The Cellular Protein Level of Parkin Is Regulated by Its Ubiquitin-like Domain*

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Parkin is a ubiquitin-protein isopeptide ligase (E3) involved in ubiquitin/proteasome-mediated protein degradation. Mutations in the parkin gene cause a loss-of-function and/or alter protein levels of parkin. As a result, the toxic build-up of parkin substrates is thought to lead to autosomal recessive juvenile Parkinsonism. To identify a role for the ubiquitin-like domain (ULD) of parkin, we created a number of hemagglutinin (HA)-tagged parkin constructs using mutational and structural information. Western blotting and immunocytochemistry showed a much stronger expression level for HA-parkin residues 77–465 (without ULD) than HA-parkin full-length (with ULD). The deletion of ULD in Drosophila parkin also caused a sharp increase in expression of the truncated form, suggesting that the function of the ULD of parkin is conserved across species. By progressive deletion analysis of parkin ULD, we found that residues 1–6 of human parkin play a crucial role in controlling the expression levels of this gene. HA-parkin residues 77–465 showed ubiquitination in vivo, demonstrating that the ULD is not critical for parkin auto-ubiquitination; ubiquitination seemed to cluster on the central domain of parkin (residues 77–313). These effects were specific for the ULD of parkin and not transfection-, toxic-, epitope tag-, and/or vector-dependent. Taken together, these data suggest that the 76 most NH2-terminal residues (ULD) dramatically regulate the protein levels of parkin.

Dopaminergic neurons found in the substantia nigra pars compacta are progressively lost in the crippling neurodegenerative movement disorder, Parkinson’s disease (1). Autosomal recessive juvenile Parkinson’s (ARJP), which recapitulates the classical parkinsonian symptoms of tremor, rigidity and bradykinesia, is characterized by an early age of onset, and it is linked to loss-of-function mutations in the parkin gene and has a recessive mode of inheritance where both alleles of parkin are mutated (2–7). Parkin has been suggested to be an E3 ligase involved in ubiquitin/proteasome-mediated protein degradation via the ubiquitin/proteosome pathway (12, 13). The lack of parkin function (i.e. mutation in both alleles) is thought to lead to the progressive accumulation of its substrates leading to cell stress, degeneration, and eventually death of dopaminergic neurons (7).

Human parkin is 465 amino acids in length (~52 kDa) with its residues 1–76 forming a ubiquitin homology-like domain (ULD) that shares 62% homology to ubiquitin and may be involved in target protein recognition (14): residues 145–232 forming a central domain with unknown function, residues 237–449 making a carboxyl-terminalRING(R1)-IBR-RING(R2) domain involved in substrate and E2 interaction (2, 10, 15–17), and the extreme three carboxyl-terminal residues creating a PDZ binding motif (18). Recently a number of proteins have been shown to interact with parkin (for reviews, see Refs. 8 and 19). These include the E2 enzymes UbcH7 and UbcH8 (10, 11, 20), a septin GTPase named CDCrel-1 (11), parkin-associated endothelin receptor-like (Pael) (9, 20), O-glycosylated o-synuclein (10), synphilin-1 (21), calcium/calmodulin-dependent serine protein kinase (CASK/Lin2) (18), and more recently HSP-70 and CHIP1 (22). Parkin has also been shown to interact with actin filaments but not microtubules in COS1 cells (23), to bind actin and the actin-binding protein, filamin (24), and be associated with synaptic vesicles (25).

In addition to ubiquitination of its substrates, parkin is also known to catalyze its own ubiquitination and proteasomal-mediated degradation, thus regulating its own cellular levels (11, 26). The E2 enzymes involved in parkin self-ubiquitination are currently unknown. Nevertheless, a set of studies have shown that E2 enzymes, UbcH7 and UbcH8, interact with the RING-IBR-RING domain parkin and are involved in the ubiquitination of parkin substrates (9–11, 14, 20, 26). More specifically, in one report both UbcH7 and UbcH8 were shown to bind to parkin with equal affinities (20). Another group have isolated a parkin-UbcH7 complex from human brain (10) and showed that UbcH8 weakly binds parkin (14). A third report suggested that parkin only interacts with UbcH8 and that the low expression level of UbcH7 in brain makes it less physiologically relevant for interaction with parkin (11). Finally, in yeast two-hybrid studies UbcH7 has been shown to interact with a human homologue of Drosophila ariadne (HHARI) and UbcH7-associated protein 1 (H7-AP1) (16, 17). Both HHARI and H7-AP1 contain a RING-IBR-RING domain and together with parkin constitute a family of parkin/ariadne like ubiquitin ligases (PAULs) (7). In these studies, the weak interaction between parkin-UbcH8 and HHARI-UbcH8 was suggested not to be physiologically relevant (17).

