Parkin Suppresses Unfolded Protein Stress-induced Cell Death through Its E3 Ubiquitin-protein Ligase Activity*

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Autosomal recessive juvenile parkinsonism (AR-JP) is caused by mutations in the parkin gene. Parkin protein is characterized by a ubiquitin-like domain at its NH2-terminus and two RING finger motifs and an IBR (in between RING fingers) at its COOH terminus (RING-IBR-RING). Here, we show that Parkin is a RING-type E3 ubiquitin-protein ligase which binds to E2 ubiquitin-conjugating enzymes, including UbcH7 and UbcH8, through its RING-IBR-RING motif. Moreover, we found that unfolded protein stress induces up-regulation of both the mRNA and protein level of Parkin. Furthermore, overexpression of Parkin, but not a set of mutants without the E3 activity, specifically suppressed unfolded protein-stress-induced cell death. These findings demonstrate that Parkin is an E3 enzyme and suggest that it is involved in the ubiquitination pathway for misfolded proteins derived from endoplasmic reticulum and contributes to protection from neurotoxicity induced by unfolded protein stresses.

AR-JP* is one of the most common forms of the familial Parkinson’s disease and is characterized by juvenile onset, a recessive mode of inheritance and selective loss of the dopaminergic neurons in the substantia nigra without Lewy bodies (intraneuronal accumulations of aggregated proteins) (1). In 1998, the gene responsible for AR-JP was identified and designated parkin (2).

Recently, several proteins with RING finger motifs have been identified as E3 ubiquitin ligases, which are responsible for substrate recognition and for promotion of substrate ubiquitination in conjunction with ubiquitin-conjugating enzymes (E2s) (3–10). In RING-type E3s, RING finger motifs serve as recruiting motifs for specific E2 ubiquitin-conjugating enzymes. These facts suggest that Parkin, which contains a RING-IBR-RING motif, is a new member of E3 ubiquitin ligases.

On the other hand, the fact that the deletion of the parkin gene causes the neuronal death of the substantia nigra in AR-JP patients suggests the cell-protective function of Parkin. Given that Parkin is involved in both the ubiquitin-proteasome pathway and cell death protection, an interesting possibility is that Parkin may inhibit a certain type of cell death through proteasome-mediated protein degradation. Accumulation of misfolded proteins in the endoplasmic reticulum (ER) would constitute an unfolded protein stress or ER stress, which may lead to cell death. Normal cells deal with unfolded protein stress by several mechanisms, including transcriptional induction of genes that facilitate protein folding or removal of misfolded proteins and degradation that is dependent on the cytosolic ubiquitin-proteasome pathway (11).

Here, we provide evidence that Parkin is a RING-type E3 ubiquitin-protein ligase. Moreover, we show that Parkin is up-regulated in response to unfolded protein stress and suppresses unfolded protein-stress-induced cell death via its E3 activity, suggesting that the physiological role of Parkin involves dealing with unfolded protein stress.

EXPERIMENTAL PROCEDURES
cDNAs and Antibodies (Abs)—cDNAs of human Parkin was a kind gift from Y. Mizuno. cDNAs of human Parkin mutants were generated using polymerase chain reaction (PCR) with wild-type human Parkin cDNA as the template and then cloned into the mammalian expression vector pcDNA3 (Invitrogen). Human ubiquitin cDNA with a hemagglutinin (HA) tag was a generous gift from S. Hatakeyama. Human ubiquitin COOH-terminal hydrolase L1 (UCH-L1) and α-synuclein cDNAs were cloned by RT-PCR. Anti-Parkin polyclonal Ab was raised against recombinant 6×His-tagged human Parkin protein produced in bacteria. Anti-Myc (9E10), anti-HA (Y-11), and anti-actin (C-2) Abs were purchased from Santa Cruz. Anti-FLAG (M2) and anti-His (Penta-His) were purchased from Sigma and Qiagen, respectively.

Transfection and Cell Death Assay—Cells were transfected with various expression vectors using the LipofectAMINE PLUS or LipofectAMINE 2000 Reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Total amounts of plasmid DNA in individual transfection experiments were adjusted using empty vector plasmid. Transfected cells were cultured for at least 24 h after transfection and used for immunoprecipitation, Western blotting, immunocytochemistry, and a cell death assay. The propidium iodide dye exclusion assay was performed as described elsewhere (12).

