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A ubiquitin ligase HRD1 promotes the degradation of Pael receptor, a substrate of Parkin

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Abbreviated title: Pael-R is a substrate of HRD1

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ABBREVIATIONS

PD, Parkinson's disease; AR-JP, autosomal recessive juvenile parkinsonism; ER, endoplasmic reticulum; ERAD, ER-associated degradation; E3, ubiquitin ligase; E2, ubiquitin-conjugating enzyme; Pael-R, Parkin-associated endothelin receptor-like receptor; HRD, HMG-CoA reductase degradation; RA, rheumatoid arthritis, SEL1, suppressor or enhancer of lin-12; SNC, substantia nigra pars compacta; UPR, unfolded protein response; XBP1, X-box binding protein 1; ATF6, activating transcription factor 6; UPS, ubiquitin-proteasome system

KEYWORDS

ubiquitin ligase; endoplasmic reticulum-associated degradation (ERAD); Parkinson's disease; endoplasmic reticulum stress; unfolded protein response (UPR); Pael-R; HRD1; ATF6

ABSTRACT

It has been proposed that in autosomal recessive juvenile parkinsonism (AR-JP), a ubiquitin ligase (E3) Parkin, which is involved in endoplasmic reticulum (ER)-associated degradation (ERAD), lacks E3 activity. The resulting accumulation of Parkin-associated endothelin receptor-like receptor (Pael-R), a substrate of Parkin, leads to ER stress, causing neuronal death. We previously reported that human E3 HRD1 in the ER protects against ER stress-induced apoptosis. This study shows that (1) HRD1 was expressed in substantia nigra pars compacta (SNC) dopaminergic neurons and (2) interacted with Pael-R through the HRD1 proline-rich region, promoting the ubiquitylation and degradation of Pael-R. Furthermore, the disruption of endogenous HRD1 by small interfering RNA (siRNA) induced Pael-R accumulation and caspase-3 activation. We also found that ATF6 overexpression, which induced HRD1, accelerated and caused Pael-R degradation; the suppression of HRD1 expression by siRNA partially prevents this degradation. These results suggest that in addition to Parkin, HRD1 is also involved in the degradation of Pael-R.

INTRODUCTION

Parkinson's disease (PD) is the most common movement disorder and the second most common neurodegenerative disease. Most PD patients are nonfamilial; only approximately 5% are familial. Autosomal recessive juvenile parkinsonism (AR-JP) occurs with increasing frequency in familial PD patients and results from parkin gene (PARK2) mutations (Kitada et al., 1998). In AR-JP patients, the loss of dopaminergic neurons and the appearance of parkinsonism symptoms occur without the formation of Lewy bodies, which are a significant characteristic of nonfamilial and some familial PD cases (Mizuno et al., 1998).

Eukaryotic cells coordinate the folding and glycosylation of secretary and membrane proteins in the endoplasmic reticulum (ER). Various stresses leading to impairment of the ER and the production of mutant proteins cause the accumulation of unfolded proteins in the ER, culminating in cell death. Unfolded proteins accumulated in the ER are degraded by the ubiquitin-proteasome system (UPS). In this ER system, termed ER-associated degradation (ERAD), unfolded proteins are initially retrotranslocated from the ER to the cytosol through the translocon, polyubiquitylated by ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), and other components, and degraded by the 26S proteasome (Hershko and Ciechanover, 1998). E3 plays an important role in the ubiquitylation of unfolded proteins, and the RING finger domain of E3 mediates the transfer of ubiquitin from E2 to substrates (Zheng et al., 2000).

Parkin is an E3 that contains two RING finger domains; AR-JP-linked Parkin

mutants have defective E3 activity. Parkin is upregulated in response to ER stress and protects against cell death caused by such stress, suggesting that it is an E3 involved in ERAD. Parkin-associated endothelin receptor-like receptor (Pael-R) has been identified as a protein that interacts with Parkin; its accumulation leads to ER stress-induced cell death. Parkin ubiquitinates and promotes the degradation of insoluble Pael-R, resulting in the suppression of cell death (Imai et al., 2001). In other words, ER stress caused by the accumulation of unfolded Pael-R might be involved in AR-JP. Furthermore, it has been recently reported that Pael-R in PD is accumulated in the core of Lewy bodies (Murakami et al., 2004) and that selective dopaminergic neurodegeneration is caused by the ectopic expression of human Pael-R in the Drosophila brain (Yang et al., 2003).

It is known that in yeast, Hrd1p/Der3p is involved in ERAD. Hrd1/Der3 is localized in the ER, encodes the RING-finger domain at the C-terminus, and ubiquitinates substrates including HMG-CoA reductase (Hmg2p) (Gardner et al., 2000, 2001; Deak and Wolf, 2001). Hrd3p is reported to regulate or stabilize Hrd1p (Plemper et al., 1999; Deak and Wolf, 2001). ER stress induces various components involved in ERAD, including Hrd1p as well as ER molecular chaperones, suggesting that ERAD involves the degradation of unfolded proteins in cooperation with ER chaperones (Travers et al., 2000; Friedlander et al., 2000). We previously reported that human HRD1 was identified and characterized as a human homolog of yeast Hrd1p (Kaneko et al., 2002). In the report, we demonstrated that HRD1 possesses E3 activity,

is induced during ER stress, and suppresses cell death caused by ER stress. Furthermore, human HRD1 is reportedly involved in the basal, and not the sterol-regulated, degradation of HMG-CoA reductase (Nadav et al., 2003; Kikkert et al., 2004) and is a pathogenic factor in rheumatoid arthritis (RA) (Amano et al., 2003).

The unfolded protein response (UPR) is required for the inhibition of further protein synthesis and the induction of ER chaperones, which reduce the number of unfolded proteins in the ER (Kaufman et al., 1999, 2002). Transcription factor ATF6 is a transmembrane protein localized in the ER (Haze et al., 1999). Under ER stress, ATF6 is cleaved to release the N-terminal fragment on the cytosolic side of the membrane; it then enters the nucleus, acts as a transcription factor, and eventually activates ER chaperone gene transcription, which enhances protein folding (Haze et al., 1999; Ye et al., 2000; Shen et. al, 2002). On the other hand, an ER-resident transmembrane protein IRE1, which possesses serine/threonine kinase and RNase domains, is dimerized and autophosphorylated during ER stress (Cox et al., 1993; Sidrauski et al., 1997). Activated IRE1 splices XBP1 mRNA and then generates an active form of XBP1 (Yoshida et al., 2001).

