

Aberrant Folding of Pathogenic Parkin Mutants

AGGREGATION VERSUS DEGRADATION*

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Loss-of-function mutations in the Parkin gene (*PARK2*) are responsible for the majority of autosomal recessive Parkinson disease. A growing body of evidence indicates that misfolding and aggregation of Parkin is a major mechanism of Parkin inactivation, accounting for the loss-of-function phenotype of various pathogenic Parkin mutants. Remarkably, wild-type Parkin is also prone to misfolding under certain cellular conditions, suggesting a more general role of Parkin in the pathogenesis of Parkinson disease. We now show that misfolding of Parkin can lead to two phenotypes: the formation of detergent-insoluble, aggregated Parkin, or destabilization of Parkin resulting in an accelerated proteasomal degradation. By combining two pathogenic Parkin mutations, we could demonstrate that destabilization of Parkin is dominant over the formation of detergent-insoluble Parkin aggregates. Furthermore, a comparative analysis with HHARI, an E3 ubiquitin ligase with an RBR domain highly homologous to that of Parkin, revealed that folding of Parkin is specifically dependent on the integrity of the C-terminal domain, but not on the presence of a putative PDZ-binding motif at the extreme C terminus.

Parkinson disease (PD)² is the second most common neurodegenerative disease after Alzheimer disease. Although most PD cases occur sporadically, familial variants share important features with sporadic PD, most notably the demise of dopaminergic neurons in the substantia nigra pars compacta. Consequently, insight into the function of PD-associated genes might promote our understanding of pathogenic mechanisms not only in familial, but also in sporadic PD. Five genes have unambiguously been linked to PD over the past decade, the genes encoding α -synuclein and LRRK2 for autosomal dominant PD, and those encoding Parkin, PINK1, and DJ-1 for autosomal recessive PD (reviewed in Refs. 1–3). So far, over a hundred different pathogenic mutations in the parkin gene have been identified, which account for the majority of autosomal

recessive PD cases. Parkin is a member of the RBR (ring between ring fingers) protein family, characterized by the presence of two RING domains (really interesting new gene), which flank a cysteine-rich in-between RINGs (IBR) domain. Similarly to other RBR proteins, Parkin has an E3 ubiquitin ligase activity, mediating the attachment of ubiquitin to substrate proteins (4–6). Parkin can obviously mediate different modes of ubiquitylation, including monoubiquitylation, multiple monoubiquitylation, and polyubiquitylation both via lysine 48 and lysine 63, depending on the experimental conditions and the putative Parkin substrate analyzed (reviewed in Refs. 7 and 8). Importantly, the neuroprotective activity of Parkin seems to be associated with its ability to promote degradation-independent ubiquitylation (9, 10).

Different lines of evidence indicate that pathogenic parkin mutations result in a loss of Parkin function. Our initial studies revealed that misfolding and aggregation is characteristic for C-terminal deletion mutants of Parkin based on the following biochemical features specific for mutant Parkin: 1) insolubility in non-ionic and ionic detergents; 2) sedimentation in a sucrose step gradient; 3) resistance to a limited proteolytic digestion; 4) loss of membrane association; and 5) formation of scattered aggregates in cells determined by immunocytochemistry (11, 12). Alterations in the detergent solubility of Parkin and formation of Parkin aggregates/inclusion bodies have also been reported for various Parkin missense mutants (13–18). We also observed that even wild-type Parkin is prone to misfolding under severe oxidative stress (12). Remarkably, insoluble, catechol-modified Parkin could be detected in the substantia nigra of patients suffering from sporadic PD, suggesting a more general role of Parkin in the pathogenesis of PD (19). In support of this concept, the E3 ligase activity of Parkin has been shown to be impaired by nitrosative stress, and there is indeed evidence for the presence of S-nitrosylated Parkin in the brains of PD patients (20, 21).

Based on our finding that the deletion of C-terminal amino acids results in misfolding and aggregation of Parkin, we performed a comparative analysis of Parkin and HHARI, an E3 ubiquitin ligase with an highly homologous RBR domain. Although Parkin and HHARI share their propensity to misfold under severe oxidative stress, HHARI tolerates C-terminal deletions. However, the C-terminal domain of HHARI cannot rescue folding of C-terminally truncated Parkin. Instead, Parkin-HHARI fusion proteins are characterized by destabilization and rapid proteasomal degradation. This observation prompted us to analyze the two different phenotypes of

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² The abbreviations used are: PD, Parkinson disease; E3, ubiquitin-protein isopeptide ligase; wt, wild type; HA, hemagglutinin; CMV, cytomegalovirus; pAb, polyclonal antibody; mAb, monoclonal antibody; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; HHARI, human homologue of *Drosophila* ariadne.

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Parkin misfolding, *i.e.* aggregation or destabilization, in more detail with a special focus on the specific role of the C terminus in Parkin folding.

EXPERIMENTAL PROCEDURES

Expression Constructs, Cell Culture, and Transfections—The following constructs were described previously: wild-type (wt) human Parkin, the W453X ($\Delta 453$ –465), and R42P Parkin mutant, HA-ubiquitin, and IKK γ -FLAG (11, 12). Amino acid deletions and substitutions of Parkin were introduced by PCR cloning techniques: Δ FDV ($\Delta 463$ –465), Parkin-*D.m.*CT (chimeric construct of human Parkin 1–450 and *Drosophila* Parkin 468–482), V465E, V465A, D464A, F463A, and R42P-W453X. All constructs were inserted into the pcDNA3.1 plasmid. The cDNA of HHARI was amplified by PCR from the RZPD clone IRATp970D0877D with the following primer pair: forward 5'-GCGGCTGAATTCGGATGGACTCGGACGAGGGCTAC-3', reverse 5'-GGAGGCGGCCGCTCAGTCCTCAATGTAC-TCCACAG-3'. The amplified fragment was digested with the restriction enzymes EcoRI and NotI and cloned into the pCMV-HA vector to obtain an N-terminally HA-tagged cDNA of HHARI. A C-terminal HHARI deletion mutant ($\Delta 377$ –557) was generated using PCR cloning techniques, as well as fusion proteins of Parkin and HHARI: Parkin 1–449/HHARI 376–557 (449/376), Parkin 1–453/HHARI 379–557 (453/379), and Parkin 1–453/HHARI 395–557 (453/395). The plasmids encoding enhanced yellow fluorescent protein and cyan fluorescent protein (control protein) were purchased from Clontech. SH-SY5Y (German Resource Centre for Biological Material number ACC 209) and HEK293T (ATCC number CRL-1573) cells were cultivated and transfected with Lipofectamine/Plus (Invitrogen) as described earlier.