Immunopurification and Western Blot Analysis—Cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, containing 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% Triton X-100, 0.5 mM N-ethylmaleimide, and 0.5 mM iodoacetamide) with protease inhibitors. Immunoprecipitations from the transfected cell lysates was performed with anti-FLAG or anti-Parkin Ab and protein G-coupled Sepharose beads (Amersham Pharmacia Biotech) and then washed four times in lysis buffer. Immunoprecipitates or total cell lysates were analyzed by Western blot analysis with ECL detection reagents (Amersham Pharmacia Biotech). The immunoblotting analysis was performed using 15% acrylamide gel. The primary antibody was rabbit anti-Parkin Ab (1:1000) and the secondary antibody was anti-rabbit HRP (1:10 000). Enhanced chemiluminescence (ECL) Western blot detection system (Amersham Pharmacia Biotech) was used to detect the immunoprecipitated Parkin.

Quantitative RT-PCR—Total RNA was isolated from SH-SY5Y cells treated with various stresses using the RNeasy Mini kit (Qiagen). Taqman EZ RT-PCR was carried out using an ABI prism 7700 sequence detector (PerkinElmer Life Sciences) according to the manufacturer’s protocol. The primers and probe sequences were as follows: Parkin

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RESULTS AND DISCUSSION

To study the physiological function of Parkin in cells, we overexpressed Parkin with NH2-terminal FLAG tag (FLAG-Parkin) in several cell lines, including human kidney-derived 293(T) cells and dopaminergic neuroblastoma-derived SH-SY5Y cells. Overexpression of FLAG-Parkin in any cell line used led to the formation of slower migrating proteins that were recognized by a Western blot using anti-FLAG Ab (Fig. 1A). As this high molecular weight smear-like appearance of FLAG-Parkin seemed to be caused by polyubiquitin, we examined whether Parkin could be covalently modified by ubiquitin. An expression plasmid encoding ubiquitin with a hemagglutinin tag (HA-Ub) was transfected into 293T cells with or without plasmid for FLAG-Parkin, followed by immunoprecipitation with anti-FLAG Ab. A Western blot analysis of immunoprecipitates with anti-HA Ab showed a high molecular weight shifted band only when HA-ubiquitin and FLAG-Parkin were co-expressed, indicating that FLAG-Parkin is heavily ubiquitinated. Western blots of the same samples using anti-FLAG Ab detected high molecular weight smear bands when FLAG-Parkin was expressed, regardless of the presence or otherwise of HA-ubiquitin. However, the average size of the smear band was smaller in the absence rather than the presence of HA-ubiquitin, suggesting that FLAG-Parkin is modified with endogenous ubiquitin in the former case (Fig. 1B, left). Overexpression of the K48R-ubiquitin mutant, which disrupts polyubiquitin chain formation, caused significant inhibition of ubiquitination of Parkin (13, 14) (Fig. 1C). The same data was obtained with α-synuclein, which was also ubiquitinated when overexpressed in 293T cells (Fig. 1B, right panel, and C). Although ubiquitin carboxyl-terminal hydrolase (UCH) family enzymes are generally thought to hydrolyze adducts with small, unfolded leaving groups, some UCHs, including Dro sophila UCH, are shown to have ability to deubiquitinate polyubiquitinated proteins (15). High molecular weight shifted FLAG-Parkin as well as ubiquitinated α-synuclein were efficiently processed by overexpressed UCH-L1 (PGP9.5), a human UCH enzyme, providing further evidence that Parkin is ubiquitinated when overexpressed in cells (Fig. 1C).

Recently, several proteins containing the RING finger motif have been shown to be E3 ubiquitin-protein ligases. Moreover ubiquitination of some of these RING-type E3s has been demonstrated previously (7, 9). Therefore, we examined whether Parkin is a RING-type E3. We first performed a co-immunoprecipitation assay using FLAG-Parkin and a set of Myc-tagged E2s co-expressed in SH-SY5Y cells. FLAG-Parkin associated with Myc-UbcH7 and closely related Myc-UbcH8, but not with Myc-UbcH5α, -H5b, -H5c, or -H6. This binding pattern was identical to that of Ariadne, a protein containing a RING-IBR-RING motif at its COOH terminus, which is structurally very similar to Parkin (16) (Fig. 2A). Next, we tried to determine the position of E2-binding domain of Parkin. We co-expressed a wild-type or several deletion/point mutants of FLAG-Parkin with Myc-UbcH7 in SH-SY5Y cells, then performed immunoprecipitation with anti-FLAG antibody followed by Western blotting by anti-Myc Ab. A deletion mutant containing only the RING-IBR-RING domain (RIR) bound to UbcH7 as potently as wild-type protein. By contrast, all the other mutated Parkin proteins, including deletion mutants missing Exons 3 and 4 (Ex3–4–), Exon 4 (Ex4–), an IBR and the second RING (Q311X), the second RING-deleted mutant (ΔRING), and a mutant with a point mutation in the first RING (T240R) completely failed to interact with Myc-UbcH7. (Fig. 2B). Essentially, the same result was obtained with Myc-UbcH8 (data not shown). These data indicate that the RING-IBR-RING domain of Parkin is responsible for binding with UbcH7, strongly suggesting that Parkin is a RING-type E3 associated with UbcH7 and H8.