Recent studies have demonstrated that Parkin knockout mice exhibit no significant change in either dopaminergic neurodegeneration or the accumulation of any Parkin substrates (Itier et al., 2003; Goldberg et al., 2003; von Coelln et al., 2004; Palacino et al., 2004; Periquet et al., 2005; Perez & Palmiter, 2005), suggesting that other unknown E3s can degrade accumulated proteins in the absence of Parkin. On the

other hand, HRD1 apparently degrades a number of unfolded proteins since overexpressed HRD1 protects against ER stress-induced cell death. This study showed that human HRD1 was located in substantia nigra pars compacta (SNC) neurons in the mouse brain. Therefore, we hypothesized that HRD1 as well as Parkin ubiquitinates and degrades the unfolded Pael-R responsible for ER stress and protects against Pael-R-induced cell death. In addition, we investigated whether ATF6-induced UPR activation promotes the degradation of Pael-R and whether UPR-induced HRD1 expression is partially involved in this degradation.

MATERIALS AND METHODS

Constructs — The expression vector for human wild-type and truncated fragments of HRD1 was tagged with myc and polyhistidine (6 × His) epitopes at the C-terminus of the inserted sequence (pcDNA6; Invitrogen Corporation, Carlsbad, CA, USA). Human Pael-R (pcDNA3), tagged with FLAG and 6 × His epitopes at the C-terminus, was a gift from Ryosuke Takahashi (RIKEN Brain Science Institute, Japan). The expression vector for wild-type human α-synuclein, tagged with hemagglutinin (HA) and 6 × His epitopes at the C-terminus, was cloned into expression vector pcDNA3.1 (Invitrogen). The expression vector for the cleaved form of ATF6 (amino acid region 1-373 of ATF6α), tagged with HA epitopes at the N-terminus was cloned into expression vector pCR3.1 (Invitrogen). The expression vector for RP-HRD1 fused at its N-terminus to glutathione S-transferase (GST) was cloned into the expression vector pGEX6p-1 (GE Healthcare Bio-sciences, Piscataway, NJ, USA).

Antibodies, chemicals, and proteins — An affinity-purified HRD1 polyclonal antibody against the KLH-conjugated synthetic peptide (C)-EDGEPDAAELRRR, corresponding to amino acid residues 594-606 of human HRD1 protein, was recognized human and mouse HRD1 (gifted from Otsuka GEN Research Institute). We also purchased anti-HRD1 polyclonal antibody (C-term) from Abgent, San Diego, CA, USA. Anti-Pael-R polyclonal antibody was used as described (Imai et al., 2001). Anti-FLAG M2 polyclonal and HRP-conjugated M2 monoclonal antibodies, and M2

affinity gel were purchased from SIGMA-ALDRICH, St Louis, MO, USA; anti-calreticulin polyclonal and anti-KDEL monoclonal antibodies were from Stressgen Biotechnologies Corporation, Ann Arbor, MI, USA; anti-myc monoclonal (9E10) antibody was from Oncogene Research Products, Cambridge, MA, USA; anti-caspase-3 (Asp175) polyclonal antibody, from Cell Signaling Technology Inc, Danvers, MA, USA; anti-GST polyclonal (Z-5) and anti-HA polyclonal (Y-11) antibodies were from Santa Cruz Biotechnology, CA, USA; MG132 was from the Peptide Institute, , Osaka, Japan,, and rabbit ubiquitin-activating enzyme (E1), GST-UbcH5c (E2), and GST-ubiquitin were from BostonBiochem, Cambridge, MA, USA. Horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare Bio-sciences) and anti-rabbit IgG (GE Healthcare Bio-sciences) were used as the secondary antibody. Bands were detected using the enhanced chemiluminescence (ECL) system (GE Healthcare Bio-sciences).

Immunohistochemistry — Mouse brains were fixed in 4% paraformaldehyde, processed on a Tissue-Tek VIP (Sakura Finetek, Tokyo, Japan), and then embedded in paraffin. The brains were sectioned into 4 μm-thick slices, mounted on silane-coated slides, and then subjected to heat treatment with 10 mM sodium citrate buffer (pH 6.0) in a pressure cooker for 3 min. Diaminobenzidin (DAB) immunostaining was performed using anti-HRD1 polyclonal antibody as the primary antibody (1:50 dilution), a peroxidase-labeled polymer-conjugated anti-rabbit antibody (Envision system; Dako,

Glostrup, Denmark), and DAB as the substrate.

Immunofluorescence staining was stained with anti-HRD1 polyclonal antibody (1:20 dilution) and either neuron-specific nuclear protein (NeuN; 1:100 dilution; CHEMICON International, Temecula, CA, USA), anti- glial fibrillary acidic protein (GFAP; 1:100 dilution; CHEMICON), or anti-tyrosine hydroxylase (TH; 1:100 dilution; CHEMICON International) monoclonal antibodies, and then with anti-mouse antibody conjugated with Alexa 546 and anti-rabbit antibody with Alexa 488 (Molecular Probes, Eugene, OR, USA). Fluorescence images were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Gottingen, Germany).

Immunocytochemistry — For the subcellular localization of HRD1 and Pael-R, COS-1 cells were transfected with HRD1-myc or a control vector (Mock) and Pael-R-FLAG using the calcium phosphate method. To visualize the effect of HRD1 degrading Pael-R, normal human embryonic kidney (HEK293) cells and those stably transfected with HRD1-myc and M-HRD1-myc were transfected with Pael-R-FLAG-pcDNA3 and DsRED-express-N1 vector (Promega, Madison, WI, USA) using LipofectAMINE 2000 (Invitrogen). At 36 h after transfection, the cells were fixed with methanol at -20°C. The cells were then stained for the presence of proteins with appropriate primary antibodies, and then with anti-mouse antibody conjugated with Alexa 488 and/or anti-rabbit antibody with Alexa 594 (Molecular Probes, Eugene, OR, USA). Fluorescence images were acquired using a Zeiss LSM 510 confocal

microscope (Carl Zeiss AG, Gottingen, Germany).