Antibodies and Reagents—The following antibodies were used: anti-Parkin rabbit polyclonal antibody (pAb) hP1 (12), anti-Parkin pAb #4230 (Cell Signaling), anti-Parkin mouse monoclonal antibody PRK8 (mAb, Millipore) anti-HA (mAb, Roche Applied Science), anti-active caspase-3 pAb (Promega, Madison, WI), Alexa 555-conjugated goat anti-rabbit pAb, anti-FLAG M2-HRP mAb, anti-HA-HRP mAb, anti- β -actin mAb (Sigma), anti-transferrin-receptor mAb (Zymed Laboratories Inc.), anti-glyceraldehyde-3-phosphate dehydrogenase mAb (Ambion), anti-LDH pAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GFP mAb (BD Biosciences), horseradish peroxidase (HRP)-conjugated anti-mouse, and anti-rabbit IgG antibody (Promega). Kainate was purchased from Calbiochem, and complete protease inhibitor mixture from Roche Applied Science.

Western Blot Analysis—Proteins were analyzed by SDS-PAGE and Western blotting using polyvinylidene difluoride membranes (Millipore). The polyvinylidene difluoride membranes were blocked with blocking solution containing 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and then incubated with the primary antibody in blocking solution for 16 h at 4 °C. After extensive washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibody for 40 min at room temperature. Following washing with TBS-T, the antigen was

detected with the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

Detergent Solubility Assay—As described earlier (22), transfected cells were harvested and lysed in detergent buffer (0.1% Triton X-100 or 0.5% Triton X-100/0.5% sodium deoxycholate in PBS). After centrifugation at $16,000 \times g$ for 20 min at 4 °C, supernatant and pellet fractions were separated. The pellet fraction was washed with lysis buffer and resuspended in Laemmli sample buffer in a volume equal to the supernatant. To compare the relative distribution of the protein of interest, equal percentages of detergent-soluble and -insoluble fractions were analyzed by Western blotting.

Metabolic Labeling of Cellular Proteins—Cells were starved for 30 min in methionine-free Dulbecco's modified Eagle's medium (Invitrogen) and then labeled with 300 μ Ci/ml Pro-mix L-[35 S] *in vitro* cell label mix (Amersham Biosciences) in methionine-free Dulbecco's modified Eagle's medium for 1 h (pulse). When indicated, the proteasomal inhibitor MG132 (Calbiochem) was present during the pulse and chase periods. For the chase, labeling medium was removed, and cells were washed twice and then incubated in complete Dulbecco's modified Eagle's medium for 1 h. Radiolabeled cells were lysed in detergent buffer and fractionated into detergent-soluble and -insoluble fractions as described above. The supernatants were precleared with protein A-Sepharose (Pierce) for 30 min, the primary antibody hP1 was added, and the samples were incubated at 4 °C for 16 h. The antigen-antibody complexes were captured by the addition of immobilized protein A and then washed three times with detergent buffer. Proteins present in the immunoprecipitates were released from the protein A-Sepharose by the addition of Laemmli sample buffer containing 1% SDS and heating at 95 °C for 5 min. Immunoprecipitates were analyzed by SDS-PAGE. Gels were impregnated with Amplify (Amersham Biosciences), dried, and exposed to film.

Ubiquitylation Assay—Parkin or Parkin mutants, HA-ubiquitin, and when indicated IKK γ -FLAG were co-transfected in HEK293T cells. One day after transfection, protein lysates were prepared in denaturing lysis buffer (50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 1% SDS, 15 units/ml DNase I, and protease inhibitor mixture) and incubated at 95 °C for 5 min. Protein extracts were diluted 1:10 with non-denaturing lysis buffer (50 mM Tris/HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitor mixture). Immunoprecipitation of Parkin was performed with hP1 pAb followed by an incubation with protein A beads (Pierce); immunoprecipitation of IKK γ -FLAG was performed with M2 FLAG-agarose (Sigma). Immunoprecipitated proteins and input samples were analyzed by Western blotting using the antibodies indicated.

Immunocytochemistry and Fluorescence Microscopy—SH-SY5Y or HEK293T cells were grown on glass coverslips, transfected, fixed 1 day after transfection in 3% paraformaldehyde/sucrose in PBS for 10 min at room temperature, and permeabilized with 0.2% Triton X-100. Fixed cells were incubated with primary antibody (diluted in 1% bovine serum albumin and 10% goat serum) for 1 h at room temperature. After

washes with PBS, the coverslips were incubated with fluorescently labeled secondary antibodies for 1 h at room temperature. Finally, cells were embedded in Mowiol mounting medium (Calbiochem). Images were obtained on a Zeiss LSM 510 confocal microscope.

Membrane Fractionation—Transfected cells were harvested, incubated in hypo-osmotic buffer, and Dounce homogenized. After a low speed spin the homogenate was mixed with 60% iodixanol (Optiprep, Axis Shield) to obtain a final iodixanol concentration of 40%. The mixture was overlaid in a SW55 tube with 2.5 ml of 28% iodixanol diluted with TNE (50 mM Tris, 150 mM NaCl, pH 7.4) and 1 ml TNE on top. After ultracentrifugation in an MLS 50 swing-out rotor (Beckman) at $165,000 \times g$ for 3 h, fractions were collected from top to bottom. Aliquots of these fractions were analyzed by Western blotting.

Fractionation of Mouse Brain Tissue—Mouse brains were isolated from 2-month-old mice, homogenized with 10 strokes in a glass potter in hypotonic buffer (20 mM citrate, 1 mM EDTA, and protease inhibitor mix). The tissue was further processed by a step of freeze-thaw using liquid nitrogen. After addition of 1% Brij-53 (Pierce), homogenates were low spin centrifuged to yield a post-nuclear supernatant. Glycerol was added to the post-nuclear supernatant to a final concentration of 5%. An ultracentrifugation step of $130,000 \times g$ for 60 min at 4 °C resulted in a cytosolic fraction and a membrane fraction. The membrane fraction was resuspended in hypotonic buffer containing 1% Triton X-100 and ultracentrifuged for another 30 min. The supernatant and cytosolic fractions were analyzed by Western blotting.

Apoptosis Assay—Activation of caspase-3 was determined as described previously (23). Briefly, SH-SY5Y cells were grown on glass coverslips. 24 h after transfection, cells were incubated with kainate (500 μ M) for 3 h. The cells were then fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 10 min at room temperature, and blocked with 1% bovine serum albumin in PBS for 1 h at room temperature. Fixed cells were incubated with anti-active caspase-3 antibody overnight at 4 °C, washed, and incubated with Alexa 555-conjugated secondary antibody for 1 h at room temperature. After extensive washing, cells were mounted onto glass slides and examined by fluorescence microscopy using a Zeiss Axioscope 2 plus microscope. To detect cells undergoing apoptosis, the number of activated caspase-3-positive cells out of at least 300 transfected cells was determined. Quantifications were based on at least three independent experiments.