To obtain direct evidence that Parkin exhibits E3 activity, we performed an in vitro ubiquitin ligase assay. Immunoprecipitates of full-length FLAG-Parkin or an Ex4– (−) deletion mutant (Ex4–Δ) from different cell types (293T and SH-SY5Y cell) were incubated with recombinant yeast E1, bacterially produced 6× histidine-tagged UbcH7 and GST-ubiquitin. Western blotting using anti-GST antibody detected a high molecular weight shifted band indicative of ubiquitinated protein(s) only with full-length Parkin, but not with Ex4– (−) or control (Fig. 2C), thereby proving the in vitro E3 activity of Parkin. The ubiquitinated substrate(s) in this reaction are thought to be cellular protein(s) associated with Parkin, but not Parkin itself, as the
Parkin Cont was performed as in mutant of the second RING.) and Myc-UbcH7. Immunoprecipitation with anti-Myc.

immunoprecipitation with anti-FLAG and detected by Western blotting and GST-Ub (167 pmol) to the immunopurified wild-type and mutant -7, -8 with ( ) or without ( ) FLAG-Parkin. The lysates were subject to immunoprecipitation with anti-FLAG Ab, Myc-UbcH5a, -5b, -5c, -6, -7, -8 with ( ) or without ( ) FLAG-Parkin. The lysates were subject to immunoprecipitation with anti-FLAG Ab ( ) or without ( ) FLAG-Parkin. The lysates were subject to immunoprecipitation with anti-FLAG Ab ( ) or without ( ).

Control cells were transfected with vector ( ) or without ( ) Myc-UbcH7. Immunoprecipitation was performed as in A. C, Parkin and its mutant were immunopurified from 293T or SH-SY5Y cells transfected with vector (Cont.), FLAG-Parkin (Parkin), and a deletion mutant of exon 4 with FLAG tag, (Ex4( )). An in vitro ubiquitin-ligase assay was carried out by adding yeast E1 (0.45 pmol) and recombinant 6 His-tagged UbcH7 (3 pmol) and GST-Ub (167 pmol) to the immunopurified wild-type and mutant Parkin proteins in reaction buffer (50 mM Tris, pH 7.4, 5 mM MgCl2, 2 mM dithiothreitol, 2 mM adenosine 5'-triphosphate) at 30 °C for 90 min (90).

The results are presented as a band size observed for Parkin and its mutant were confirmed on the same membrane by Western blotting with anti-GST Ab. The amount of actin

protein of unknown nature, which was reproducibly detected by anti-Parkin Ab only in tunicamycin-treated samples; it may represent a processed form of Parkin. ER stress response was assessed by the induction of GRP78 (BiP). Total protein level of each sample was confirmed by the amount of actin.

During the preparation of this manuscript, Shimura et al. (17) reported that Parkin is an E3 protein with characteristics identical to those shown here, except that overexpressed Parkin was not ubiquitinated in their system. Although the reasons for this apparent discrepancy are not clear, it is conceivable that better expression in our cells enabled us to detect the ubiquitination of Parkin.

As dysfunction of Parkin is thought to lead to selective neuronal cell death, we sought evidence for a role for Parkin in neuronal cell death induced by various stresses including unfolded protein stress. mRNA levels of Parkin in SH-SY5Y cells cultured under various stress conditions for 5 h were measured by quantitative RT-PCR (Fig. 3A). No change or only a slight reduction was observed in the Parkin mRNA level under a variety of stress treatments, including hydrogen peroxide (H2O2; 600 μM), high osmolarity (0.3 M sorbitol), the DNA alkylation agent (methyl methanesulfonate (MMS); 100 μg ml-1), or the reducing agent 2-mercaptoethanol (2-ME; 7.5 mM), either of which is an effective inducer of unfolded protein stress (18–20), resulted in significant up-regulation of Parkin mRNA compared with no treatment control (p < 0.001). Next, we examined the expression levels of Parkin mRNA in cells treated with tunicamycin or 2-ME for up to 24 h. Specific up-regulation of Parkin mRNA was observed in tunicamycin- or 2-ME-treated cells (p < 0.001), whereas no significant change was seen in untreated cells at 24 h (Fig. 3B). Consistent with the observation of Parkin mRNA up-regulation, the Parkin protein expression level was increased severalfold during the unfolded protein stress tests (Fig. 3C).