Immunoprecipitation and Western blotting — Transfected HEK293 cells were lysed in a lysis buffer [20 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM EDTA, 10% glycerol, and 1% Triton X-100 with complete protease inhibitors (Roche Diagnostics K.K., Basel, Switzerland)]. Immunoprecipitation was carried out by incubating the supernatant with the indicated antibodies for 16 h and then with Protein G Sepharose Fast Flow (GE Healthcare Bio-sciences) for 1 h. For immunoprecipitation with an anti-FLAG antibody, the supernatant was incubated with anti-FLAG M2 affinity gel for 16 h. The immune complex was rinsed with a washing buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, and 1% Triton X-100].

Pulse-Chase experiment — Neuro2a cells were transfected with Pael-R-FLAG and either a control vector, HRD1-myc or M-HRD1-myc. At 36 h after transfection, the cells were starved for 1 h in methionine/cysteine-free Dulbecco's modified Eagle's medium (DMEM; SIGMA) containing 5% dialyzed fetal calf serum (FCS), and then labeled for 1 h at 37°C with 100 μCi/ml [^{35}S]-methionine/cysteine (Redivue Pro-mix $_{L}$-[^{35}S] *in vitro* cell labeling mix; GE Healthcare Bio-sciences). The cells were then washed and incubated in DMEM containing 10% FCS for the indicated periods. The cell lysates were immunoprecipitated with the anti-FLAG antibody, subjected to SDS-PAGE, and visualized using an imaging analyzer (BAS-2500, FUJIFILM, Tokyo,

Japan). The metabolically labeled Pael-R was quantified using Image Gauge software (FUJIFILM).

Cell death assay — Normal HEK293 cells and those stably expressing HRD1-myc or M-HRD1-myc were transfected with a control vector or Pael-R-FLAG and incubated for 24 h. The cells were washed with PBS and then stained by crystal violet (0.1% crystal violet, WAKO Pure Chemical Industries, Osaka, Japan), and the wells were washed with water and air-dried. The dye was eluted with water containing 0.5% SDS, and optical density was measured at 590 nm.

In vitro ubiquitylation assay — RING-proline (RP)-HRD1-myc and Pael-R-FLAG were produced by T_NT quick-coupled transcription/translation systems (Promega). Sixteen microliters of T_NT reaction lysates producing RP-HRD1 or Pael-R-FLAG were mixed with other components including E1 (25 ng), E2 (GST-UbcH5c, 400 ng), and GST-ubiquitin (7 ng) in 100 μl of reaction buffer (40 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP, and 2 mM DTT). The reaction mixtures were incubated at 30°C for 90 min, immunoprecipitated with anti-FLAG antibody, subjected to SDS-PAGE, and analyzed by Western blotting using the anti-GST polyclonal antibody.

In vitro binding assay — RP-HRD1 was cloned into the pGEX 6p-1 vector (GE Healthcare Bio-sciences). GST-RP-HRD1 and GST were expressed by culturing

Escherichia coli DH5α with 0.5 mM isopropyl-β-$_D$-thiogalactopyranoside (IPTG) for 4 h at 37°C. The cells were collected and lysed in a lysis buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EGTA, 10%, 0.5% Triton X-100 with 1.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The supernatants were mixed with glutathione-Sepharose 4B (GE Healthcare Bio-sciences) for 16 h at 4°C. The beads were washed with lysis buffer and eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM reduced glutathione, and the eluted fraction was dialyzed against PBS.

Equal amounts of purified GST or GST-RP-HRD1 were applied to Glutathione Sepharose 4B in a binding buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.25% gelatin, and 1% Triton X-100 at 4°C for 16 h, and then washed with the buffer. T_NT reaction lysates producing ^{35}S-labeled Pael-R-FLAG were incubated with aliquots of GST- or GST-RP-HRD1-coupled glutathione-Sepharose 4B for 2 h at 4°C in the binding buffer. After extensive washing of the column with a washing buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Triton X-100, the proteins recovered from the resin were subjected to SDS-PAGE followed by Coomassie blue staining and then visualized using an imaging analyzer (BAS-2500, FUJIFILM, Tokyo, Japan).

RNA interference — For HRD1 knockdown by RNA interference, siGENOME SMARTpools of four oligoduplexes targeted against HRD1 (M-007090-00; Dharmacon Research, Lafayette, CO, USA) were used. Small interfering RNA (siRNA) transfection

was performed using 100 pmol of siRNA and 7.5 μl of LipofectAMINE 2000 reagent (Invitrogen) in 6 cm dishes.

RESULTS

Localization of HRD1 and Pael-R in the murine brain and cellular ER

Since HRD1 has been shown to be highly expressed in the human fetal brain by RT-PCR-ELISA (Nagase et al., 2001), we immunohistochemically examined where HRD1 is localized in the murine brain. DAB staining showed HRD1 expression was observed in SNC neurons, which are selectively degenerated in PD (Fig. 1A), as well as in pyramidal cells of the hippocampus and Purkinje cells of the cerebellum (data not shown). Fluorescence staining using anti-NeuN and anti-GFAP antibodies showed that HRD1 was widely expressed in neuronal cells but not glial cells (Fig. 1B). Furthermore, HRD1-immunoreactive cells were partially tyrosine hydroxylase-positive, indicating that HRD1 was expressed in dopaminergic neurons in the SNC (Fig. 1C). Thus, we hypothesized that HRD1 exists in the substantia nigra together with Pael-R since Pael-R is expressed in SNC dopaminergic neurons (Imai et al., 2001). To examine the subcellular localizations of HRD1 and Pael-R, expression vectors for HRD1-myc or the control vector (Mock) and Pael-R-FLAG were transfected into COS-1 cells. The localization of HRD1 (*green*) almost completely overlapped that of endogenous calreticulin (*red*) as revealed by an ER marker (Fig. 1D, lower). Pael-R (*red*) was widely localized in the ER as well as the cell surface and partially colocalized with HRD1 (*green*) in the ER (Fig. 1D, upper). Furthermore, endogenous HRD1 (*green*) was partially colocalized with Pael-R (*red*) in Pael-R-FLAG-expressing SH-SY5Y cells (Fig. 1E).