Stress Treatment and Proteasomal Inhibition—To induce oxidative stress, transfected cells were incubated with 10 or 20 mM H_2O_2 in PBS for 30 min, harvested, and lysed in detergent buffer containing 0.5% Triton X-100/sodium deoxycholate in PBS. For inhibition of the proteasome, cells were treated with 5 μ M MG132 (Calbiochem) for 16 h or 30 μ M for 1 h during metabolic labeling.

Statistical Analysis—Data were expressed as means \pm S.E. All transfections were performed in triplicates and repeated at least three times. Quantification of Western blots was performed with the FluoChem 8900 detection system and the Alpha Ease FC software. Statistical analysis among

groups was performed using one-way analysis of variance. *p* values were <0.05 .

RESULTS

HHARI Is Sensitive to Stress-induced Misfolding but Tolerates C-terminal Deletions—Another E3 ubiquitin ligase harboring an RBR domain is encoded by the human homologue of *Drosophila* ariadne (HHARI) gene. In comparison to Parkin, HHARI contains a glycine-rich region but lacks the N-terminal UBL domain (Fig. 1A). Recently, the solution structure of HHARI RING2 was determined by NMR studies, revealing a distinct topology of RING2 from classic RINGs (24). Based on the high sequence homology of RING2 between HHARI and Parkin, Capili and coworkers proposed a similar fold for Parkin RING2. To test whether the propensity of Parkin to misfold is a common feature of RBR proteins or a unique feature of Parkin, we generated a deletion mutant of HHARI (HHARI P378X), which was truncated behind the RBR domain, similarly to the pathogenic Parkin W453X mutant. A detergent solubility assay after expression of the mutants in HEK293T cells revealed that in comparison to wild-type (wt) HHARI there was no significant increase in the detergent-insoluble fraction when P378X was analyzed (Fig. 1B, right panel). In contrast, the Parkin W453X mutant almost quantitatively adopted an insoluble conformation under the same conditions (Fig. 1B, left panel), indicating that HHARI is significantly less sensitive to C-terminal truncations. Next we analyzed the propensity of Parkin and HHARI to misfold under severe oxidative stress. HEK293T cells transiently expressing wt Parkin or wt HHARI were subjected to hydrogen peroxide treatment and analyzed by the detergent solubility assay. As shown in Fig. 1C, high level oxidative stress induced the misfolding of both Parkin and HHARI, whereas yellow fluorescent protein was not affected under the same conditions. In conclusion, Parkin and HHARI share the sensitivity to stress-induced misfolding, which seems to be attributable to the cysteine-rich RBR domain. The arrangement of cysteines within RING domains is crucial to stabilize the native conformation, thus oxidation of critical cysteine residues will result in a collapse of the tertiary structure. However, the sensitivity to C-terminal deletions seems to be specific to Parkin.

The C-terminal Portion Adjacent to RING2 of HHARI Cannot Replace That of Parkin—To better understand the role of the C terminus in Parkin folding, we addressed the question of whether the C-terminal domain distal to RING2 of HHARI can compensate for the folding defect of C-terminally truncated Parkin. The rationale of this approach was the observation that folding of non-classic RING domains extends into the adjacent C-terminal region to stabilize RING2 (24). Because RING2 of Parkin and HHARI are supposed to share the tertiary structure, it is conceivable that the C-terminal domain of HHARI allows stabilization of Parkin RING2. To address this possibility experimentally, we generated three Parkin-HHARI chimeric constructs, differing in the length of either the Parkin or HHARI fragment (449/376, 453/379, and 453/395, Fig. 1D). HEK293T cells were transiently transfected with the chimeric constructs and analyzed by a detergent solubility assay 1 day after transfection. Surprisingly, we could not detect either of the chimeric proteins in a Western blot analysis. To test the

