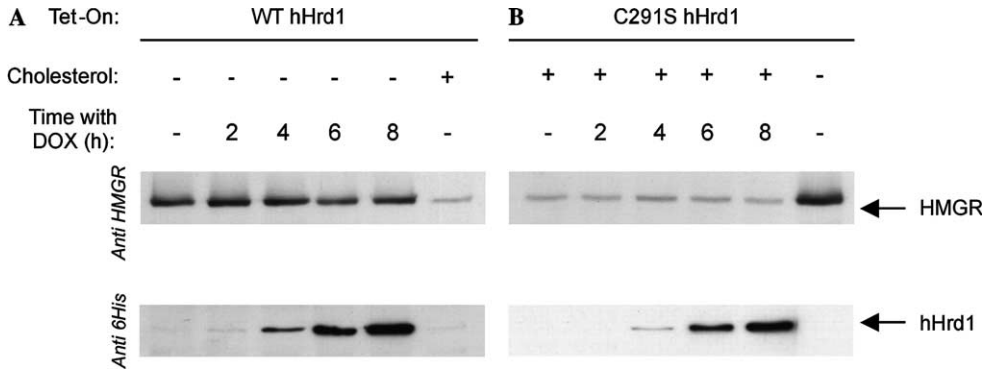

Fig. 6. Induced expression of hHrd1 does not affect HMGR steady-state levels. (A) Tet-On CHO cells expressing doxycycline-induced (DOX) 6× His tagged wild-type hHrd1 were cultured in cholesterol-deprived conditions (−). The right lane is a control dish that was cultured in high-cholesterol medium (+). After 24 h, expression of hHrd1 was induced by addition of DOX (1 μM) for the indicated time periods. The levels of HMGR and induced hHrd1 were detected by Western blots. (B) Tet-On CHO cells expressing DOX-induced 6× His tagged C291S hHrd1 were cultured in high-cholesterol medium (+). The right lane is a control dish that was cultured in cholesterol-deprived conditions (−). After 24 h, expression of C291S hHrd1 was induced by addition of DOX (1 μM) for the indicated time periods. The levels of HMGR and induced hHrd1 were detected by Western blots.

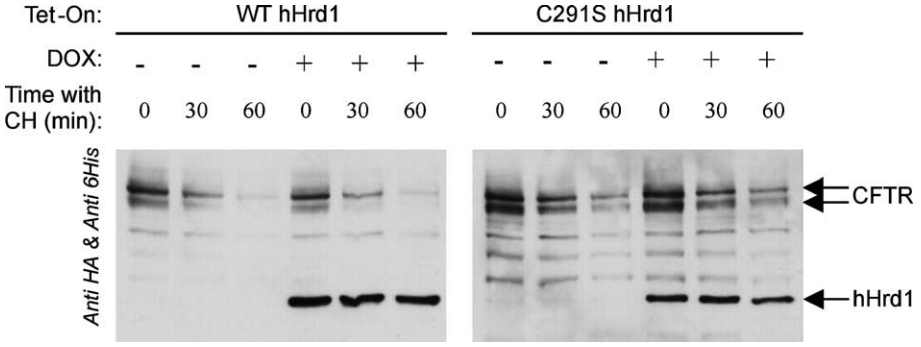

Fig. 7. Degradation of CFTRΔF508 in the presence of hHrd1. Tet-On CHO cells expressing either inducible 6× His wild-type or C291S hHrd1 were transiently transfected with HA tagged CFTRΔF508. After 24 h, DOX (1 μM) was added as indicated and the cells were further cultured for additional 24 h. To monitor the kinetics of CFTRΔF508 degradation, cycloheximide (CH) was added (100 μg/ml) 30 or 60 min prior to harvesting. Equal protein amounts of cell lysates were subjected to Western blot with anti-HA (CFTR) and anti-6× His (hHrd1) antibodies.

the kinetics of CFTR degradation. Experiments with wild-type CFTR or transfections to other cell types led to the same conclusion (data not shown).

The role of the human homologue of the yeast Hrd1 remains unknown. While initial results indicated its involvement in UPR [16], it is clear that the enzyme is not

involved in degradation of all misfolded proteins, as CFTR remains unstable in the presence of inactive hHrd1. The important challenges now are to identify the E3(s) involved in HMGR and CFTR degradation and to reveal which are the substrates of hHrd1.

Acknowledgments

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