HRD1 interacts with unfolded Pael-R

When Pael-R was overexpressed in HEK293 cells, Pael-R proteins migrated as high molecular mass broad smears (Fig. 2A, lane 2), suggesting that they had undergone covalent modifications (glycosylation, ubiquitylation, etc.) (Imai et al., 2001); however, in the transfection of Pael-R with HA-Ub, the ubiquitylation of Pael-R was barely observed in the absence of proteasome inhibitor MG132 (Fig. 2A, lane 5). Therefore, we presumed that the high molecular mass broad smears observed were the result of the aggregate formation of detergent-insoluble Pael-R rather than ubiquitylated Pael-R. Next, we used the immunoprecipitation method to investigate whether HRD1 interacts with Pael-R. HRD1 protein was detected in anti-FLAG antibody immunoprecipitates from cells cotransfected with HRD1-myc and Pael-R-FLAG (Fig. 2B, lane 15). In addition, Pael-R protein was detected in immunoprecipitates with an anti-myc antibody (Fig. 2B, lane 3), indicating that HRD1 interacts with Pael-R.

Furthermore, we performed coimmunoprecipitation in SH-SY5Y cells that stably expressed Pael-R-FLAG. The endogenous HRD1 protein was detected in immunoprecipitates with overexpressed aggregated Pael-R (Fig. 2C, upper and lower, lane 4). To investigate the interaction between HRD1 and Pael-R under a wider range of physiological conditions, the endogenous proteins in dopaminergic neuroblastoma SH-SY5Y cells were coimmunoprecipitated with the anti-Pael-R antibody; however, HRD1 was not coimmunoprecipitated with Pael-R (Fig. 2D, lane 3) under normal

conditions. Since Pael-R is easily unfolded and becomes insoluble under ER stress, we investigated the interaction between Pael-R and HRD1 in native SH-SY5Y cells under the condition of ER stress. Under ER stress, HRD1 was precipitated with Pael-R that tends to exist in an unfolded state under ER stress conditions (Fig. 2D, lane 4); this indicates that HRD1 interacts with the unfolded form of Pael-R.

HRD1 interacts with and ubiquitinates Pael-R through the proline-rich region

To investigate which HRD1 region interacts with Pael-R, a series of HRD1 mutants was prepared (Fig. 3A). HEK293 cells were transiently transfected with Pael-R-FLAG along with an empty vector (Mock), wild-type (wt)-HRD1-myc, Δmembrane (ΔM)-HRD1-myc, or membrane-RING (MR)-HRD1-myc. Wt-HRD1 and ΔM-HRD1 were detected in immunoprecipitates with anti-FLAG whereas membrane (M)-HRD1 and (MR)-HRD1 were not detected (Fig. 3B, upper, lanes 3, 5), suggesting that HRD1 requires a proline-rich region for association with Pael-R. We examined whether Pael-R interacts with the proline-rich region of HRD1 *in vitro* (Fig. 4A). In an *in vitro* GST pull-down assay, RP-HRD1 bound to both the native and aggregated forms of Pael-R (Fig. 4A, upper, lane 5). Thus, HRD1 may directly interact with Pael-R through the proline-rich region.

We then evaluated whether HRD1 ubiquitinates Pael-R through its E3 activity *in vitro*. Using RP-HRD1-myc and Pael-R-FLAG generated by *in vitro* translations (Fig. 4B), we examined whether Pael-R is ubiquitylated by RP-HRD1 *in vitro*.

Recombinant E2 UbcH5c was used in this assay since HRD1 is shown to be ubiquitylated by UbcH5c *in vitro* (Nadav et al., 2003; Kikkert et al., 2004). *In vitro* transcription/translation reaction lysates containing RP-HRD1 and Pael-R were incubated with other components including E1 (rabbit), E2 (GST-UbcH5c), and GST-ubiquitin. Pael-R-FLAG proteins were ubiquitylated only in the presence of RP-HRD1 along with all other components (Fig. 4C, lane 6), indicating that HRD1 directly interacts with and ubiquitinates Pael-R.

HRD1 degrades unfolded Pael-R

We investigated whether HRD1 accelerates Pael-R degradation via the UPS. Normal HEK293 cells and those stably expressing wt-HRD1 or M-HRD1 were transiently transfected with Pael-R-FLAG. Equal amounts of proteins were immunoprecipitated with anti-FLAG monoclonal antibody and subjected to Western blotting. Pael-R and its high molecular mass broad smears were markedly decreased in wt-HRD1-expressing cells (Fig. 5A, first panel, lanes 5, 6). MG132 inhibited the decrease of Pael-R protein (Fig. 5A, first panel, lane 7), indicating that HRD1 promoted the degradation of Pael-R via the UPS. In contrast, Pael-R was not degraded by M-HRD1, which has no RING-finger domain and lacks E3 activity (Fig. 5A, first panel, lanes 8, 9). To confirm that these results were not caused by a decrease in the transfection or transcription efficiency of Pael-R, the expression level of Pael-R mRNA was examined by RT-PCR using the total RNA of the cells used in Western blotting.

In each clone, the expression levels of transfected Pael-R were almost equal (Fig. 5A, third panel); furthermore, another clone stably expressing wt-HRD1 degraded Pael-R (data not shown).

To immunocytochemically visualize the degradation of Pael-R by HRD1, normal HEK293 cells and those stably expressing wt-HRD1 or M-HRD1 were transfected with Pael-R-FLAG and DsRED, a red fluorescent protein. The amount of Pael-R-FLAG protein decreased in cells expressing wt-HRD1-myc compared with control cells, whereas the amount of Pael-R-FLAG protein in cells expressing M-HRD1-myc and in control cells was similar (Fig. 5B, upper, *green*). The red signals (lower panels) were DsRED proteins cotransfected with Pael-R-FLAG for use as transfection controls. These results indicate that HRD1 degrades Pael-R by its E3 activity.