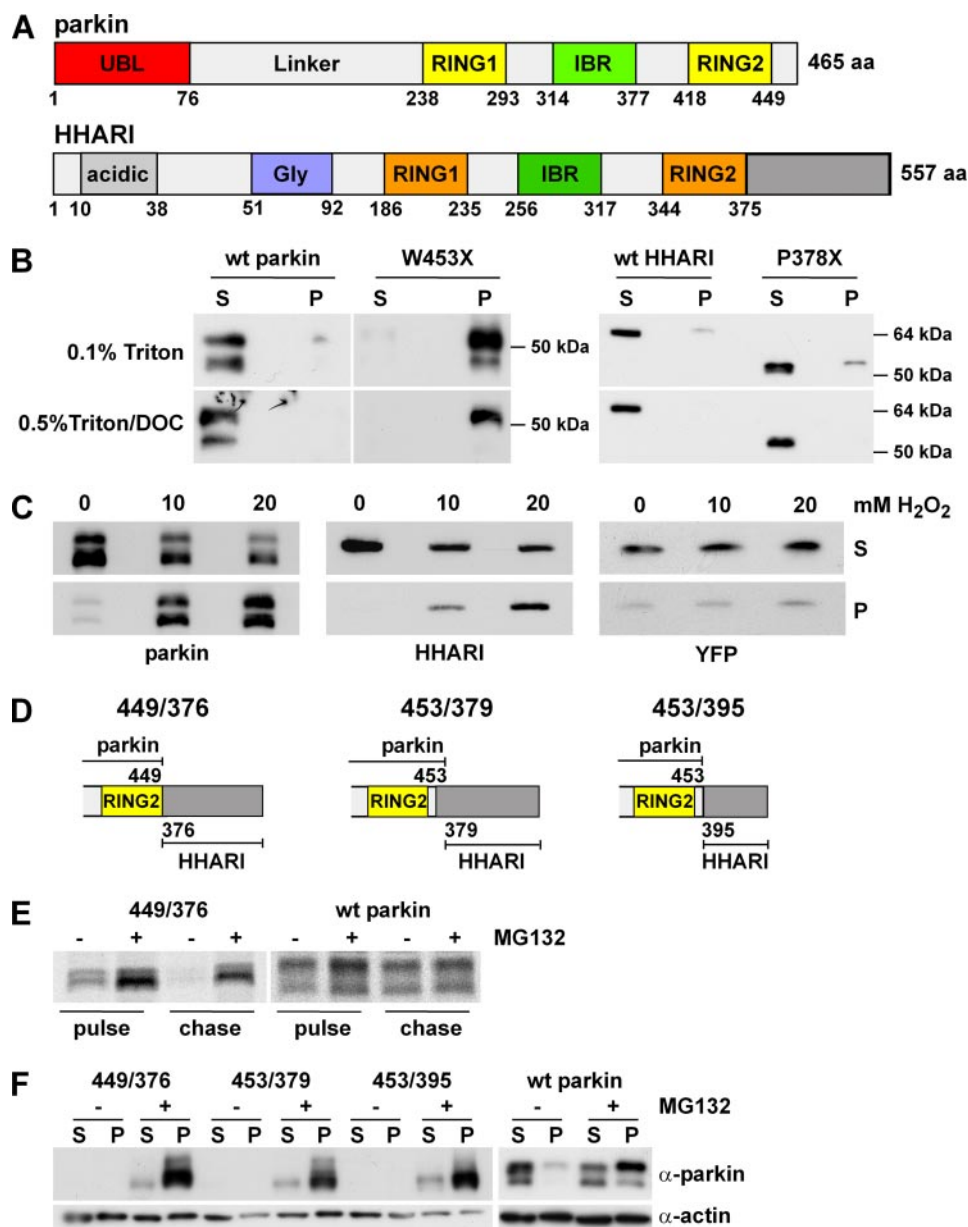


FIGURE 1. Folding characteristics of Parkin and HHARI. *A*, modular structure of Parkin and HHARI. *B*, Parkin is particularly vulnerable to C-terminal truncations. HEK293T cells were transiently transfected with wild-type (wt) and truncated mutants of Parkin or HA-tagged HHARI, harvested, lysed in 0.1% Triton X-100 or 0.5% Triton X-100/0.5% deoxycholate (DOC). Detergent-soluble (S) and insoluble (P) fractions were obtained by centrifugation and analyzed by Western blotting. Parkin was detected by the hP1 antiserum, and HHARI by an anti-HA monoclonal antibody. *C*, Parkin and HHARI are similarly vulnerable to misfolding induced by oxidative stress. HEK293T cells transiently expressing Parkin, HHARI, or yellow fluorescent protein (as a non-RBR protein) were treated with H₂O₂ at the concentrations indicated. After 30 min, cells were harvested, lysed in 0.5% Triton X-100/0.5% deoxycholate (DOC), and analyzed by the detergent solubility assay and Western blotting. *D–F*, chimeric Parkin/HHARI proteins containing the C-terminal portion of HHARI adjacent to RING2 are detergent-insoluble and rapidly degraded by the proteasome. *D*, schematic presentation of the chimeric Parkin/HHARI proteins. *E*, HEK 293T cells transiently expressing the Parkin-HHARI construct 449/376 or wt Parkin were metabolically labeled with medium containing [³⁵S]methionine for 1 h (pulse) and chased for 1 h in the presence or absence of the proteasomal inhibitor MG132. The proteins were immunoprecipitated using the anti-Parkin antiserum hP1. *F*, the three chimeric Parkin/HHARI proteins and wt Parkin were transiently expressed in HEK293T cells in the presence or absence of the proteasomal inhibitor MG132 (5 μM, 16 h) and analyzed by the detergent solubility assay as described under *B*.

possibility of an increased proteasomal degradation of the chimeric proteins, we performed pulse/chase experiments in 449/376-expressing cells metabolically labeled with [³⁵S]methionine and analyzed the chimeric protein by immunoprecipitation. Indeed, >50% of the chimeric protein was degraded dur-

ing the pulse (60 min), and after a chase of 60 min the protein could only be detected in the presence of MG132 (Fig. 1E). We then repeated the Western blot analysis and incubated the transfected cells in the presence of the proteasomal inhibitor MG132 for 16 h. It turned out that the chimeric proteins could be stabilized by MG132, but all of them were present in a predominantly detergent-insoluble conformation (Fig. 1F). Thus, the C-terminal domain of HHARI could not compensate for the folding defect observed for C-terminal deletion mutants of Parkin. Moreover, replacement of the C-terminal domain of Parkin next to RING2 by that of HHARI not only induced the formation of detergent-insoluble Parkin, but also led to a destabilization of the chimeric protein. These observations indicate that the C-terminal domain of Parkin exerts an essential and specific function in folding and stabilization of Parkin.

Misfolding of Pathogenic Parkin Mutants Can Lead to Aggregation or Destabilization of Parkin—The destabilization of Parkin-HHARI fusion proteins reminded us of some pathogenic Parkin mutants, which are also characterized by an accelerated proteasomal degradation. We reported previously that missense mutations within the N-terminal UBL domain induce a destabilization of Parkin (11). We now wondered whether this destabilization might be another manifestation of Parkin misfolding. To test this possibility, we expressed the R42P Parkin mutant in the absence and in the presence of the proteasomal inhibitor MG132. Indeed, the larger Parkin species (52 kDa), which comprises the UBL and thus the pathogenic mutation, was only detectable in the presence of the proteasomal inhibitor and predominantly occurred in the detergent-insoluble fraction (Fig. 2A).

Please note that the smaller Parkin species (42 kDa) is generated due to the presence of an internal translation initiation site at codon 80, consequently, the smaller Parkin species lacks the UBL domain (11). This experiment indicated that the R42P mutant is degraded by the proteasome, because it obviously