FIG. 3. Parkin is up-regulated by unfolded protein stress. A, SH-SY5Y cells were treated with a variety of stresses. After 5 h, total RNA was extracted and subject to quantitative RT-PCR using a Parkin-specific probe and primers. Messenger RNA level of each sample was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level. The mRNA levels of Parkin represent the -fold increase compared with the amount obtained from no treatment (Control). The error bars represent the S.D. calculated from triplicate samples. MMS, methyl methanesulfonate. *, p < 0.001. B, SH-SY5Y cells were treated with tunicamycin (10 μg ml-1) or 2-ME (7.5 mM) for the indicated times. The Parkin mRNA was measured and calculated as in A. *, p < 0.001 versus time 0 h. C, total cell lysates from SH-SY5Y cells in B were analyzed by Western blotting with anti-Parkin Ab. An arrowhead indicates the protein of unknown nature, which was reproducibly detected by anti-Parkin Ab only in tunicamycin-treated samples; it may represent a processed form of Parkin. ER stress response was assessed by the induction of GRP78 (BiP). Total protein level of each sample was confirmed by the amount of actin.

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Based on this observation, we anticipated that the up-regulation of Parkin might protect cells from unfolded protein stress. To investigate whether Parkin suppresses unfolded protein stress-induced cell death in neuronal cells, we performed an unfolded protein stress-induced cell death assay using SH-SY5Y cells (Fig. 4, A and B). Parkin-transfected cells were significantly more resistant to unfolded protein stress-induced cell death following treatment with either 3 mM 2-ME or 10 μg ml-1 tunicamycin for 24 h (p < 0.01 versus control cells). In contrast, mutated Parkin proteins that had no E2 recruiting activity (Ex4(-), T240R) had no anti-cell death activity at all, indicating that the E3 ubiquitin ligase activity of Parkin is
essential for the suppression of unfolded protein stress-induced cell death (Fig. 2B). No differences among the percentages of dead cells in wild-type Parkin, mutated Parkin cDNAs, and empty vector transfectants treated with H$_2$O$_2$ for 24 h were observed. On the other hand, XIAP, an endogenous inhibitor of cell death proteases (caspase-3/-7/-9) (21), suppressed cell death induced by any of these stress treatments. These results indicate that Parkin specifically blocks unfolded protein stress-induced cell death rather than acting as a general cell death inhibitor. Consistent with the idea that Parkin exerts an anti-cell death function through ubiquitin-proteasome-mediated protein degradation, the protective effect of Parkin over 2-ME or tunicamycin-induced cell death was significantly reduced by treatment with lactacystin, a potent proteasome inhibitor ($p < 0.01$ versus untreated cells) (Fig. 4C).

In response to stress in the ER, the unfolded protein response (UPR), which regulates gene expression, is induced. A very recent DNA microarray study revealed that UPR up-regulates multiple ER and secretory pathway genes, including ER-associated protein degradation (ERAD)-related genes in yeast (22). The ERAD system eliminates misfolded ER proteins via degradation in the cytosol. ERAD substrates are retrotranslocated across the ER membrane into the cytosol, where they are degraded through the ubiquitin-proteasome pathway (23). It is an interesting hypothesis that Parkin-E3 may be involved in ERAD, since it is located in microsomal fractions in addition to the cytosol and Golgi fractions (24). The deletion of the parkin gene may lead to accumulation of misfolded substrate protein(s) in the ER, resulting in the nigral cell death that causes AR-JP.

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FIG. 4. Parkin suppresses unfolded protein stress-induced cell death. A. SH-SY5Y cells were transfected with empty plasmid (vector), FLAG-Parkin, Ex4(–), T240R, or XIAP with pEFGP vector. After 24 h, cells were treated with or without $\Delta$No$_2$, $\Delta$O$_2$ (600 µM), 2-ME (3 mM), or tunicamycin (Tuni, 10 µg/ml) for 24 h, then about 300 GFP-positive cells were counted. Of them, cells with round or shrunken shapes were counted as dead cells (Morphology), or propidium iodide dye exclusion assay was performed (PI). The error bars represent the S.D. calculated from triplicate samples. **, $p < 0.01$ versus respective control. B, morphology of the GFP-positive cells in B. C, the effect of proteasome inhibitor on cell death suppression by Parkin overexpression. SH-SY5Y cells transfected with FLAG-Parkin were treated with 2-ME or tunicamycin as in A. At 12 h prior to the cell death assay, cells were treated with (+) or without (–) 50 µM lactacystin. Cell death assay was performed as in A. **, $p < 0.01$. 

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