Next, the degradation of Pael-R by HRD1 was examined by performing a pulse-chase experiment. The levels of ^{35}S-labeled Pael-R were plotted relative to the amount present at time 0 (Fig. 5C). Following a 3 h chase, 54.4% and 52.0% of *de novo* synthesized Pael-R remained in cells transfected with Mock and M-HRD1, respectively. In contrast, Pael-R degradation in HRD1-transfected cells was accelerated such that at 3 h, 28.7% of proteins remained, indicating that HRD1 accelerates the degradation of newly synthesized Pael-R protein.

Furthermore, to investigate whether HRD1 is involved in the physiological degradation of Pael-R, we examined the effect of HRD1 suppression by siRNA on

Pael-R accumulation in SH-SY5Y cells stably expressing Pael-R-FLAG. The amount of the aggregated form of Pael-R was increased by the suppression of HRD1 expression (Fig. 5D, upper, lane 2) whereas the native form was not affected markedly; thus, it is possible that endogenous HRD1 preferentially degrades aggregated Pael-R but not native Pael-R.

α-Synuclein is a component of Lewy bodies in PD (Trojanowski et al., 1998), and 22-kilodalton glycosylated form of α-synuclein is reported to be ubiquitylated by Parkin (Shimura et al., 2001), and are ubiquitylated when overexpressed in cells (Imai et al., 2000). Unfolded α-synuclein can be degraded by the 20S proteasome *in vitro* (Tofaris et al., 2001). We examined whether α-synuclein, like Pael-R, is a substrate of HRD1. Normal HEK293 cells and those stably expressing wt- or M-HRD1 were transiently transfected with α-synuclein-HA. The protein levels of α-synuclein were not changed by HRD1 (Fig. 5E, upper), indicating that α-synuclein is not a substrate of HRD1.

HRD1 suppresses Pael-R-induced cell death

The accumulation of Pael-R causes ER stress and subsequent cell death. We investigated whether HRD1 suppresses Pael-R-induced cell death. Normal HEK293 cells and those stably expressing wt- or M-HRD1 were transiently transfected with a control vector (Mock) or Pael-R-FLAG and incubated for 24 h. The cell death of HEK293 was compared with that of cells transfected with the control vector. The

crystal violet assay showed that wt-HRD1-expressing cells were more resistant to Pael-R overexpression than control and M-HRD1 cells (control, 34.3%; wt-HRD1, 20.8%; M-HRD1, 33.4%) (Fig. 6). Furthermore, we found that the accumulation of aggregated Pael-R induced by the repression of HRD1 in SH-SY5Y cells that stably expressed Pael-R-FLAG promoted a decrease in pro-caspase-3 and an increase in cleaved caspase-3 (Fig. 5D, third panel, lane 2), which indicates the activation of caspase-3 and subsequent apoptosis. These results indicate that HRD1 suppresses apoptosis induced by Pael-R accumulation.

Involvement of HRD1 in the degradation of Pael-R induced by ATF6

We found that ATF6 induced the expression of HRD1 (Kaneko et al., 2002; unpublished data). Since ATF6-mediated UPR possibly induces a number of ERAD genes, we speculated that the degradation of Pael-R is promoted by ATF6. HEK293 cells were transiently transfected with Pael-R-FLAG and either an empty vector (Mock) or HA-ATF6 (1-373; cytoplasmic domain worked as a transcription factor), and incubated for 48 h in the presence or absence of MG132. The amount of both native and aggregated Pael-R decreased in cells expressing ATF6 (Fig. 7A, upper, lane 4); moreover, MG132 inhibited the decrease in Pael-R protein by ATF6 overexpression (Fig. 7A, upper, lane 5). The increased expression of glucose-regulated proteins GRP78 and GRP94 indicates the induction of UPR by ATF6 (Fig. 7A, lower, lanes 4, 5). These results indicate that the upregulation of UPR by ATF6 leads to the degradation of Pael-R

proteins via the UPS; however, it is not known which proteins induced by ATF6 are involved in this degradation.

To determine whether HRD1 is involved in the degradation of Pael-R induced by UPR upregulation, we investigated the effect of HRD1 suppression by siRNA on degradation. HEK293 cells were transiently transfected with Pael-R-FLAG, HA-ATF6, and either GFP (siRNA) or HRD1 (siRNA). ATF6 induced HRD1 expression (Fig. 7B, lower, lane 3), whereas HRD1 repression partilly suppressed the ATF6-induced decrease in the number of Pael-R aggregates, but not the amount of the native form (Fig. 7B, upper, lane 6), suggesting that UPR-induced HRD1 preferentially promotes the degradation of unfolded Pael-R.

DISCUSSION

In this report, we found that HRD1 was expressed in the dopaminergic neurons of the SNC, colocalized with Pael-R in the ER, and directly interacted with Pael-R at the proline-rich region of HRD1. We showed that HRD1 promoted the ubiquitylation and degradation of Pael-R; additionally, the activation of UPR by ATF6 induced Pael-R degradation, which partially depends on HRD1.

First, we found that HRD1 was locally expressed in SNC neurons, including dopaminergic neurons, of the murine brain. Pael-R is reportedly expressed in SNC neurons, implying that HRD1 and Pael-R are colocalized in dopaminergic neurons in the SNC. Parkin, an E3, is upregulated in response to ER stress and protects cells via ERAD from ER stress-induced apoptosis (Imai et al., 2000). Pael-R accumulates in the brains of AR-JP patients and induces ER stress, possibly because of Parkin mutation (Imai et al., 2001). Furthermore, it has been reported that Pael-R overexpression causes the selective degeneration of dopaminergic neurons in *Drosophila* and that the coexpression of human Parkin suppresses Pael-R toxicity by degrading Pael-R. It has also been reported that interference in endogenous *Drosophila* Parkin functions enhances Pael-R toxicity (Yang et al., 2003). On the other hand, we previously reported that human HRD1 is upregulated in response to ER stress. It possesses E3 activity and protects against ER stress-induced cell death (Kaneko et al., 2002), suggesting that HRD1 can degrade protein substrates accumulated during ER stress; however, there is little information regarding these substrates, with the exception of

CD-3α and TCR-α, and HMG-CoA reductase (Kikkert et al., 2004). We showed that HRD1 was colocalized in the ER with Pael-R and they interacted at endogenous levels as well as overexpression levels. We therefore hypothesized that HRD1, like Parkin, may degrade Pael-R and suppress cell death caused by Pael-R accumulation.