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‡ The abbreviations used are: ARJP, autosomal recessive juvenile Parkinson’s; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating protein; E3, ubiquitin-protein isopeptide ligase; ULD, ubiquitin-like domain; GST, glutathione S-transferase.
Regulating Protein Levels of Parkin

The structural domains within parkin that may regulate its expression and/or self-ubiquitination have not been clearly investigated. In addition, the E2 enzymes involved in parkin ubiquitination and control its degradation rate also remain unclear. Our present study investigates some of the factors that may control cellular protein levels of parkin. Here, we demonstrate that the ULD of parkin, specifically its first six residues, is important for regulating protein levels of parkin in the cell. These data suggest that residues 77–313 of parkin play an important role in self-ubiquitination.

EXPERIMENTAL PROCEDURES

Plasmids Construction and Molecular Biology—cDNA fragments of parkin (human clone AB009973; Drosophila clone AE003593) corresponding to the residues indicated in Fig. 1 were amplified using PCR and subcloned into the mammalian expression vector pCl (Promega, Madison, WI) or into the Sf9/baculovirus GST tag expression vector pGEX-PreScission (Amersham Biosciences) by standard cloning procedures. Amino acid residue mutations and deletions were introduced in Drosophila cDNA using QuickChange™ kit (Stratagene, Amsterdam, Netherlands). A hemagglutinin (HA) or a Myc epitope tag was added to the NH2 terminus of human parkin or Drosophila parkin, respectively. Similar procedures were used for the creation of Myc-UbcH7 (human clone X92962) and Myc-UbcH8 (human clone AF031141) mammalian expression constructs. Isolation of all DNA from transformed Escherichia coli was performed using Qiagen plasmid kits (Qiagen, Basel, Switzerland). The integrity of constructs was verified by DNA sequencing as described by manufactures (Applied Biosystems-PerkinElmer Life Sciences, Rotkreuz, Switzerland).

Cell Culture and Biochemistry—HEK-293 cells were grown in 80-cm2 flasks using Dulbecco’s modified Eagle’s medium/Ham’s F-12 culture media (3.151 g/liter glucose, with 0.524 g/liter L-alanyl-L-glutamine (Invitrogen, Basel, Switzerland) supplemented with 10% dialyzed fetal calf serum (Amimed, Basel, Switzerland) at 37 °C in a 5% CO2 incubator. HEK-293 cells were transfected as described previously (27) with 0.5–1 μg each plasmid DNA in the presence of Opti-MEM using LipofectAMINEPLUS reagent (Invitrogen). The cells were used ~48 h after transfection. For expression analysis the cells were scraped from multwell plates, the cell pellets solubilized in 50–100 μl of radioimmune precipitation assay buffer, and the samples (10–20 μl) denatured. Electrophoretic separation was carried out on 10 or 12% SDS-polyacrylamide gels (BioWhittaker, Allschwill, Switzerland) as described previously (27). The blots were developed either by incubating in alkaline phosphatase buffer (Promega), and immunoreactivity was visualized as a blue-purple color or by incubating in enhanced chemiluminescence (ECL) supersignal west memto substrate reagent (Pierce, Bonn, Germany) and then exposed to film and developed. For immunocytochemistry, transfected HEK-293 cells were grown on coverslips and immunocytochemistry performed as described previously (27).

Antibodies—Polyclonal rabbit primary antibodies include anti-parkin (AbCam, Cambridge, UK), anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-ubiquitin (Calbiochem, Schwalbach, Germany). Monoclonal mouse primary antibodies include anti-Myc (Onoguen, Boston, MA) and anti-HA (Santa Cruz Biotechnology). The secondary antibodies that were alkaline phosphatase-conjugated were goat anti-rabbit IgG (Promega) and goat anti-mouse IgG (Promega). The secondary antibodies that were horseradish peroxidase-conjugated were goat anti-rabbit IgG (Sigma) or goat anti-mouse IgG (Sigma). The secondary antibodies used in immunocytochemistry were Texas Red-X goat anti-rabbit IgG (Molecular Probes, Eugene, OR) or Texas Red-X goat anti-mouse IgG (Molecular Probes). All antibodies were used at dilutions recommended by the manufacturer. Protein concentrations were determined using the Bio-Rad protein assay kit using serum bovine albumin as a standard (Bio-Rad, Munchen, Germany).

