Identification of a Novel Zn\(^{2+}\)-binding Domain in the Autosomal Recessive Juvenile Parkinson-related E3 Ligase Parkin*3

Ventzislava A. Hristova†‡, Steven A. Beasley†, R. Jane Rylett*†‡, and Gary S. Shaw†‡

From the Departments of †Biochemistry and *Physiology and Pharmacology, University of Western Ontario, London, Ontario N6A 5C1, Canada and the †Molecular Brain Research Group, Robarts Research Laboratory, London, Ontario N6A 5K8, Canada

Missense mutations in park2, encoding the parkin protein, account for ~50% of autosomal recessive juvenile Parkinson disease (ARJP) cases. Parkin belongs to the family of RBR (RING-between-RING) E3 ligases involved in the ubiquitin-mediated degradation and trafficking of proteins such as Pael-R and synphilin-1. The proposed architecture of parkin, based largely on sequence similarity studies, consists of N-terminal ubiquitin-like and C-terminal RBR domains. These domains are separated by a ~160-residue unique parkin sequence having no recognizable domain structure. We used limited proteolysis experiments on bacterially expressed and purified parkin to identify a new domain (RING0) within the unique parkin domain sequence. RING0 comprises two distinct, conserved cysteine-rich clusters between Cys\(^{150}\)-Cys\(^{169}\) and Cys\(^{196}\)-His\(^{197}\) consisting of CX\(_2\)-CX\(_1\)-CX\(_2\)-C and CX\(_4\)-eCX\(_{10-16}\)-CX\(_{3\text{ (H/C) motifs. The positions of the cysteine/histidine residues in this region bear similarity to parkin RING1 and RING2 domains, as well as other E3 ligase RING domains. However, in parkin a 26-residue linker region separates the motifs, which is not typical of other RING domain structures. Further, the RING0 domain includes all but one of the known ARJP mutation sites between the ubiquitin-like and RBR regions of parkin. Using electrospray ionization mass spectrometry and inductively coupled plasma-atomic emission spectrometry analysis, we determined that the RING0, RING1, IBR, and RING2 domains bind two Zn\(^{2+}\) ions, the first observation of an E3 ligase with the ability to bind eight metal ions. Removal of the zinc from parkin causes near complete unfolding of the protein, an observation that rationalizes cysteine-based ARJP mutations found throughout parkin, including RING0 (C212Y) that form cellular inclusions and/or are defective for ubiquitination likely because of poor zinc binding and misfolding. The identification of the RING0 domain in parkin provides a new overall domain structure for the protein that will be important in assessing the roles of ARJP mutations and designing experiments aimed at understanding the disease.

Autosomal recessive juvenile Parkinson disease (ARJP) is a neurodegenerative disorder arising from the loss of dopaminergic neurons in the substantia nigra of the midbrain. ARJP is characterized by the onset of Parkinsonian symptoms such as tremors, rigidity, and bradykinesia. It is distinguished from the idiopathic form of Parkinson disease by the onset of symptoms, prior to the age of forty. The hereditary nature of ARJP implicates a number of mutations in the genes encoding the proteins parkin, PINK1, LRRK2, and DJ-1 as the cause of dopaminergic neurodegeneration (1–4). A variety of deletion, truncation, and point mutations distributed throughout the park2 gene, which encodes the protein parkin, have been reported in ARJP patients (1, 5–18).

Parkin functions as a ubiquitin ligase (E3) and belongs to a family of RBR (RING-between-RING) ubiquitin ligase enzymes involved in proteosome-mediated protein degradation (19–21). The currently accepted domain architecture of parkin, deduced from multiple sequence alignment, shows that the C terminus of the protein is characterized by two ~50-residue RING (really interesting new gene) domains separated by a 51-residue IBR (In-Between-RING) domain (22, 23). The RING domains of parkin are proposed to interact with the ubiquitin-conjugating enzymes UbcH7, UbcH8, Ubc7, and Ubc13 and control parkin-mediated ubiquitination of a variety of substrates such as Pael-R, synphilin-1, Sept5, and PICK1 among others (24–31). Other members of the RBR family include the human homolog of Drosophila Ariadne (HHARI), DORFIN, and HOIL-1, which share close domain architecture (32–35). Traditionally, RING domains coordinate two Zn\(^{2+}\) ions through a C\(_3\)H\(_4\)C\(_4\) metal-binding consensus sequence. However, the RING2 domain of HHARI binds a single Zn\(^{2+}\) (36), and because this is the only RING2 structure available for an RBR protein, it suggests that there may be variability in the number of Zn\(^{2+}\) ions coordinated by different RING domains. The recent three-dimensional structure of the parkin IBR domain (23) revealed a two-site zinc-binding motif with a novel fold compared with other zinc-binding motifs (37). However, despite the potential importance of zinc binding to the RING

* This work was supported by research grants from the Canadian Institutes of Health Research (to G. S. S. and R. J. R.) and the Canada Research Chairs (to V. A. H.).