We found that endogenous HRD1 interacted with not only overexpressed Pael-R but also endogenous Pael-R under ER stress conditions. Pael-R tends to exist in an unfolded state when it is overexpressed or when subjected to ER stress; therefore, it is likely that HRD1 preferentially interacts with the unfolded form of Pael-R but not the with the normally folded form. Therefore, it can be speculated that unfolded Pael-R is recognized by acceptors of terminally misfolded glycoproteins, such as ER degradation-enhancing alpha-mannosidase-like protein (EDEM), and is destined to be eliminated from the ER (Molinari et al., 2003 ; Oda et al., 2003); HRD1 then binds to Pael-R passing through the translocon in the ER membrane by its proline-rich region and ubiquitinates the unfolded form of Pael-R. If this is true, it is unlikely that HRD1 directly associates with and ubiquitinates native Pael-R on the ER membrane without mediation of the translocon.

On the other hand, we showed that the high molecular mass broad smears of Pael-R mostly comprised not ubiquitylated forms, but possibly glycosylated or aggregated forms, as previously reported (Imai et al., 2001). The inhibition of HRD1 expression by siRNA induced the accumulation of smears and the activation of caspase-3. Therefore, it is likely that HRD1 preferentially ubiquitinates and degrades

unfolded Pael-R to prevent the accumulation of aggregated Pael-R that leads to ER stress-induced apoptosis.

We further showed that HRD1 interacted with Pael-R at its proline-rich region and ubiquitylated Pael-R *in vitro*, indicating direct interaction between the proline-rich region of HRD1 and Pael-R. Yeast Hrd1p has no proline-rich region whereas human HRD1 contains a proline-rich region similar to that seen in the Cbl family of ubiquitin ligases (Fujita et al., 2002). It has been reported that the proline-rich region is essential for protein-protein interaction and that the RING-finger and proline-rich regions are sufficient for the binding and ubiquitylation of substrates (Fang et al., 2001). Therefore, human HRD1 appears to interact with substrates at the proline-rich region and ubiquitinates the substrates at the RING-finger domain. On the other hand, Hrd1p degrades Hmg2p, one of the yeast isozymes of HMG-CoA reductase, despite the lack of a proline-rich region (Gardner et al., 2000). Thus, we propose that in the course of evolution, human HRD1 acquired a proline-rich region to interact with and ubiquitinate a variety of substrates; however, whether other substrates are bound to the proline-rich region remains to be determined.

We investigated whether α-synuclein is a substrate of HRD1. An α-synuclein mutant (Ala53Thr or Ala30Pro) has been reported in the brain of PD patients, promoting protofibril formation relative to wild-type α-synuclein (Conway et al., 2000). Parkin ubiquitinates the *O*-glycosylated form (αSp22) (Shimura et al., 2001) and suppresses the toxicity of normal or pathogenic alpha-synuclein (Petrucelli et al., 2002;

Yang et al., 2003; Lo Bianco et al., 2004; A. F. Haywood & B. E. Staveley, 2004). HRD1 did not degrade wild-type α-synuclein, probably due to the different localization or binding ability of HRD1 and α-synuclein; however, whether HRD1 degrades α-synuclein mutants or the *O*-glycosylated form remains to be clarified. On the other hand, Hrd3p, another UPR-inducible ERAD protein, has been reported to interact with Hrd1p and mediate the regulation of Hrd1p stability and activity in yeast (Gardner et al., 2000). We have identified SEL1 as a candidate human homologue of Hrd3p and have found that SEL1 interacted with human HRD1 (data not shown). We have further found that HRD1 did not degrade SEL1 despite this interaction; rather, the amount of SEL1 increased in the presence of HRD1 (data not shown). Based on these observations, we speculate that HRD1 specifically increases the degradation of proteins.

When unfolded proteins accumulate in the ER, the UPR is activated by ATF6 and IRE1, resulting in the induction of several ER chaperones and ERAD components (Travers et al., 2000; Lee et al., 2003). Therefore, we hypothesized that ATF6 promotes the degradation of Pael-R by inducing UPR genes including HRD1, although ATF6 can induce a variety of genes in addition to HRD1. Interestingly, ATF6 induced the degradation of both aggregated and unaggregated Pael-R, whereas the suppression of ATF6-induced HRD1 expression by siRNA caused an increase in the aggregated form. Thus, it is likely that endogenous HRD1 preferentially recognizes and degrades the unfolded forms of Pael-R. Based on these results, we propose that after the accumulation of unfolded Pael-R due to stress or Parkin mutation, ATF6 and/or

IRE1-XBP1 pathways are activated and induce UPR genes including HRD1; this promotes the folding or degradation of unfolded Pael-R to prevent unfolded Pael-R-induced cell death (Fig. 8).

It has been reported that Parkin knockout mice exhibit little change in movement ability and the neurons of the substantia nigra (Itier et al., 2003; von Coelln et al., 2004; Goldberg et al., 2003; Perez & Palmiter, 2005). We therefore speculate that HRD1 degrades Pael-R and possibly other proteins in order to balance the unfolded protein accumulation caused by Parkin gene mutation; nonetheless, it is possible that other unknown E3s participate in this degradation in the absence of Parkin, although the reason behind the loss of dopaminergic neurons in AR-JP patients but not in Parkin knockout mice remains unknown despite the similarity in the functional loss of Parkin. On the other hand, it is likely that HRD1 ubiquitinates not only Pael-R but also other substrates related to conformational diseases caused by the accumulation of unfolded proteins since HRD1 can suppress global ER stress induced by various chemical reagents.

