Relative Sensitivity of Parkin and Other Cysteine-containing Enzymes to Stress-induced Solubility Alterations

Received for publication, October 6, 2006, and in revised form, February 8, 2007 Published, JBC Papers in Press, February 28, 2007, DOI 10.1074/jbc.M609466200

Esther S. P. Wong,1,2 Jeanne M. M. Tan3,4, Cheng Wang5, Zhengui Zhang, Shiam-Peng Tay, Norane Zaiden, Han Seok Ko, Valina L. Dawson,6,7 Ted M. Dawson5,12, and Kah-Leong Lim*4,11

From the 4Neurodegeneration Research Laboratory and 9Parkinson’s Disease and Movement Disorders Center, National Neuroscience Institute, Singapore 308433, Singapore 8Institute for Cell Engineering, Departments of Neuroscience, Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the Departments of 4Biological Sciences and 10Microbiology, National University of Singapore 119077, Singapore

Loss of parkin function is a predominant cause of familial Parkinsonism. Emerging evidence also suggests that parkin expression variability may confer a risk for sporadic Parkinson disease. We have recently demonstrated that a wide variety of Parkinson disease-linked stressors, including dopamine (DA), induce parkin solubility alterations and promote its aggregation within the cell, a phenomenon that may underlie the progressive susceptibility of the brain to degeneration. The vulnerability of parkin to stress-induced modification is likely due to its abundance of cysteine residues. Here, we performed a comprehensive mutational analysis and demonstrate that Cys residues residing both within and outside of the RING-IBR (in between RING fingers)-RING domain of parkin are important in maintaining its solubility. The majority of these Cys residues are highly conserved in parkin across different species and potentially fulfill important structural roles. Further, we found that both parkin and HHARI (human homologue of Drosophila arida), another RING-IBR-RING-type ubiquitin ligase, are comparably more susceptible to solubility alterations induced by oxidative and nitrosative stress when compared with other non-RING-IBR-RING Cys-containing enzymes. However, parkin appears to be uniquely sensitive to DA-mediated stress, the specificity of which is likely due to DA modification of 2 Cys residues on parkin (Cys-268 and Cys-323) that are distinct from other RING-IBR-RING members.

Parkinson disease (PD) is the most common neurodegenerative movement disorder characterized pathologically by the rather selective loss of midbrain dopaminergic neurons in the substantia nigra pars compacta and the presence of intraneuronal protein inclusions known as Lewy bodies. Although most cases of PD occur in a sporadic manner, a subset of PD cases is inheritable and attributable to mutations in specific genes. These familial PD-linked genes include α-synuclein, parkin, DJ-1, PINK1, and LRRK2 (1, 2). Of these, mutations in the parkin gene are currently recognized to be a predominant cause of familial, early onset PD (3–5). Further, emerging evidence also suggests that parkin expression variability may confer a risk for the development of the more common, sporadic form of PD (6, 7).

The importance of functional parkin to dopaminergic neuronal survival is probably related to the multitude of neuroprotective roles it serves (8, 9). Parkin functions as a ubiquitin ligase associated with protein homeostasis and apparently confers protection to neurons against a diverse range of cellular insults (8, 9). Recently, we have demonstrated that a wide variety of PD-linked stressors, including dopamine (DA), induce parkin solubility alterations and promote its aggregation within the cell (10). Our observations corroborated with a similar study conducted by LaVoie et al. (11), who further showed that DA covalently modifies parkin via its Cys residues, although the number and location of the Cys targeted by DA remain unknown. Since parkin functions as a broad spectrum neuroprotectant, the effects on parkin brought about by these oxidative stressors could deplete the availability of soluble parkin in the brain, and as such, may underlie the progressive susceptibility of the brain to degeneration.

We have previously speculated that enzymes whose structure and function are dependent on catalytic Cys are more susceptible to the consequence of oxidative modification (10). Although the active site Cys-dependent tyrosine phosphatase family of enzymes provides one example (12), the RING finger-containing ubiquitin ligases, all of which are characterized by their Cys-rich catalytic moieties, potentially represent another. In particular, the abundance of Cys residues residing on RING-IBR-RING-containing proteins, such as parkin, conceivably could present a ready source of targets for oxidative modification. As the majority of these horseradish peroxidase; HA, hemagglutinin; HMW, high molecular weight; CHIP, carboxyl terminus of the Hsc70-interacting protein; HHARI, human homologue of Drosophila aridae; UBL, ubiquitin-like; IBR, in between RING fingers.
highly conserved RING finger Cys residues are thought to fulfill important structural roles (13, 14), it is conceivable that their modification by oxidation could disrupt the overall structural integrity of the protein, leading to alterations in their biochemical properties.