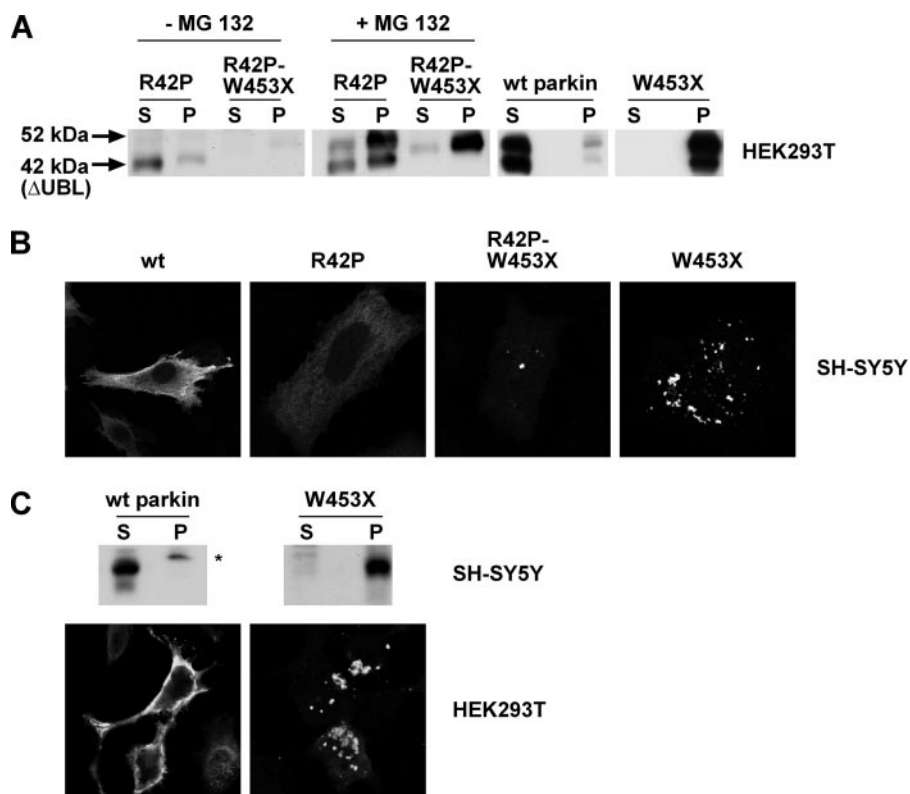


FIGURE 2. Two phenotypes of Parkin misfolding. A, HEK293T cells were transiently transfected with wt or mutant Parkin and treated the proteasomal inhibitor MG132 (5 μ M, 16 h). Parkin present in the soluble and insoluble fraction was analyzed by the Western blotting as described under Fig. 1B. B, SH-SY5Y cells transiently expressing wt Parkin or the mutants indicated were analyzed by indirect immunofluorescence using the anti-parkin antiserum hP1. C, the misfolding phenotype of mutant Parkin is not cell type-specific. Upper panel: SH-SY5Y were transiently transfected with either wt or W453X Parkin. A detergent solubility assay was performed as described under Fig. 1B. The asterisk (left panel) indicates a non-specific band. Lower panel: HEK293T cells transiently expressing wt or W453X Parkin were analyzed by indirect immunofluorescence using the anti-Parkin antiserum hP1.

does not adopt a native conformation, similarly to the chimeric Parkin-HHARI constructs. Given that the formation of relatively stable detergent-insoluble aggregates is also a consequence of Parkin misfolding, exemplified by C-terminal deletion mutants and various point mutants, we asked which phenotype of Parkin misfolding might be dominant over the other. To this end, we generated the R42P-W453X double Parkin mutant. To facilitate the interpretation of the results, the internal translation initiation site, *i.e.* methionine at position 80 was replaced by threonine, which occurs at position 80 in murine Parkin. Remarkably, the R42P-W453X double Parkin mutant could be detected only in the presence of the proteasomal inhibitor MG132 in an almost exclusively detergent-insoluble conformation, indicating that destabilization of Parkin is dominant over the formation of stable aggregates (Fig. 2A). In line with the detergent solubility assay, immunofluorescence experiments performed with SH-SY5Y cells showed typical scattered aggregates of the W453X Parkin mutant, but almost no expression of the R42P-W453X double Parkin mutant under steady state conditions (Fig. 2B). The weak staining of R42P-expressing cells can be explained by the presence of the smaller Parkin species, which lacks the R42P mutation and therefore is not misfolded. We used SH-SY5Y cells for immunocytochemistry, because HEK293T cells display a disadvantageous nucleus-to-cytoplasm ratio for the analysis of cytosolic

proteins. To provide evidence that the misfolding phenotype of Parkin mutants is not a cell type-specific phenomenon, we performed immunofluorescence experiments also in HEK293T cells and detergent solubility assays in SH-SY5Y cells (Fig. 2C).

The Putative PDZ-binding Motif at the C Terminus Is Dispensable for Parkin Folding—In previous studies we showed that pathogenic C-terminal truncations lead to misfolding and aggregation of Parkin (11, 12). Remarkably, the deletion of three C-terminal amino acids was sufficient to drastically interfere with the native folding of Parkin, leading to the formation of detergent-insoluble, aggregated Parkin (Fig. 3A). It has been suggested that the three amino acids (FDV) at the extreme C terminus of Parkin function as a PDZ-binding motif, which can mediate an interaction with the PDZ domain-containing proteins CASK and PICK1 (25, 26). We therefore addressed the question of whether this motif is necessary for Parkin to obtain or stabilize its native conformation. Interestingly, whereas other functional domains of Parkin, such as the UBL, RING1,

RING2, and the IBR, are highly conserved between species, the FDV motif is present only in mammalian species, but not in non-mammalian vertebrates (*Gallus gallus*, *Fugu rubripes*, and *Danio rerio*) or non-vertebrates (*Drosophila melanogaster*, *Anopheles gambiae*, and *Caenorhabditis elegans*, Fig. 3B). The FDV sequence of Parkin falls into the class II PDZ-binding motif Φ -X- Φ , where Φ is a hydrophobic amino acid and X is any amino acid. To destroy the putative PDZ-binding motif, we replaced valine at position 465 by glutamic acid (V465E), a mutation that has been reported previously to disrupt PDZ-dependent interactions (27). In addition, we replaced the C-terminal domain of human Parkin (amino acids 451–465) by that of *Drosophila melanogaster* Parkin (amino acids 468–482), which lacks the C-terminal valine residue and thus a functional PDZ-binding motif (Parkin-*D.m.CT*). The detergent solubility assay revealed that both PDZ mutants, V465E and Parkin-*D.m.CT*, were almost entirely found in the detergent-soluble fraction, similarly to wt Parkin (Fig. 3C, upper panel). In line with this observation, the indirect immunofluorescence analysis did not show differences in the cellular distribution of wt Parkin and the C-terminal Parkin mutants (Fig. 3C, lower panel). To determine which amino acid of the FDV motif is crucial for Parkin folding, we replaced either phenylalanine, aspartate, or valine with alanine (V465A, D464A, and F463A). We observed that only the F463A mutant adopts a detergent-