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FIGURE LEGENDS

Figure 1. Brain distribution and subcellular localization of Pael-R and HRD1. (A) Immunolocalization of HRD1 in the coronal section of the murine brain. HRD1 localization was detected by DAB immunostaining using anti-HRD1 polyclonal antibody (pAb). The magnification of the box in the left panel (x12.5) is part of the substantia nigra pars compacta (SNC), which is shown in the right panel (x400). The arrowheads indicate the expression of HRD1. (B) Localization of HRD1 in neurons and glia. The *green* signal (HRD1) was obtained with anti-HRD1 pAb and anti-rabbit IgG Alexa 488-conjugated secondary Ab, while the *red* signal (Pael-R) was obtained with either anti-NeuN monoclonal Ab (mAb; dentate gyrus; upper panel) or anti-GFAP mAb (CA4; lower panel) and anti-mouse IgG Alexa 546-conjugated secondary Ab. (C) Colocalization of HRD1 and tyrosine hydroxylase in the SNC of the coronal section of murine brain. HRD1 was detected by anti-HRD1 pAb (*green*); tyrosine hydroxylase was detected by anti-tyrosine hydroxylase mAb (*red*). *Yellow* indicates the expression of HRD1 in the SNC. (D) Colocalization of HRD1 and Pael-R in the ER. COS-1 cells were transiently transfected with HRD1-myc and Pael-R-FLAG. At 24 h after transfection, the cells were fixed and subjected to indirect immunofluorescence staining with anti-myc mAb and anti-FLAG pAb, or anti-calreticulin pAb. The *green* signal (HRD1) was obtained with anti-mouse IgG Alexa 488-conjugated secondary Ab, while the *red* signal (Pael-R or calreticulin) was obtained with anti-rabbit IgG Alexa 594-conjugated secondary Ab. Superimposing the two colors (merge) resulted in a

yellow signal, indicating the colocalization of the two proteins. (E) Colocalization of HRD1 and Pael-R in the ER of SH-SY5Y cells. The SH-SY5Y cells expressing Pael-R-FLAG were fixed and subjected to indirect immunofluorescence staining with anti-FLAG mAb (*red*) and anti-HRD1 pAb (*green*). The *green* signal (HRD1) was obtained with anti-rabbit IgG Alexa 488-conjugated secondary Ab, while the *red* signal (Pael-R) was obtained with anti-mouse IgG Alexa 594-conjugated secondary Ab.

Figure 2. Interaction of HRD1 with aggregated Pael-R. (A) Pael-R tends to exist in an aggregated form. HEK293 cells were transiently transfected with HA-ubiquitin and an empty vector (Mock) or Pael-R-FLAG and incubated in the presence or absence of 5 μM MG132 (proteasome inhibitor). Equal amounts of proteins were immunoprecipitated with anti-FLAG mAb, and the immune complex was then analyzed by Western blotting using anti-FLAG mAb (left) or anti-HA pAb (right). (B) Interaction of Pael-R with HRD1 in HEK293 cells. HRD1 and Pael-R were coimmunoprecipitated in HEK293 cells transiently transfected with or without Pael-R-FLAG and HRD1-myc. At 48 h after transfection, the total cell lysates (Input) were analyzed by Western blotting to check the expression of Pael-R and HRD1 proteins. Equal amounts of the proteins were immunoprecipitated with normal mouse IgG, anti-myc mAb, or anti-FLAG mAb. The immune complex was lysed in SDS sample buffer, resolved by SDS-PAGE, and analyzed by Western blotting using anti-myc mAb or anti-FLAG mAb. (C) Endogenous HRD1 interacts with Pael-R in

neuroblastoma SH-SY5Y cells stably expressing Pael-R-FLAG. The total cell lysates that stably expressed Pael-R-FLAG in neuroblastoma SH-SY5Y cells were analyzed by Western blotting using anti-HRD1 pAb (Input, right panel). Equal amounts of the proteins were immunoprecipitated with normal mouse IgG (I) or anti-FLAG mAb (F), and the immune complex was then analyzed by Western blotting using anti-HRD1 pAb (left panel, upper) or anti-FLAG mAb (right panel, lower). (D) Endogenous interaction of HRD1 with Pael-R in tunicamycin-treated neuroblastoma SH-SY5Y cells. The SH-SY5Y cells were either untreated (control) or treated (tunicamycin) with 2.5 μg/ml tunicamycin for 24 h. The total cell lysates (Input, lanes 1 and 2) were analyzed by western blotting to check the expression of HRD1 proteins. Equal amounts of proteins were immunoprecipitated with anti-Pael-R pAb. The immune complex was analyzed by western blotting by using anti-HRD1 pAb (IP, lanes 3 and 4; Abgent).

Figure 3. Interaction of Pael-R with HRD1 and its mutants. (A) Schematic representation of the HRD1 constructs. The panel diagrammatically represents wild-type HRD1 and a variety of HRD1 mutants used to determine the Pael-R binding domain. Numbers in parentheses indicate the corresponding amino acid residues of HRD1. (B) Coimmunoprecipitation of Pael-R and a variety of HRD1 mutants. Coimmunoprecipitation was performed in HEK293 cells transiently transfected with Pael-R-FLAG and an empty vector (Mock), wild-type (wt)-HRD1-myc, membrane (M)-HRD1-myc, Δmembrane (ΔM)-HRD1-myc or membrane-RING (MR)-HRD1-myc.

At 48 h after transfection, the total cell lysates (Input) were analyzed by Western blotting using anti-myc mAb (lower). Equal amounts of the proteins were immunoprecipitated with anti-FLAG mAb, and the immune complex was then analyzed by Western blotting using anti-myc mAb (upper).