† To whom correspondence should be addressed. Tel.: 519-661-4021; Fax: 519-661-3175; E-mail: gshaw1@uwo.ca.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

2 The abbreviations used are: ARJP, autosomal recessive juvenile Parkinson; E1, ubiquitin-activating enzyme; E3, ubiquitin-protein isopeptide ligase; UPD, unique parkin domain; ESI-MS, electrospray ionization mass spectrometry; ICP-AES, inductively coupled plasma-atomic emission spectrometry; GST, glutathione S-transferase; LC-MS, liquid chromatography-mass spectrometry.
domains (or other portions) of parkin, the ability and capacity for zinc coordination or its impact on structure has not been identified for parkin.

The N terminus of parkin comprises a ubiquitin-like domain (UbID) proposed to facilitate the delivery and degradation of ubiquitinated substrates by the 26 S proteasome via interactions with the 26 S a subunit (38, 39). The central ~150 residues of parkin separating the UbID from the RBR region are referred to as the unique parkin domain (UPD). This segment of parkin is essential for function, and ARIP associated mutations within this region have been shown to lead to dysfunction of parkin E3 ligase activity (40, 41). However, the absence of any sequence similarity to other proteins or the identification of a distinct domain within the UPD has made these experiments difficult to interpret. Other than the isolated UbID and IBR domains of parkin, there has been limited success with the purification and characterization of parkin, especially when lacking affinity tags in the final purified form. Bacterially expressed parkin typically shows a heterogeneous mixture of full-length and degraded protein species, making characterization of the protein difficult (42). In this work we have used purified parkin to identify a novel zinc-binding CaCa(C/H) domain upstream of the RBR region and within the UPD. We have used limited proteolysis and electrospray ionization mass spectrometry (ESI-MS) to show that this domain coordinates two Zn2+ ions in addition to six other Zn2+ ions in the RBR C terminus. The presence of a new parkin-specific zinc-binding domain provides insight into the structure of parkin and opens the door to establish the importance of this domain in ARIP for this new subclass of RBR E3 ligases.

EXPERIMENTAL PROCEDURES

Recombinant Parkin—Human parkin was cloned from the Human Fetal Brain Marathon-Ready cDNA library (Clontech) and ligated into the PCR Blunt-TOPO vector using the Zero Blunt TOPO PCR kit from Invitrogen. The human parkin sequence was then transferred to the pGEX-4T3 bacteriophage expression vector. Rat parkin cloned into a pGEX-4T1 vector was received from Dr. Ted Fon (McGill University).

Protein Expression and Purification—The pGEX-4T1 and pGEX-4T3 parkin constructs were overexpressed in Escherichia coli BL21 Codon Plus competent cells and grown at 37 °C in LB containing 50 mg/liter ampicillin until an A600 of 0.6 was recorded. Protein expression was induced by the addition of 50 mM isopropyl D-1-thiogalactopyranoside (IPTG) into the culture, with rotation at room temperature for 30 min. Removal of the IPTG tag from human and rat parkin cloned into the pGEX-4T3 and pGEX-4T1 vectors, respectively, resulted in short residual sequences preceding the N-terminal sequence of parkin. Human parkin is preceded by a GSPNS amino acid sequence, and a GSPGPARCHAPATTLPVT sequence is found N-terminal to methionine at position one of rat parkin. Parkin was separated from the GST tag on the 20-ml GST Prep FF 16/10 affinity column, using a flow rate of 1 ml/min. The flow through was concentrated to 500 µl using Amicon Ultra concentrators with a mass cut-off of 10 kDa (Millipore) and separated on a 120-µl Superdex HiLoad 16/60 gel filtration column (GE Healthcare) at a flow rate of 0.3 ml/min.

In Vitro Ubiquitination Assay—Auto-ubiquitination assays of GST-parkin and parkin were carried out in a 20 mM Tris (pH 7.4), 120 mM NaCl, 1 mM TCEP buffer using standard ubiquitin conjugation reagents and enzymes (Boston Biochem) unless indicated otherwise. Bacterially expressed GST-parkin and parkin at concentrations of 25 µM each were incubated with 90 nM of E1 enzyme, 0.2 mM ubiquitin, 2.5 µM of Ubc7 (expressed and purified as a His6-Ubc7 protein from a pET28a construct), 4 mM MgATP, and 1X reaction buffer. The reactions were carried out at 37 °C for 1 h, inhibited with E1 inhibition buffer, and then heated to 100 °C with Laemmli sample buffer prior to analysis by SDS-PAGE gel electrophoresis. Ubiquitination was identified by Western blotting with anti-ubiquitin polyclonal antibody, used according to the manufacturers protocol.