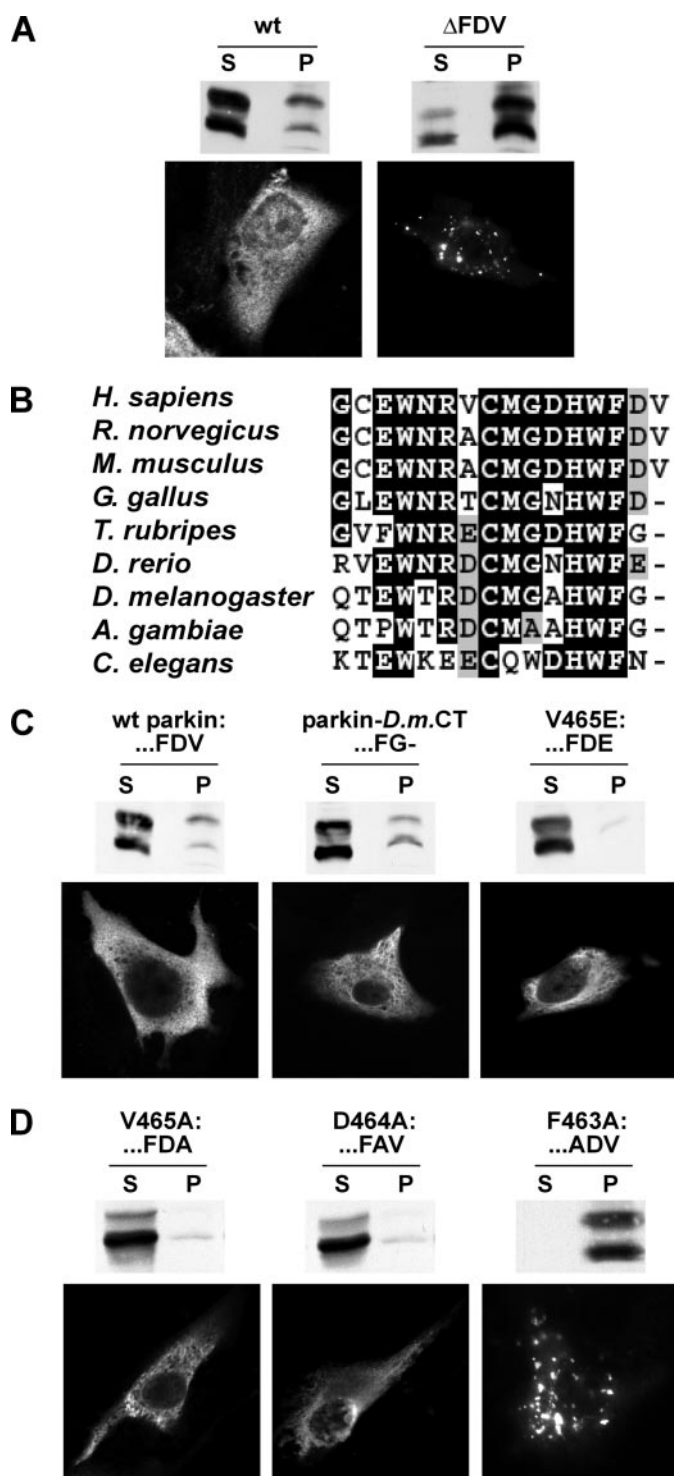


FIGURE 3. Role of the putative PDZ-binding motif in Parkin folding. A, deletion of the last three amino acids (FDV), comprising a putative PDZ-binding motif, leads to misfolding of Parkin. HEK293T (detergent solubility assay) or SH-SY5Y cells (indirect immunofluorescence) were transfected with wt Parkin or C-terminally truncated Parkin (ΔFDV) and analyzed as described in Fig. 2 (A and B). B, alignment of Parkin C termini of different species. The FDV motif is conserved only in mammalian species. C, the integrity of the FDV motif is dispensable for Parkin folding. HEK293T and SH-SY5Y cells were transiently transfected with wt Parkin or the Parkin mutants Parkin-*D.m.CT* (the last 15 amino acids of human wt Parkin replaced by the respective C-terminal amino acids of *Drosophila* parkin) or V465E (putative PDZ-binding motif disrupted) and analyzed by the detergent solubility assay and indirect immunofluorescence. D, the presence of phenylalanine at position 463 is crucial for Parkin folding. HEK293T and SH-SY5Y cells were analyzed as described above.

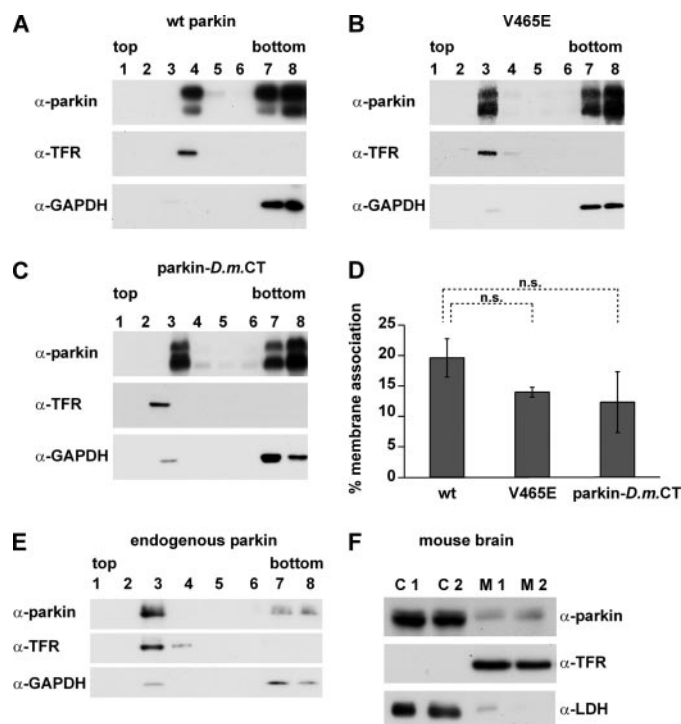


FIGURE 4. The putative PDZ-binding motif is not necessary for the binding of Parkin to cellular membranes. A–C, HEK293T cells were transiently transfected with wt Parkin or the Parkin mutants indicated. Total cell homogenates were subjected to density gradient centrifugation, and eight fractions were analyzed by Western blotting using antibodies against Parkin, the cytosolic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or the transmembrane protein transferrin receptor (TFR). D, quantification of membrane-associated Parkin or Parkin mutants (three independent experiments). Error bars indicate \pm S.E.; n.s., not significant. E and F, membrane association of endogenous Parkin. E, homogenates of SH-SY5Y cells were subjected to density gradient centrifugation and analyzed as described above. F, homogenates of mouse brain tissue were differentially centrifuged to enrich a microsomal fraction. To identify cytosolic (C)- and membrane (M)-enriched fractions, transferrin receptor (TFR) and lactate dehydrogenase (LDH) antibodies were used.

insoluble conformation and forms aggregates in cells, indicating that the phenylalanine at position 463 is essential (Fig. 3D). Of note, in contrast to the putative PDZ-binding motif, this phenylalanine is conserved between all species (Fig. 3B).