RESULTS AND DISCUSSION

The ULD of Parkin Regulates Parkin Expression but Not Its Ubiquitination—The parkin gene (PARK2) was identified by positional cloning from genetic materials by Japanese families affected by ARJP (2). It was shown that ARJP has a recessive mode of inheritance where both alleles of parkin are mutated (4). To date several mutations in parkin have been linked to ARJP (2–7). We generated a number of parkin constructs to examine the function of the parkin domains and the effects of disease-causing mutations on parkin expression (Fig. 1A). When equivalent amounts of cDNA were transfected we found that cellular levels of NH2-terminaI HA-tagged full-length parkin, HA-parkin-FL, proteins (either wild type or mutants) were too low for detection by Western blot analysis (Fig. 1B). In contrast, all the constructs where residues 1–76 (ULD) were removed, HA-parkin-77 deletion mutants, showed high levels of expression in HEK-293 cells (Fig. 1B). None of the point mutations tested were found to significantly alter the...
expression levels of HA-parkin-FL or HA-parkin-77.

The self-ubiquitination of parkin is an event that controls parkin degradation rates (14, 24). In some reports, overexpressed parkin has been found ubiquitinated in vivo (20), while in other studies parkin was not ubiquitinated (14). To explain these results, it has been suggested that the higher expression levels of parkin lead to the better detection of ubiquitinated parkin (20). The molecular mass of full-length parkin is ~52 kDa, the mass of HA-parkin-77 is ~43 kDa, and the molecular mass of ubiquitin is ~8.5 kDa. In our study, HA-parkin-77-expressed proteins showed band sizes corresponding to the molecular masses of native (~43 kDa) and monoubiquitinated (~51 kDa) HA-parkin-77 (Fig. 1B). We also detected band smears for some (but not all) HA-parkin-77 proteins, indicating these band patterns are specific for particular forms of HA-parkin-77 (Fig. 1B). Of interest, when HA-parkin-77-transfected cells were resuspended, sonicated, and rotated in PT×E buffer (phosphate-buffered saline, 1% Triton X-100, 0.1 mM EDTA, pH 7.4; see Ref. 27), these band patterns were found predominantly in the cell pellet fraction as compared with the cell sonicate. As reported previously, we suggest these high molecular weight band patterns to represent polyubiquitinated parkin (20). Specifically, we found HA-parkin constructs encoding only the central domain and RING1 (residues 77–313) were mono- and polyubiquitinated, whereas those encoding the central domain alone (residues 77–237) were mono- but not polyubiquitinated (Fig. 1B). Taken together, this data indicate that residues 1–76 (the ULD) of parkin regulate its protein levels, and as a result the high protein levels of HA-parkin-77 lead to elevated ubiquitination. Furthermore, the removal of the ULD of parkin does not significantly alter parkin ubiquitination and that parkin polyubiquitination requires the presence of central and RING1 domains but not the ULD.

To examine the effects of UbcH7 and UbcH8 on the protein levels and ubiquitination of parkin we co-expressed them with UbcH7 or UbcH8. Overexpression of UbcH7 or UbcH8 appeared neither to increase parkin ubiquitination nor to regulate the expression of HA-parkin-FL or HA-parkin-77 (data not shown). Interestingly, the proteasome inhibitor MG132 also showed no significant effects on the expression levels of HA-parkin-FL or HA-parkin-77 (data not shown). These results suggest that the ubiquitination of parkin may be associated with a regulation of its function rather than proteolysis-associated ubiquitination or that proteolysis-associated ubiquitination of overexpressed HA-parkin-FL or HA-parkin-77 may not be the (only) mechanism controlling their cellular protein levels. Next, we performed a number of in vitro ubiquitination assays to identify E2 enzymes involved in the auto-ubiquitination of HA-parkin-77. Despite observing the ubiquitination of p27 (as control), we did not observe any evidence of parkin self-ubiquitination using the E2 enzyme UbcH7 or other E2 enzymes UbcH2, -3, -5a, -5b, -6, -7, and -10 (data not shown). These results are in agreement with a previous report suggesting that UbcH7, and perhaps UbcH8, are involved in parkin-substrate ubiquitination, but these two enzymes do not directly affect parkin self-ubiquitination (20). We also attempted to clarify conflicting data from previous studies suggesting that parkin interacts with only UbcH7, or UbcH8, or both (11, 14, 20). In yeast two-hybrid assays (and co-immunoprecipitation studies) we were unable to observe any specific interaction between parkin and UbcH7 or UbcH8, despite identifying novel interacting proteins for full-length parkin (parkin-FL) in a large-scale yeast two-hybrid study.2 Interestingly, in yeast two-hybrid studies, we found that the N-terminal deletion mutant parkin 77–465 (but not parkin-FL or parkin 1–76) showed a strong activation of β-galactosidase, suggesting that residues 1–76 of parkin may also play a negative role in gene transcription (data not shown). In agreement, parkin has been suggested to play a role in transcription and/or in ubiquitinating a nuclear substrate (28).