In this study, we systematically mutate almost all of parkin’s Cys residues and demonstrate that conserved Cys residues residing both within and outside of the RING-IBR-RING domain of parkin are important for maintaining its solubility, although modification of Cys residues within parkin RING-IBR-RING domain also resulted in a significantly higher tendency for the protein to form aggresome-like structures within the cell. Further, we found that both parkin and HHARI, another RING-IBR-RING ubiquitin ligase, are comparably more susceptible to solubility alterations induced by oxidative and nitrosative stress when compared with other non-RING-IBR-RING Cys-containing enzymes examined. However, parkin appears to be uniquely sensitive to DA-mediated stress. Further mutational analysis suggests the involvement of 2 non-conserved Cys residues on parkin, Cys-268 and Cys-323, in DA-mediated parkin insolubility. As these residues are distinct from other RING-IBR-RING members, including HHARI, they could potentially explain for the enhanced sensitivity of parkin to modification by DA.

MATERIALS AND METHODS

Antibodies and Reagents—The following antibodies were used: monoclonal anti-FLAG-HRP (Sigma), monoclonal anti-Myc-HRP (Roche Applied Science), monoclonal anti-HA-HRP (Roche Applied Science), monoclonal anti-β actin (Sigma), rhodamine-conjugated anti-mouse IgG (Molecular Probes), monoclonal anti-UCH-L1 (Dako), polyclonal anti-HHARI (Abcam), polyclonal anti-c-Cbl (Cell Signaling), and polyclonal anti-UCH-L1 (Dako). The FLAG-tagged wild-type parkin and Myc-tagged CHIP expression constructs were gifts from L. Petrucelli. The FLAG-tagged wild-type parkin or various C-terminal parkin mutants were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. At 48 h after transfection, the cells were harvested for sequential fractionation of the cell lysates into Triton-X-soluble and SDS-soluble fractions as described previously (18). An equivalent amount of proteins among different Triton-X-soluble and SDS-soluble fractions was resolved by means of SDS-PAGE, and the levels of various proteins were analyzed by means of Western blotting procedures using ECL detection reagents (Amersham Biosciences). For stress treatments, plasmids containing FLAG-parkin species, HA-HHARI, HA-c-Cbl, Myc-CHIP, or Myc-UCH-L1 were transfected using Lipofectamine Plus reagent (Invitrogen) at 48 h after transfection, the cells were transfected with 20 mM H₂O₂ for 30 min, 0.5–1 mM DA for 24 h, and 0.25–0.5 mM NOC-18 for 24 h.

Immunocytochemistry and Confocal Microscopy—5 × 10⁴ SH-SY5Y cells were seeded on coverslips for subsequent transfection with FLAG-tagged wild-type parkin or selected C-terminal parkin mutants using Lipofectamine Plus reagent (Invitrogen). At 48 h after transfection, the cells were harvested for sequential fractionation of the cell lysates into Triton-X-soluble and SDS-soluble fractions as described previously (18). An equivalent amount of proteins among different Triton-X-soluble and SDS-soluble fractions was resolved by means of SDS-PAGE, and the levels of various proteins were analyzed by means of Western blotting procedures using ECL detection reagents (Amersham Biosciences). For stress treatments, plasmids containing FLAG-parkin species, HA-HHARI, HA-c-Cbl, Myc-CHIP, or Myc-UCH-L1 were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. At 48 h after transfection, the cells were harvested for sequential fractionation of the cell lysates into Triton-X-soluble and SDS-soluble fractions as described previously (18). An equivalent amount of proteins among different Triton-X-soluble and SDS-soluble fractions was resolved by means of SDS-PAGE, and the levels of various proteins were analyzed by means of Western blotting procedures using ECL detection reagents (Amersham Biosciences). For stress treatments, plasmids containing FLAG-parkin species, HA-HHARI, HA-c-Cbl, Myc-CHIP, or Myc-UCH-L1 were transfected using Lipofectamine Plus reagent (Invitrogen) at 48 h after transfection, the cells were transfected with 20 mM H₂O₂ for 30 min, 0.5–1 mM DA for 12 h, or 0.25–0.5 mM NOC-18 for 24 h.

RESULTS

Conservation and Structural Implication of Parkin’s Cysteine Residues—Inspection of the amino acid sequence of human parkin reveals a total of 35 Cys residues, the majority of which (23 out of 35) reside within the RING-IBR-RING domain of the protein (Fig. 1A). Further, comparison of human parkin protein sequence with orthologous sequences from rodent, fish, insect, and worm reveals that most of the parkin’s Cys residues are absolutely conserved across these diverse species, a feature that suggests their importance to the protein’s structure and/or function. Additionally, the Cys residues are highly conserved across these species, suggesting that they may play important roles in protein function. Further, comparison of human parkin protein sequence with orthologous sequences from rodent, fish, insect, and worm reveals that most of the parkin’s Cys residues are absolutely conserved across these diverse species, a feature that suggests their importance to the protein’s structure and/or function.

Susceptibility of Parkin to Stress-induced Alterations

Site-directed Mutagenesis and Generation of Parkin Deletion Constructs—All the Cys → Ala (C → A) parkin mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions and verified via DNA sequencing. Parkin 1–137 and 1–237 deletion constructs were generated by means of PCR amplification of the designated regions using wild-type FLAG-parkin cDNA as a template and subcloned into pCDNA3 plasmid. For parkin Δ77–237, the UBL and RING-IBR-RING regions of parkin were amplified separately from wild-type FLAG-parkin cDNA by means of PCR and subsequently cloned in-frame into pCDNA3.