Membrane Association of Parkin Is Independent on the Integrity of the Putative PDZ-binding Motif—PDZ domains act as modules and scaffolds for protein-protein interactions and play a prominent role in organizing protein complexes at the plasma membrane. We and others have observed that Parkin can associate with membranes (11, 25, 28–30), which is recapitulated here for endogenous Parkin in SH-SY5Y cells (Fig. 4E) and mouse Parkin in brain lysates (Fig. 4F). Therefore, we analyzed a possible role of the putative PDZ-binding motif in targeting Parkin to membranes. HEK293T cells were transiently transfected with wt Parkin or the C-terminal PDZ Parkin mutants (V465E, Parkin-*D.m.CT*), and cell homogenates were fractionated by a density gradient centrifugation. The majority of wt Parkin was found in the bottom fractions, representing the cytosolic fractions. However, a fraction of wt Parkin colocalized with the transferrin receptor, indicating membrane association of Parkin (Fig. 4A). The Parkin constructs with a mutated PDZ-binding motif, Parkin-*D.m.CT* and V465E, were not significantly impaired in their ability to associate with membranes,

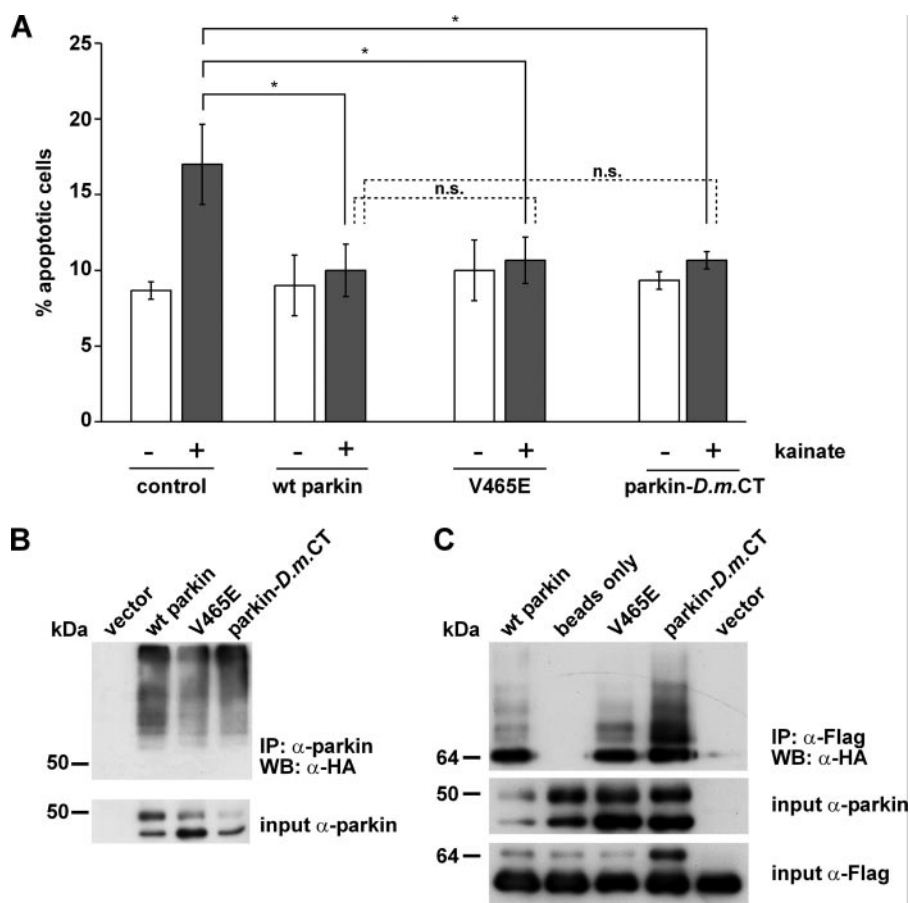


FIGURE 5. A, the neuroprotective activity of Parkin is not dependent on the putative PDZ-binding motif. SH-SY5Y cells were co-transfected with the indicated Parkin construct and yellow fluorescent protein to visualize transfected cells. 24 h after transfection, cells were incubated with 500 μ M kainate for 3 h at 37 $^{\circ}$ C, fixed, permeabilized, and analyzed by indirect immunofluorescence using an antibody against active caspase 3. Shown is the percentage of apoptotic cells among the transfected cells. *, $p < 0.05$; n.s., not significant. B, ubiquitylation of Parkin and PDZ Parkin mutants. HEK293T cells were transfected with the indicated Parkin constructs and HA-tagged ubiquitin. Parkin was immunoprecipitated under denaturing conditions and analyzed by Western blotting using an anti-HA antibody. C, ubiquitylation of IKK γ by Parkin and PDZ Parkin mutants. HEK293T cells co-expressing wt or mutant Parkin, IKK γ -FLAG, and HA-ubiquitin and were lysed, and IKK γ was immunoprecipitated with an anti-FLAG antibody under denaturing conditions. The immunocomplexes were analyzed by Western blotting using an anti-HA antibody.

suggesting that the integrity of the putative PDZ binding motif is not essential for membrane targeting, at least under steady-state conditions (Fig. 4, B–D). Of note, the fraction of membrane-associated endogenous parkin might even be higher, due to saturation of Parkin-binding sites at the membrane.

Parkin Lacking a Functional PDZ-binding Motif at the C Terminus Is Not Impaired in Its Neuroprotective Capacity—Parkin has been shown to protect neurons from stress-induced cell death in various model systems (reviewed in Refs. 7 and 31). We recently showed that activation of the NF- κ B signaling pathway is an essential prerequisite for the neuroprotective capacity of Parkin (10). Given that PDZ domains have the capacity to assemble components of signaling pathways to specific subcellular sites, we reasoned that the putative PDZ-binding domain might play a role in orchestrating signaling pathways that mediate the neuroprotective activity of Parkin. To test this hypothesis, we analyzed the cytoprotective activity of Parkin mutants with a non-functional PDZ-binding motif. For this assay we used SH-SY5Y cells, which are characterized by a neuron-like phenotype and a dopaminergic capacity. SH-SY5Y cells tran-

siently expressing wt or mutant Parkin were incubated in the presence or absence of kainate, an excitotoxin that activates ionotropic glutamate receptors. Apoptotic cells were identified by indirect immunofluorescence using an antibody specific for activated caspase-3. As shown in Fig. 5A, the protective activity of V465E and Parkin-D.m.CT in response to kainate treatment was comparable to that of wt Parkin. Thus, the putative PDZ-binding motif at the C terminus of Parkin seems not to contribute to the neuroprotective activity of Parkin. To extend the functional analysis, we performed ubiquitylation experiments. Wt or mutant Parkin was co-expressed with HA-tagged ubiquitin in HEK293T cells, immunoprecipitated with an anti-Parkin antibody, and subjected to Western blotting using an anti-HA antibody. No significant differences in the amount of ubiquitylated Parkin could be detected (Fig. 5B). Next we tested the activity of wt and mutant Parkin to enhance ubiquitylation of IKK γ /NEMO (10). HEK293T cells were co-transfected with wt or mutant Parkin and FLAG-tagged IKK γ /NEMO and HA-tagged ubiquitin. Immunoprecipitation under denaturing conditions was performed with an anti-FLAG antibody, and precipitated proteins were subjected to a Western blot

analysis using an anti-HA antibody. This experiment revealed that destroying the putative PDZ-binding motif at the C terminus does not interfere with the capacity of Parkin to promote ubiquitylation of IKK γ /NEMO (Fig. 5C). Ubiquitylation of IKK γ /NEMO was even higher in the presence of the C-terminal Parkin mutants; however, this observation can be explained by differences in the Parkin expression level. Nevertheless, the ubiquitylation activity of Parkin seems not to be dependent on the presence of the C-terminal PDZ-binding motif.