Figure 4. HRD1 ubiquitinates Pael-R *in vitro*. (A) Coimmunoprecipitation of Pael-R with RING-proline (RP)-HRD1 *in vitro*. GST-fused RP-HRD1 or GST alone was bound to glutathione beads incubated with [^{35}S]-labeled Pael-R generated by an *in vitro* transcription/translation system. After extensive washing, the protein-bound beads were resolved by SDS-PAGE followed by Coomassie blue staining (lower), and detected by autoradiography (upper). (B) Protein products of Pael-R and RP-HRD1. Western blotting analysis of the components used for *in vitro* ubiquitylation assays. RP-HRD1-myc and Pael-R-FLAG were produced by a T_NT quick-coupled transcription/translation system. T_NT reaction lysates containing RP-HRD1 were analyzed by Western blotting using anti-myc mAb (left), whereas T_NT reaction lysates containing Pael-R were immunoprecipitated with anti-FLAG mAb. The immune complex was then analyzed by Western blotting using anti-FLAG mAb (right). (C) *In vitro* ubiquitylation assay. Western blotting analysis of the *in vitro* ubiquitylation reactions mediated by HRD1 with anti-GST pAb. T_NT reaction lysates containing RP-HRD1 and Pael-R were mixed with other components including E1 (rabbit), E2 (GST-UbcH5c), or GST-ubiquitin in the reaction buffer. The reaction lysates were

then incubated at 30°C for 90 min, immunoprecipitated with anti-FLAG mAb, and analyzed by Western blotting using anti-GST pAb.

Figure 5. Degradation of Pael-R by HRD1. (A) HEK293 cells stably expressing wt-HRD1 or M-HRD1 were transiently transfected with Pael-R-FLAG and incubated for the indicated periods in the presence or absence of 5 μM MG132, which was added 12 h before cell harvest. The total cell lysates (Input) were analyzed by Western blotting using anti-myc mAb (middle). Equal amounts of the proteins were immunoprecipitated with anti-FLAG mAb, and the immune complex was then analyzed by Western blotting using anti-FLAG mAb (upper). The total RNA of the cells used in Western blotting was prepared and subjected to RT-PCR (lower). (B) HEK293 cells stably expressing wt-HRD1 or M-HRD1 were transiently transfected with Pael-R-FLAG and DsRED (red fluorescent protein). At 36 h after transfection, the cells were fixed and subjected to indirect immunofluorescence staining with anti-FLAG mAb (upper). The *green* signal (Pael-R) was obtained with anti-mouse IgG Alexa 488-conjugated secondary Ab while the *red* signal (lower) shows DsRED proteins used as a transfection control. (C) Pulse-chase assay. Neuro2a cells were transiently transfected with Pael-R-FLAG and an empty vector (Mock), wt-HRD1, or M-HRD1. At 36 h after transfection, cells were pulse-labeled with [^{35}S]-methionine/cysteine and chased for the indicated periods. Equal amounts of [^{35}S]-labeled Pael-R and M-HRD1 were immunoprecipitated with anti-FLAG mAb; the immune complex was then lysed in

SDS sample buffer, resolved by SDS-PAGE, detected by autoradiography, and quantified by phosphorimaging. The levels of [^{35}S]-labeled Pael-R are plotted relative to the amount present at time 0. (D) Induction of aggregated Pael-R accumulation and caspase activation by inhibition of HRD1 expression. SH-SY5Y cells (clone #11) stably expressing Pael-R-FLAG were transiently transfected with the siRNA of enhanced green fluorescent protein (EGFP, control) or HRD1, and incubated for 72 h. The total cell lysates were analyzed by Western blotting using anti-HRD1 pAb (2nd panel), anti-caspase-3 pAb (3rd panel), and anti-α-tubulin mAb (5th panel). Equal amounts of the proteins were immunoprecipitated with anti-FLAG mAb, and the immune complex was then analyzed by Western blotting using anti-FLAG mAb (1st panel). (E) HRD1 did not degrade α-synuclein. HEK293 cells stably expressing wt-HRD1 or M-HRD1 were transiently transfected with α-synuclein-HA and incubated for the indicated periods. The total cell lysates were analyzed by Western blotting using anti-HA pAb (upper) or anti-myc mAb (lower).

Figure 6. HRD1 protects against Pael-R-induced cell death. HEK293 cells (control) and HEK 293 cells stably expressing wt-HRD1 or M-HRD1 were transiently transfected with a control vector (Mock) or Pael-R-FLAG and incubated for 24 h. The cells were washed with PBS and then stained with crystal violet (0.1%) for 20 min, and the wells were washed with water and air-dried. The dye was eluted with water containing 0.5% SDS for 20 min, and the optical density at 590 nm was measured. The percentage of

cell death was calculated as follows: 100 – [(OD for assay/OD for control well) × 100]. The results obtained from each cell transfected with Pael-R-FLAG were compared with those obtained from cells transfected with Mock. The results are expressed as the means ± S.D. (three independent experiments in duplicate). Statistical analysis was performed with Student's *t*-test (**, $p < 0.01$; versus normal).

Figure 7. Degradation of Pael-R promoted by ATF6. (A) HEK293 cells were transiently transfected with an empty vector (Mock) or Pael-R-FLAG with or without HA-ATF6, (cleaved form); they were incubated for 48 h in the presence or absence of 5 μM MG132 (12 h incubation). The total cell lysates (Input) were analyzed by Western blotting using anti-KDEL mAb (lower). Equal amounts of the proteins were immunoprecipitated with anti-FLAG mAb, and then analyzed by Western blotting using anti-FLAG mAb (upper). (B) Involvement of HRD1 in ATF6-mediated Pael-R degradation. HEK293 cells were transiently transfected with Pael-R-FLAG and HA-ATF6 (cleaved form) and siRNA of EGFP (control) or HRD1 and incubated for 30 h. The total cell lysates (Input) were analyzed by Western blotting using anti-HRD1 pAb (middle) or anti-α-tubulin mAb (lower). Equal amounts of the proteins were immunoprecipitated with anti-FLAG mAb, and the immune complex was then analyzed by Western blotting using anti-FLAG mAb (upper).

Figure 8. A hypothetical model demonstrating how HRD1 participates in the

degradation of unfolded Pael-R. When unfolded Pael-R is accumulated in the ER, ATF6 and IRE1-XBP1 pathways are activated, and UPR genes including HRD1 are then induced. HRD1 degrades unfolded Pael-R and suppresses Pael-R-induced cell death.