Limited Proteolysis—Parkin was subjected to limited proteolysis with both trypsin and V8 proteases. Six 140-µl samples of 50 µM parkin were incubated at 4 °C with 0.4 µg of trypsin or V8 each for 5, 10, 15, 30, 60, 90, or 120 min. Upon completion 20 µl from each sample were heated to 100 °C with Laemmli sample buffer and analyzed by SDS-PAGE with Coomassie Blue staining. Protease activity in the remainder of each reaction was inhibited with 5% formic acid; the sample was then desalted by centrifugation with protein desalting spin columns (Pierce). These samples were analyzed by ESI-MS (University of Western Ontario Biological Mass Spectrometry Laboratory). Mass fragments detected by ESI-MS were assigned to parkin sequences using the ProteinInfo amino acid sequence analysis tool PROWL.

Bioinformatics—A comprehensive list of parkin orthologs was compiled from a search of the RefSeq nonredundant protein data base (Release 29) and the Uniprot databases, resulting in a list of proteins from 28 separate species based on human isoform 1. The multiple sequence alignment was originally created using ClustalW and manually edited using Jalview. The list was parsed based the exclusion of alternate isoforms, as well as...
sequences containing probable errors such as the *Bos taurus* parkin that possesses an extended C terminus. The full-length sequences for parkin from human, mouse, rat, fly, worm, and HOIL-1 were submitted for PONDR VSL1 analysis.

Circular Dichroism Spectropolarimetry—Parkin samples in the range of 3–5 μM were analyzed using a Jasco J-810 circular dichroism spectropolarimeter. A 0.1-mm cell was used to measure protein absorbance in the UV range between 240 and 190 nm, keeping high tension voltage below 600 V. Fifty scans were collected per sample at 4 °C. EDTA was titrated into each parkin sample and equilibrated for 15 min after each addition prior to data collection. A CD spectrum of the buffer in the presence and absence of EDTA was collected at the beginning of each experiment and used for baseline correction.

Zinc Analysis by ICP-AES and Quadrupole Time-of-flight ESI-MS—Parkin, eluted from the Superdex HiLoad 16/10 gel filtration column, was submitted for ICP-AES Zn²⁺ analysis to determine the concentration of Zn²⁺ in the sample (Laboratory for Geochemical Analysis, University of Western Ontario). The concentration of parkin in the sample was identified by the guanidine hydrochloride denaturation method and amino acid analysis done at the Amino Acid Analysis Facility at the Hospital for Sick Children in Toronto, Canada. The total number of Zn²⁺ ions bound to parkin was determined by quadrupole time-of-flight ESI-MS (University of Western Ontario Biological Mass Spectrometry Laboratory) done on full-length parkin eluted from the Superdex HiLoad 16/10 column and dialyzed 1000-fold against 120 mM ammonium acetate at a pH of 7.4.

RESULTS

**Differential Stability of Rat and Human Parkin**—Stability has proven to be the major limitation in the large scale purification of bacterially expressed parkin (42). To identify the source of instability in the parkin protein, the human and rat forms of the protein were expressed as GST fusion proteins (pGEX-4T3 and pGEX-4T1) in *E. coli* BL21 Codon Plus cells. Following GST-glutathione affinity chromatography, thrombin cleavage, and a second GST-glutathione affinity step, the protein was analyzed by gel filtration chromatography. SDS-PAGE analysis of the elution profile showed the presence of bands near 52 kDa (supplemental Fig. S1) that were confirmed by ESI-MS as the rat protein (observed molecular mass, 53165.1 ± 2.8 Da; calculated molecular mass, 53167.6 Da) and human protein (observed molecular mass, 52063.6 ± 2.5 Da; calculated molecular mass, 52063.0). Although the rat form of parkin reproducibly eluted as a single purified form, the human protein consistently showed the presence of other bands, notably a band near 12 kDa. ESI-MS analysis showed this band had a mass of 11980.1 ± 2.5 Da, very close to that calculated for a C-terminal cleavage at residue Thr¹⁰³ within the UPD of human parkin by an unspecified source. The presence of this cleavage product is very similar to that previously observed by Rankin and co-workers (42). This fragment incorporated the entire UbID (residues 1–75) and the first 28 residues from the UPD. This region is the most divergent between the rat and human orthologs possessing only 38/53 (71%) similarity between residues Asp⁸⁵ and Arg¹⁴⁰. Further, sequence analysis of parkin using the program PONDR predicted a disordered state for residues 75–141 of the UPD where cleavage was noted. Based on our results, the sequence difference in rat parkin within this region prevents its cleavage and allows for the purification of milligram levels of full-length parkin lacking fusion protein tags.

**Ubiquitin Ligase Activity of in Vitro Purified Parkin**—Parkin is a ubiquitin ligase capable of auto-ubiquitination, as well as ubiquitination of other substrate proteins including PAEL-R, synphilin, and Sept5 (26, 28, 29). A number of studies have demonstrated auto-ubiquitination of parkin and various ARJP parkin mutants in vitro (13, 26, 31, 43–45) using GST, maltose-binding protein, and a variety of other N-terminally tagged parkin proteins. The availability of full-length parkin lacking a fusion tag enabled us to compare in vitro auto-ubiquitination of GST-parkin and parkin to determine whether the bacterially purified protein was properly folded in the absence of an N-terminal carrier protein.