DISCUSSION

The formation of misfolded protein conformers is a common pathological denominator in various neurodegenerative diseases. PD is a paradigm for the possible consequences of protein misfolding. Misfolding can induce a gain of toxic function, exemplified by α -synuclein, a loss of physiological function, as shown for Parkin, or even a combination of both, which seems to apply to α -synuclein (reviewed in Ref. 32). Misfolding of Parkin induced by pathogenic mutations or cellular stress has been established as a major mechanism of Parkin inactivation,

accentuating a possible pathological role of Parkin even in sporadic PD (11–21, 33–35). It is obvious that the high cysteine content found in the RBR domain predisposes Parkin to oxidative stress-induced inactivation and misfolding, a phenomenon that has recently been shown experimentally (36). Interestingly, in comparison to other RBR proteins Parkin seems to be uniquely sensitive to dopamine-induced inactivation (33, 36).

Unfortunately, the three-dimensional structure of full-length Parkin has not been determined so far, which might be due to the high propensity of Parkin to misfold and the consequent difficulties in generating sufficient amounts of natively folded recombinant Parkin. In this study we describe two different manifestations of Parkin misfolding, leading to a loss of Parkin function. Pathogenic mutations leading to conformational alterations can induce either the formation of detergent-insoluble, aggregated Parkin, or the destabilization of Parkin, resulting in its rapid proteasomal degradation. In addition, we addressed intrinsic determinants of Parkin folding with a special focus on the role of the C-terminal domain.

Role of the Putative PDZ-binding Motif in Parkin Folding—Based on our previous observation that the deletion of more than two C-terminal amino acids drastically interfered with the folding of Parkin, we concentrated on a possible functional role of the last three C-terminal amino acids of Parkin, which have been proposed to constitute a PDZ-binding motif (25). Whereas all the other domains are highly conserved between species, the putative PDZ-binding motif can only be found in mammalian species, compatible with an additional function evolved in mammals. Replacing the C-terminal domain of human Parkin by that of *Drosophila* Parkin (Parkin-*D.m.CT*), which lacks a PDZ-binding motif as well as converting the predicted PDZ-binding motif into a non-functional one (V465E), did not alter the folding properties of Parkin. Because interactions between PDZ proteins and PDZ-binding motifs mediate the assembly of protein complexes specifically at membranes, we expected that the putative PDZ-binding domain of Parkin is involved in the targeting of Parkin to membranes. However, we could not detect significant differences between wild-type Parkin and mutant Parkin (Parkin-*D.m.CT*, V465E) in binding to cellular membranes determined by density gradient centrifugation under steady-state conditions. This observation does not exclude the possibility that, under certain conditions, for example in response to a specific stimulus, a transient PDZ-dependent interaction occurs. To determine the functional relevance of the putative PDZ-binding domain, we tested the neuroprotective capacity of the PDZ Parkin mutants in comparison to wild-type Parkin. Both mutants lacking a functional PDZ-binding domain (Parkin-*D.m.CT* and V465E) protected neuronal cells from stress-induced cell death similarly to wild-type Parkin. In line with this observation, the ubiquitylation activity of the PDZ Parkin mutants was not impaired in comparison to wild-type Parkin. Our results allow two interpretations. Either the C-terminal FDV motif is not an authentic PDZ-binding domain (no conservation between species, only low stringency consensus sequence), or an interaction of Parkin with PDZ proteins, such as CASK or PICK1 (25, 26), is associated with other properties or activities of Parkin

than those we addressed in our study (folding, membrane association, neuroprotective activity).

The Two Facets of Parkin Misfolding: Aggregation and Degradation—To further address the role of the C-terminal domain in Parkin folding, we performed a comparative analysis between Parkin and HHARI, which also contains an RBR domain close to the C terminus. We found that HHARI is sensitive to oxidative stress-induced misfolding similarly to Parkin, which is in line with recent observations (33, 36). However, the propensity to misfold upon C-terminal truncations was specific for Parkin. We then replaced the C-terminal portion of Parkin by that of HHARI, to test whether the tolerance to C-terminal truncations can be transferred to Parkin. This strategy was based on the commonly held view that RING2 of the RBR domain extends into the adjacent C-terminal region to stabilize its fold (24). Surprisingly, different strategies to generate such a chimeric Parkin-HHARI protein resulted in the formation of unstable, misfolded conformers. Thus, although the RBR domain of Parkin and HHARI show a high degree of homology and possibly the same fold, the role of the C terminus in Parkin folding is unique.

Destabilization of Parkin is also induced by some pathogenic missense mutations within the UBL domain (11). The behavior of the chimeric Parkin-HHARI proteins led us to uncover the reason for the instability of the R42P mutant, which we described previously as the most unstable UBL mutant. Our results indicate that this mutant is rapidly degraded by the proteasome due to the formation of a non-native conformer. Our findings are in line with a recent study on the folding and structure of the UBL domain of Parkin. Safadi and Shaw showed by NMR spectroscopy that the R42P mutation causes the complete unfolding of the UBL (37).

In conclusion, our study demonstrates that conformational alterations of Parkin induced by pathogenic mutations can lead to either a decrease in detergent solubility and aggregation or destabilization of Parkin. Although our observations are based on overexpression of pathogenic Parkin mutants and aggregate formation not necessarily occurs in patients (38), there are consistent biochemical differences between wild-type Parkin and mutant Parkin, which are not dependent on expression levels: alterations in detergent solubility, sedimentation in a sucrose gradient, and resistance to a limited proteolytic digestion (C-terminal deletion mutants) as well as rapid proteasomal degradation (R42P). The fact that misfolding of Parkin can occur in two facets, aggregation or destabilization, is an interesting feature, which needs further mechanistic analysis. Conceptually, pathogenic mutations might induce the formation of different Parkin conformers or might affect Parkin folding at distinct stages of the folding pathway.

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Aberrant Folding of Pathogenic Parkin Mutants: AGGREGATION VERSUS DEGRADATION

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