Parkin Expression Is Not Transfection-, Toxic-, Epitope Tag-,
and/or Vector-dependent—Numerous investigators have successfully expressed full-length wild type and mutant parkin using different tags in HEK-293 cells and neuroblastoma cells (18, 20, 21). Within these studies, some differences have been noted in terms of E2 enzymes that interact with parkin and the ubiquitination of overexpressed parkin (compare Refs. 10–11, 14, and 20). These differences have previously been linked, in part, to unknown cell type-specific factors, possibly E2 enzymes, co-factors, and/or associated proteins (20). Although reasons for the poor expression of HA-parkin-FL is presently unclear, our current studies have enabled to detect differences between HA-parkin-FL and HA-parkin-77 and thus determine the effects of ULD on parkin expression. To further validate our hypothesis that the ULD of parkin plays a role in regulating its cellular protein levels, we have performed a number of control experiments.

First, as control, we observed a single, specific, and antibody concentration-dependent band for native full-length parkin in a variety of cell types and brain tissue regions (Fig. 2A). Of note, we did not find endogenous full-length parkin to be ubiquitinated (Fig. 2A). Second, to determine whether the differences in HA-parkin-77 and HA-parkin-FL protein expression were due to differences in transfection methods, we tested a number of transfection protocols (Fig. 2B). LipofectAMINE and NeuroPorter transfection complex mixtures gave similar results showing that HA-parkin-77 was highly expressed compared with that of HA-parkin-FL and that these results were not due to differences in transfection methods (Fig. 2B). Third, to exclude the possibility that lack of parkin-FL expression is not due to epitope tag- and/or vector type-dependent, we tested the role of ULD in a Myc-tagged Drosophila parkin. In HEK-293 cells, we found that Drosophila Myc-parkin showed no expression whereas Drosophila Myc-parkin-93 (lacking the ULD, residues 1–92) showed high expression levels (Fig. 2B). Finally, to confirm that the lack of parkin-FL expression was not due to detection problems caused by our parkin gene sequence or by antibody recognition, we used a large batch non-mammalian cellular expression system. Using SB/baculovirus expression, we still found that GST-parkin-FL was poorly expressed as compared with GST-parkin-77 (Fig. 2C). However, by using large culture volumes and affinity purification we were able to observe small amounts of GST-parkin-FL, further indicating that the differences in GST-parkin-77 and GST-parkin-FL are not due to the epitope tag and/or vector type used (Fig. 2C).

Taken together, these results indicate that the ULD of parkin regulates not only the protein levels of human parkin but also Drosophila parkin and that these effects are not dependent on the epitope tags and/or vectors used.

To further examine the role of the ULD of parkin, we performed immunocytochemistry to observe the distribution patterns of parkin 77–465 on a single cell level and to determine any low level expression of parkin-FL. In HEK cells, the distribution pattern of endogenous parkin was found to be diffuse and cytoplasmic (Fig. 2D). No oddities (for example clusters, endoplasmic reticulum retention, and/or nuclear build up) were found in the distribution patterns of transfected HA-tagged human parkin constructs (Fig. 2D). In agreement with Western blotting results, in these studies, we found low protein levels of HA-parkin-FL as compared with the high expression levels of

2 K. K. Dev, unpublished data.
HA-parkin-77. Similar results were found using Myc-tagged 
Drosophila parkin, where full-length Myc-parkin showed a 
lower expression level as compared with Myc-parkin-93 (Fig. 2D). As control, the E2 enzymes showed similar expression 
levels as human HA-parkin-77 and Drosophila Myc-parkin-93, 
indicating that expression was not non-specifically dependent 
on the used vector or epitope tag (Fig. 2D).

To test for differences in toxicity between HA-parkin-FL and 
HA-parkin-77, we also determined whether transient expres-
sion of HA-parkin-FL and HA-parkin-77 altered cell viability. 
HA-parkin constructs, together with a luciferase reporter plas-
mid, were transiently transfected into HEK-293 cells. Cellular 
viability was quantified in terms of luciferase activity. When 
compared with empty pCI vector-transfected cells (taken as 
100%), HA-parkin-FL (138 ± 12%) and HA-parkin-77 (134 ± 
13%) had no drastic effects on cellular viability and in some 
cases showed a slight protective effect as suggested previously 
by other reports (9, 20, 22, 29). In contrast the transfection of 
Bax cDNA (15 ± 2%) and caspase cDNA (2 ± 0) into HEK-293 
cells resulted in cellular death. These results indicate that 
differences in HA-parkin-FL and HA-parkin-77 expression can-
not be explained by some intrinsic cytotoxicity related to these 
expression constructs (data not shown).