Auto-ubiquitination reactions using GST-parkin and parkin were assessed using different combinations of ubiquitin, E1 enzyme, and the E2 ubiquitin conjugation enzyme Ubc7. For both GST-parkin and parkin a broad high molecular mass ladder was observed above the parent protein as monitored by either SDS-PAGE or Western blot analysis (Fig. 1). This ladder is characteristic of covalent modification by ubiquitin resulting in a distribution of multiple ubiquitinated forms of either GST-parkin or parkin proteins. In the absence of E1, ubiquitin, or parkin, the higher molecular mass species corresponding to a ubiquitinated parkin protein was not observed. These results indicate that both the bacterially expressed GST-parkin and parkin generated in this study are functional and capable of carrying out auto-ubiquitination in vitro. Although several studies have shown that bacterially expressed parkin carrying an N-terminal tag is functional in auto-ubiquitination assays, this work is the first to show that the untagged protein also participates in this function. This observation demonstrates that full-length parkin lacking an N-terminal tag adopts a correctly folded structure that can be used for further characterization of this E3 ligase protein.
A New Domain in Parkin Identified by V8 and Trypsin Limited Proteolysis—Sequence similarity and alignment methods have identified the N-terminal UblD (1–75) and C-terminal RBR domains (238–465) in parkin, whereas the central UPD (76–238) conforms to no known domain sequence or structural motif (22, 46). However, to date only the UblD and IBR domains (23, 38, 39) of parkin have actually been purified and characterized by protein chemistry methods. The purification of parkin now allows a more comprehensive examination of the remaining domain architecture within this E3 ligase protein, possibly establishing new domains and/or refining theoretical boundaries. To identify the structured domains in parkin and in particular in the UPD, we conducted limited proteolysis of the full-length protein using V8 and trypsin proteases.

The formation and disappearance of proteolytic fragments upon treatment of parkin with V8 protease (Fig. 2) and trypsin (Fig. 3) were used to identify domain boundaries. In both cases, the time-dependent, enzyme-mediated fragmentation of parkin showed an initial 53-kDa band, corresponding to the full-length protein that was confirmed by ESI-MS. In the absence of V8 or trypsin, proteolysis of parkin was not observed, indicating that cleavage is enzyme-dependent. During the initial stages of the digestions, a prominent band at 40 kDa formed that gradually gave way to lower molecular mass bands. At about 60 min for V8 (Fig. 2A) and 5 min for trypsin (Fig. 3A), the band for full-length parkin is no longer observable, indicating that trypsin is more efficient at cleaving the parkin protein. Identification of the proteolytic fragments of parkin at different time points was achieved through LC-MS by comparing the observed and theoretical masses specific for the potential 52 V8 and 43 trypsin proteolysis sites within the protein (Figs. 2B and 3B). For example, the addition of V8 protease to parkin showed the disappearance of the full-length protein at 53,168.1 Da (Fig. 2C) and the rapid formation of two major fragments with masses of 12,038.4 Da and 41,146.5 Da (Fig. 2D and E), corresponding to residues 1–93 and 94–464, respectively (Fig. 2B). The smaller of these fragments was identified as the UblD that underwent further proteolysis to residues 1–79 (10668.5 Da) (Fig. 2B and E). Proteolytic fragments from the trypsin digestion were also identified for residues 1–76 (10314.2 Da) and 1–75 (10186.3 Da) (Fig. 3B, D, and E). Based on the V8 and trypsin limited proteolysis data for parkin, the most susceptible region to proteolysis and therefore the least structured was found at the N terminus of the UPD where V8 and trypsin rapidly cleaved at Glu93 and Asp126 and at Arg104, respectively. This result is in agreement with our stability experiments (supplemental Fig. S1) showing that human parkin is susceptible to proteolysis in this region.

Larger fragments containing the UPD, RING1, IBR, and RING2 domains were observed at intermediate time points with stable peptides spanning the RING1-IBR-RING2 (26,065.8 Da) and portions of the UPD-RING1-IBR (25,414.4 and 29,989.7 Da) regions identified for V8 proteolysis (Fig. 2, B and D). Unlike the observation of a stable UblD fragment, the LC-MS data did not reveal V8 or trypsin fragments that consisted solely of the proposed RING1, IBR, or RING2 domains, indicating that these regions, when isolated, were rapidly degraded. Of particular interest were proteolytic fragments
noted from V8 digestion that corresponded to residues 94–322 (25,414.4 Da) and 127–399 (29,989.7 Da) and from trypsin cleavage identified as residues 105–464 (39,864.2 Da) and 141–349 (23,294.9 Da). These fragments all included a section N-terminal to the parkin RBR, but within the UPD, not previously recognized as a structured region. In addition, trypsin-mediated proteolysis yielded a stable parkin fragment with a molecular mass of 10,336.5 Da identified as residues 141–234 of the UPD that was detected by LC-MS at 90–120 min (Fig. 3, B and E). This isolated domain directly upstream of RING1 was observed despite the fact that it contains nine trypsin recognition sites, indicating that it must retain a tightly folded conformation that is resistant to trypsin cleavage.

The RING0 Domain Is Conserved in Parkin—Stable proteolytic fragments encompassing residues 127–399 from the V8 digest and residues 141–349 from the trypsin digest experiments contain the predicted RING1 and IBR domains. The observation that both of these fragments contained a considerable region N-terminal to RING1, and the isolation of a stable fragment spanning residues Pro141–Ser234 indicated that a previously unidentified structured domain exists within the UPD. To investigate this possibility we analyzed the sequence between residues Pro141–Ser234 using a multiple sequence alignment for all parkin orthologs in the RefSeq data base (Fig. 4). Surprisingly, this analysis revealed a remarkable degree of conservation for cysteine and histidine residues between residues Ser145 and His215 not previously identified by biochemical or bioinformatics experiments. In particular, two distinct clusters between Cys150 and Cys169 and between Cys196 and His215 consisting of C<sub>4</sub>C<sub>3</sub>(H/C) motifs, respectively, were identified as potential Zn<sup>2+</sup>-coordinating sites. Located ahead of RING1 in the parkin sequence, this C<sub>4</sub>C<sub>3</sub>(H/C) region is hereon referred to as RING0.

The potential Zn<sup>2+</sup>-binding sites within the RING0 domain have a unique organization (C<sub>4</sub>C<sub>3</sub>(H/C)) compared with other double Zn<sup>2+</sup>-binding motifs such as the RING (C<sub>3</sub>H<sub>4</sub>C<sub>4</sub>) and IBR (C<sub>2</sub>H<sub>5</sub>C<sub>5</sub>) domains. The observation that both of these fragments contained a considerable region N-terminal to RING1, and the isolation of a stable fragment spanning residues Pro141–Ser234 indicated that a previously unidentified structured domain exists within the UPD. To investigate this possibility we analyzed the sequence between residues Pro141–Ser234 using a multiple sequence alignment for all parkin orthologs in the RefSeq data base (Fig. 4). Surprisingly, this analysis revealed a remarkable degree of conservation for cysteine and histidine residues between residues Ser145 and His215 not previously identified by biochemical or bioinformatics experiments. In particular, two distinct clusters between Cys150 and Cys169 and between Cys196 and His215 consisting of C<sub>4</sub>C<sub>3</sub>(H/C) motifs, respectively, were identified as potential Zn<sup>2+</sup>-coordinating sites. Located ahead of RING1 in the parkin sequence, this C<sub>4</sub>C<sub>3</sub>(H/C) region is hereon referred to as RING0.

The potential Zn<sup>2+</sup>-binding sites within the RING0 domain have a unique organization (C<sub>4</sub>C<sub>3</sub>(H/C)) compared with other double Zn<sup>2+</sup>-binding motifs such as the RING (C<sub>3</sub>H<sub>4</sub>C<sub>4</sub>) and IBR (C<sub>2</sub>H<sub>5</sub>C<sub>5</sub>) domains. Extensive sequence comparisons of RBR proteins uncovered HOIL-1 as the only
other member with an additional Zn\(^{2+}\)-binding motif upstream of the RING1 domain. HOIL-1 contains a single NZF-like zinc finger ubiquitin-interacting motif in a position similar to the putative RING0 C\(_4\) zinc-binding site (47) but lacks the subsequent C\(_4\)(H/C) region identified in parkin. As such, this site in parkin and HOIL-1 would be expected to coordinate a single zinc ion in the absence of the other site (in parkin). Therefore, RING0 likely binds Zn\(^{2+}\) in a linear fashion similar to that observed in the LIM domain as opposed to the RING or PHD-like domains where the first and third pairs and the second and fourth pairs of cysteine residues bind in a cross-brace manner (36, 37). Mutation of the predicted Zn\(^{2+}\)-binding cysteines in RING0 may result in loss of parkin solubility and/or E3 ligase activity as demonstrated for other RING domains in the RBR region (7, 13, 41, 48, 49).

**Parkin Is an Octuple Coordinating Zinc-binding Protein**—
The RING1, IBR, and RING2 domains are each predicted to bind two Zn\(^{2+}\) ions. Of these, the only experimental evidence exists for the IBR domain that has been shown to coordinate two Zn\(^{2+}\) ions (23). The RING2 domain of the RBR family member HHARI has been observed to coordinate only a single Zn\(^{2+}\) ion, although it was originally thought to ligate two metal ions (36). The presence of the RING0 domain, which could potentially bind two additional Zn\(^{2+}\) ions, indicates that parkin could coordinate between five and eight Zn\(^{2+}\) ions throughout the RING0, RING1, IBR, and RING2 domains.