**Residues 1–6 of the ULD of Parkin Regulate Protein Expression**—To further characterize the exact site within the ULD of parkin responsible for controlling its protein levels, a series of 
progressive deletion mutants within the ULD of parkin were 
created and tested for expression (Fig. 3A). Using the same 
concentrations of cDNA for transfections, we found that re-

**Fig. 2.** A, native parkin was observed in a variety of cell types and brain tissue regions. Western blotting was performed using parkin antibody. B, LipofectAMINE (see “Experimental Procedures”) or NeuroPorter (see the manufacturer’s instructions, Gene Therapy Systems Inc., San Diego, CA) transfection methods were tested. Western blotting was performed using HA rabbit antibody or Myc mouse antibody. The two methods gave similar results for both human parkin and Drosophila parkin, indicating that the ULD of parkin reduces parkin expression. C, GST-(PreScission)–parkin was prepared using Sf9/baculovirus expression. After large batch culture the GST-(PreScission)–parkin was purified by standard GST 
affinity chromatography and treated with PreScission (see the manufacturer’s instructions, Amersham Biosciences). The PreScission-cleaved parkin (parkin-FL or parkin-77) was collected and processed for Western blotting using a parkin antibody. The expression of parkin-FL was only observed after longer exposure. D, single cell expression levels of parkin in HEK-293 cells. Endogenous parkin was found in HEK-293 cells as detected by parkin rabbit antibody followed by Texas Red-conjugated secondary antibody. Transiently transfected HA-tagged parkin proteins were detected by HA rabbit antibody followed by Texas Red-conjugated secondary antibody. HA-tagged human parkin (wild type) showed a low level of expression at the single cell level. On comparison parkin residues 77–465 (no ULD) showed high expression levels. Similar results were observed for Drosophila parkin. Myc-UbcH7 and Myc-UbcH8 showed similar high expression levels as human and Drosophila parkin without its ULD.

**Fig. 3.** Effects of residues 1–6 (MIVFVR) of the ULD of parkin on protein levels. A, a schematic representation of a series of deletions in the UDL of parkin is shown and was made to further investigate the site within the UDL that may regulate parkin expression levels. B, only the deletion mutant lacking the residues FAATMIVFVR (of which FAAT belong the linker sequence and MIVFVR belong to parkin) and the mutant lacking MIVFVR were expressed, suggesting that MIVFVR regulates the levels of parkin.
moval of the first six residues, namely MIVFVR, of parkin (see deletion constructs Δ1 or Δ1a) resulted in its expression detectable by Western blotting (Fig. 3B). In contrast, any parkin construct encoding the first 6 residues showed no detectable expression (Fig. 3B). These results suggest that the first 6 residues of the ULD of mammalian parkin play an important role in regulating its protein levels. Since the ULD of Drosophila parkin also appears to regulate its expression levels similar to that seen for the ULD of its human counterpart (Fig. 2B), we searched for this motif in Drosophila. Although this linear epitope is conserved in human, mouse, and rat sequences of parkin, it was not found in the Drosophila parkin (residues 1–6 correspond to MLLELLQ). Interestingly, Drosophila parkin also lacks the PDZ binding motif found at the extreme carboxyl terminus of human, mouse, and rat sequences of parkin. This PDZ binding motif is thought to be critical for parkin interaction with CASK (18). Therefore it appears that Drosophila parkin has some (but not complete) identity in its structural/functional domains as compared with mammalian parkin. Taken together, we propose that the ULD (although variable in sequence) is likely to have a conserved function through evolution in terms of regulating parkin levels in the cell.

In summary, in this study we identify two structural/functional domains within parkin, namely the ULD (residues 1–76, specifically the MIVFVR epitope) as a site that controls parkin expression and the central + RING1 domain (residues 77–313) as a site that plays a role in parkin ubiquitination. The molecular mechanism regulating parkin protein levels is not well understood. We propose that the ULD of parkin could play a role in protein-protein interaction events where this domain is recognized either directly or indirectly by an associated protein that targets parkin for proteolysis. Under normal conditions, polyubiquitination of parkin could alter proteins interacting with parkin, modulate parkin function, and/or regulate the rate parkin proteolysis. In disease or under experimental conditions an altered ULD may result in parkin no longer being recognized by accessory protein(s) and/or being degraded and thus leading to changes of parkin protein levels in the cell.

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