To determine the ability of the RING0 domain to coordinate Zn\(^{2+}\) ions and to identify the stoichiometry of Zn\(^{2+}\)-binding to parkin, we analyzed the purified protein by ESI-MS under benign and denaturing conditions (Fig. 5). Under native conditions a signal at 53676.0 Da was observed (Fig. 5A) in excellent agreement with the expected mass for parkin bound to eight Zn\(^{2+}\) ions (53674.8 Da). To confirm that the increase in mass was a result of eight equivalent ions having a net mass increase of 63.4 Da/Zn\(^{2+}\), dilute formic acid was added to the sample to generate partially denaturing conditions (Fig. 5B). Under these conditions, a near symmetric spectrum was detected comprised of eight resolved peaks each separated by about 63 Da. The symmetric profile of these mass peaks indicates the near Gaussian distribution expected for all possible Zn\(^{2+}\)-bound protein forms between one and eight bound ions. Upon complete denaturation of parkin (Fig. 5C), a single peak with mass of 53,169.8 Da was observed corresponding to the zinc-free protein.

The metal ion content for parkin was also determined using ICP-AES coupled to quantitative amino acid analysis. The ratio of these two measurements provided a stoichiometry of 8.2 Zn\(^{2+}\) ions/parkin protein, confirming the mass spectrometry data showing that eight Zn\(^{2+}\) ions are bound to the parkin protein. These results represent the first in-depth analysis of the metal composition of full-length parkin and show that the RING0, RING1, IBR, and RING2 domains each coordinate two Zn\(^{2+}\) ions.

**Zinc Binding Is Required for Proper Parkin Folding**—ARJP parkin mutations such as C253Y, C289G, and C431F substitute cysteine residues with nonligating residues within the C\(_4\)(H/C) Zn\(^{2+}\)-binding sequences of the RING1 and RING2 domains. These mutations would be expected to alter Zn\(^{2+}\) binding and have been shown to decrease parkin E3 ligase activity, indicating that Zn\(^{2+}\) coordination is crucial for protein function. In addition, removal of Zn\(^{2+}\) ions by EDTA addition to the IBR domain (23) or the HHARI RING2 domain (36) causes complete unfolding of these domains. In light of this, we investigated the effect of EDTA-induced Zn\(^{2+}\) removal on the secondary structure of parkin by CD spectropolarimetry.

The CD spectrum of parkin (Fig. 6) was consistent with a protein having ~30% \(\alpha\)-helical and 30% \(\beta\)-sheet character. EDTA was titrated against the parkin sample to determine whether the protein undergoes a gradual change in secondary structure or whether the partial removal of Zn\(^{2+}\) results in the complete unfolding of parkin. Following the addition of four molar equivalents of EDTA, the CD spectrum of parkin showed a decrease in ellipticity at 205 and 220 nm corresponding to a loss of \(\alpha\)-helical and \(\beta\)-sheet structure in the protein (Fig. 6). This change in the CD spectrum implicates a transition to a higher random coil composition upon the removal of Zn\(^{2+}\) ions. When eight molar equivalents of EDTA were added to parkin, the protein reached a point where the spectrum was no longer susceptible to EDTA-induced changes (Fig. 6), evident by the similarity of this spectrum with that where a 3-fold excess of EDTA (not shown) was added. The gradual unfolding of parkin upon the removal of Zn\(^{2+}\) ions by EDTA shows that the loss of Zn\(^{2+}\) at a single site does not result in the complete unfolding of the protein. Denaturation of parkin with 6 M gua-
A Novel Zn$^{2+}$-binding Domain in Parkin

**DISCUSSION**

This work reports the first isolation of bacterially expressed, full-length parkin lacking an N-terminal carrier tag. Further, we have used this protein to identify a novel Zn$^{2+}$-binding domain (RING0) within the UPD, previously thought to have no recognizable domain structure. The establishment of the RING0 domain redefines the widely accepted domain architecture of parkin, which has been based on multiple sequence alignment data of the UblD (residues 1–75) and RBR (residues 238–465) domains. Further, the RING0 domain identity helps shed light on previous functional experiments that focused on this region of the protein but lacked information on the domain or its zinc binding ability. The new domain structure for parkin is shown in Fig. 7.

The RING0 domain (residues 145–215) observed as a stable folded domain in trypsin and V8 limited proteolysis contains a $\text{C}_X_2\text{C}_X_3\text{C}_X_1\text{X}_2\text{C}_X_4\text{C}_X_4\text{C}_X_1\text{X}_2\text{C}_X_3\text{C}_X_4\text{C}_X_4\text{H/C}$ Zn$^{2+}$-binding consensus sequence that is highly conserved in parkin among species. Each of the zinc-binding motifs in RING0 closely matches those of other RING domains (including RING1 and RING2 in parkin), which typically consist of $\text{C}_X_2\text{C}_X_3\text{C}_X_4\text{C}_X_4\text{C}_X_1\text{X}_2\text{C}_X_3\text{C}_X_4\text{C}_X_4\text{H/C}$ patterns. Structures of individual RING domains have revealed the zinc ions are coordinated in a cross-brace fashion where the first ion is bound via the first and third pairs of cysteines, and the second ion is bound using the second and fourth pairs of cysteine/histidine residues (36, 37). A distinguishing feature between these structures and sequences is that RING0 contains an unusual 26-residue linker between its two zinc sites. Based on this it seems unlikely the RING0 domain adopts a similar cross-brace structure like other RING domains. Further, the N-terminal portion of RING0 resembles the sequence of the NZF-like zinc finger in HOIL-1 (Fig. 4) that lacks the C-terminal $\text{C}_X_4\text{C}_X_4\text{C}_X_1\text{X}_2\text{C}_X_3\text{C}_X_4\text{H/C}$ region. This would suggest that each of the N- and C-terminal portions of the parkin RING0 domain coordinate zinc separately forming a linear two-zinc domain.

This work is the first to demonstrate the Zn$^{2+}$ stoichiometry of parkin, showing that full-length parkin binds eight Zn$^{2+}$ ions, two in each of the RING0, RING1, IBR, and RING2 domains. Coordination of zinc ions is critical to the maintenance of the three-dimensional fold for parkin because removal of these metal ions causes unfolding of most of the protein. Although this has not been previously demonstrated for full-length parkin, it has been shown that removal of zinc from the IBR domain (23) or from the RING2 domain of the RBR protein HHARI (36) leads to unfolding of these domains. The necessity for zinc binding to parkin to maintain its three-dimensional structure also indicates that ARJP mutations that compromise zinc-coordinating residues would also lead to unfolding of the protein. In fact several proposed zinc coordinating residues in RING0 (Cys$^{212}$), RING1 (Cys$^{53}$ and Cys$^{789}$), and RING2 (Cys$^{418}$, Cys$^{431}$, and Cys$^{441}$) are sites of ARJP mutations.

It is also interesting that although ARJP missense mutations are generally found throughout the parkin protein, all of the mutations except one (A82E) within the previously defined UPD domain exist within the RING0 domain. ARJP-associated mutations within the RING0 domain include K161N, S167N, M192V/L, S193I, K211R/N, and C212G/Y (Fig. 7). However, prior to the identification of the RING0 domain, interpretation of the functional outcomes of these mutations (6, 8, 15–18, 53, 54) has been limited. The affects of these mutations can be classified into two groups based on their affects on parkin solubility and/or ubiquitination. The ARJP mutants K161N, M192L, and K211N exhibit cellular expression and localization that are similar to those of wild type parkin (13, 55, 56). Some experiments show that K161N also yields a protein with lower solubility that does not form inclusions (55, 56). In contrast, parkin containing a C212Y mutation yields an insoluble form of parkin, which forms aggresome-like inclusions in SH-SY5Y cells (55). Based on the zinc-binding motif and properties identified for the RING0 domain, Cys$^{212}$ is expected to be a key zinc-coordinating residue. Loss of zinc binding by the C212Y ARJP mutation likely causes its unfolding and aggregation. A corollary of this conclusion is that the ARJP mutations C289G,
C431F, and C441R found in the parkin RING1 and RING2 domains are also expected to be important zinc coordinating residues because our work shows that these domains each bind two zinc ions. These proteins and several other cysteine substituted parkin derivatives also form insoluble aggresomes consistent with disruption of zinc binding to the RING1, IBR, and RING2 domains (55, 57). In particular, a C166A substitution caused increased aggregation and insolubility compared with wild type parkin consistent with a structural zinc-binding role in RING0 (57).

Some studies have shown that ARJP mutations (K161N and K211N) within the RING0 domain have similar ubiquitination and substrate binding properties to wild type parkin, whereas other experiments show the K161N mutation causes decreased aggregation and insolubility compared with wild type parkin consistent with a structural zinc-binding role in RING0 (57).

Acknowledgments—We thank Dr. Ted Fon (McGill University) for the pGEX-4T1 vector used to express parkin and the pET28a His6-Lbc7 construct and Kathryn Barber for help with the purification of Lbc7 and technical assistance. We are also grateful to Paula Pittok, Siya Liu, and Dr. Gilles Lajoie in the University of Western Ontario Biological Mass Spectrometry Lab for help and expertise in acquiring and analyzing mass spectral data.

REFERENCES

1. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998) Nature 392, 605–608
2. Valente, E. M., Abou-Sleiman, P. M., Caputo, V., Mujit, M. M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A. R., Healy, D. G., Albanese, A., Nussbaum, R., Gonzalez-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W. P., Latchman, D. S., Harvey, R. J., Dallapiccola, B., Auburger, G., and Wood, N. W. (2004) Science 304, 1158–1160
3. Bonifati, V., Rizzu, P., van Baren, M. J., Schaap, O., Breedveld, G. J., Krieger, E., Dekker, M. C., Squitieri, F., Ibanez, P., Joosse, M., van Dongen, J. W., van Wijnen, N., van Swieten, J. C., Brice, A., Meco, G., van Duijn, C. M., Oostra, B. A., and Heutink, P. (2003) Science 299, 256–259
4. Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., Kachergus, J., Hulihan, M., Uitti, R. J., Calne, D. B., Stoessl, A. J., Pfeiffer, R. F., Patenge, N., Carabajal, I. C., Vieregge, P., Asnus, F., Muller-Myskow, B., Dickson, D. W., Meitering, T., Strom, T. M., Wszolek, Z. K., and Gasser, T. (2004) Neuron 44, 601–607
5. Hattori, N., Matsumine, H., Asakawa, S., Kitada, T., Yoshihino, H., Elbolb, B., Brookes, A. J., Yamamura, Y., Kobayashi, T., Wang, M., Yoritaka, A., Minoshima, S., Shimizu, N., and Mizuno, Y. (1998) Biochem. Biophys. Res. Commun. 249, 754–758
6. Foroud, T., Uniakec, S. K., Liu, L., Pankratz, N., Rudolph, A., Halter, C., Shults, C., Marder, K., Conneally, P. M., Nichols, W. C., and Parkinsons Study Group (2003) Neurology 60, 796–801
7. Gu, W. J., Corti, O., Araujo, F., Hampe, C., Jacquier, S., Lucking, C. B., Abbas, N., Duyckaerts, C., Roooney, T., Pradier, L., Ruberg, M., and Brice, A. (2003) Neurobiol. Dis. 14, 357–364
8. Lucking, C. B., Durr, A., Bonifati, V., Vaughan, J., De Michele, G., Gasser, T., Harhangi, B. S., Meco, G., Denefle, P., Wood, N. W., Agid, Y., and Brice, A. (2000) Engl. J. Med. 342, 1560–1567
9. West, A., Periquet, M., Lincoln, S., Lucking, C. B., Nicholl, D., Bonifati, V., Rawal, N., Gasser, T., Lohmann, E., Deleuze, J. F., Maraganore, D., Levey, A., Wood, N., Durr, A., Hardy, J., Brice, A., Farrer, M., and French Parkinson’s Disease Genetics Study Group and the European Consortium on Genetic Susceptibility on Parkinson’s Disease (2002) Am. J. Med. Genet. 114, 584–591
10. Periquet, M., Latouche, M., Lohmann, E., Rawal, N., De Michele, G., Ricard, S., Teive, H., Fraix, V., Vidalhelith, M., Nicholl, D., Barone, P., Wood, N. W., Raskin, S., Deleuze, J. F., Agid, Y., Durr, A., and Brice, A. (2003) Brain 126, 1271–1278
11. Lohmann, E., Periquet, M., Bonifati, V., Wood, N. W., De Michele, G., Bonnet, A. M., Fraix, V., Broussolle, E., Worstink, M. W., Vidalhelith, M., Verpillat, P., Gasser, T., Nicholl, D., Teive, H., Raskin, S., Rascol, O., Destee, A., Ruberg, M., Gasparini, F., Meco, G., Agid, Y., Durr, A., Brice, A., French Parkinson’s Disease Genetics Study Group, and the European Consortium on Genetic Susceptibility in Parkinson’s Disease (2003) Ann. Neurol. 54, 176–185
12. Henn, I. H., Gostner, J. M., Lackner, P., Tatzelt, J., and Winklhofer, K. F. (2005) J. Neurochem. 92, 114–122
13. Hampe, C., Ardila-Osorio, H., Fournier, M., Brice, A., and Corti, O. (2006) Hum. Mol. Genet. 15, 2059–2075
14. von Coelln, R., Dawson, V. L., and Dawson, T. M. (2004) Cell Tissue Res. 318, 175–184
15. Shyu, W. C., Lin, S. Z., Chiang, M. F., Pang, C. Y., Chen, S. Y., Hsin, Y. L., Thajeb, P., Lee, Y. J., and Li, H. (2005) Parkinsonism Relat. Disord. 11, 173–180
16. Satoh, J., and Kuroda, Y. (1999) Neuroreport. 10, 2735–2739
17. Abbas, N., Lucking, C. B., Ricard, S., Durr, A., Bonifati, V., De Michele, G., Bouley, S., Vaughan, J. R., Gasser, T., Marconi, R., Broussolle, E., Brefel-Courbon, C., Harhangi, B. S., Oostra, B. A., Fabrizio, E., Bohme, G. A., Pradier, L., Wood, N. W., Filla, A., Meco, G., Denefle, P., Agid, Y., and Brice, A. (1999) Hum. Mol. Genet. 8, 567–574
18. Pineda-Trujillo, N., Carvalaj-Carmona, L. G., Buriotica, O., Moreno, S., Uribe, C., Pineda, D., Toro, M., Garcia, F., Arias, W., Bedoya, G., Lopera, F., and Ruiz-Linares, A. (2001) Neurosci. Lett. 298, 87–90
19. Marin, I., and Ferrus, A. (2002) Mol. Biol. Evol. 19, 2039–2050