Cell Culture, Treatment with Various Stressors, and Western Blot Analysis—SH-SY5Y neuroblastoma cells were seeded at 2 × 10⁵ cell density for all transfections. pCDNA3 plasmid bearing FLAG-tagged wild-type parkin or various C → A parkin mutants were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. At 48 h after transfection, the cells were harvested for sequential fractionation of the cell lysates into Triton-X-soluble and SDS-soluble fractions as described previously (18). An equivalent amount of proteins among different Triton-X-soluble and SDS-soluble fractions was resolved by means of SDS-PAGE, and the levels of various proteins were analyzed by means of Western blotting procedures using ECL detection reagents (Amersham Biosciences). For stress treatments, plasmids containing FLAG-parkin species, HA-HHARI, HA-c-Cbl, Myc-CHIP, or Myc-UCH-L1 were transfected using Lipofectamine Plus reagent (Invitrogen) at 48 h after transfection, the cells were transfected with 20 mM H₂O₂ for 30 min, 0.5–1 mM DA for 12 h, or 0.25–0.5 mM NOC-18 for 24 h.

Immunocytochemistry and Confocal Microscopy—5 × 10⁴ SH-SY5Y cells were seeded on coverslips for subsequent transfection with FLAG-tagged wild-type parkin or selected C → A parkin mutants using Lipofectamine Plus reagent (Invitrogen). At 48 h after transfection, the cells were harvested for sequential fractionation of the cell lysates into Triton-X-soluble and SDS-soluble fractions as described previously (18). An equivalent amount of proteins among different Triton-X-soluble and SDS-soluble fractions was resolved by means of SDS-PAGE, and the levels of various proteins were analyzed by means of Western blotting procedures using ECL detection reagents (Amersham Biosciences). For stress treatments, plasmids containing FLAG-parkin species, HA-HHARI, HA-c-Cbl, Myc-CHIP, or Myc-UCH-L1 were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. At 48 h after transfection, the cells were transfected with 20 mM H₂O₂ for 30 min, 0.5–1 mM DA for 12 h, or 0.25–0.5 mM NOC-18 for 24 h.

Human Tissues and Statistical Analysis—Aliquots of previously described (10) human brain lysates prepared from post-mortem brains of control and PD individuals that were stored at −80 °C were used in this study. Statistical significance for all the quantitative data obtained was analyzed using Student’s t test (*, p < 0.05, **, p < 0.001) unless otherwise stated.
Conserved Cysteine Residues on Parkin Residing Both Within and Outside the RING-IBR-RING Domain Are Important in Maintaining Its Solubility

To examine whether the modification of parkin’s Cys residues would influence its solubility, we generated a large series of parkin Cys→Ala (C→A) point mutants that cover the length of the protein via site-directed mutagenesis and expressed each function (Fig. 1A and supplemental Fig. S1). Although the invariant Cys residues in parkin across different species are largely found within the catalytically important RING-IBR-RING domain, a number of such highly conserved Cys residues, such as Cys-150, Cys-166, Cys-212, and Cys-457, are notably also found along the length of the protein outside of this domain (Fig. 1A).

To gain insights into the importance of individual Cys residue on parkin to its overall tertiary architecture, it is essential to elucidate the three-dimensional structure of the protein, information of which is currently lacking. However, the structure of both RING1 and RING2 in related proteins, c-Cbl and HHARI, respectively, have previously been reported (13, 14). Accordingly, we used the structure of c-Cbl RING1 and HHARI RING2 as templates to model parkin RING1 and RING2 domains, respectively. Homology models of parkin RING1 and RING2 so obtained reveal the coordination of Cys-238, Cys-241, Cys-260, and Cys-263 to a zinc atom in RING1 and the coordination of Cys-418, Cys-421, Cys-436, and Cys-441 to another zinc atom in RING2 (Fig. 1B). Although our program failed to model a small stretch of parkin sequence containing Cys-289 and Cys-293 residues accurately, these residues, together with Cys-253 and His-257, should coordinate a second zinc atom in RING1 in view of their high sequence homology to c-Cbl RING1 (14) (Fig. 1B). Not surprisingly, all of these structurally important Cys residues are absolutely conserved in parkin across different species (Fig. 1A and supplemental Fig. S1). On the other hand, Cys-268, which resides on a solvent-exposed surface (supplemental Fig. S2A) and does not appear to have a critical structural role in RING1 (Fig. 1B), is replaced by a leucine (Leu) in C. elegans parkin (supplemental Fig. S1) and by other amino acid residues in related proteins such as RBCK1 (isoleucine) and ariadne-2 (phenylalanine) (19).

Similarly, the presumably non-structural Cys-451 residue proximal to RING 2 (Fig. 1B) is poorly conserved among parkin from different species (supplemental Fig. S1). Since the structure of parkin’s UBL domain is known (20), we also inspected the structural position of Cys-59 and found that this Cys residue, like Cys-268, is located at a solvent-exposed loop on the surface of the UBL domain and not within its core (Fig. 1C), thereby offering some structural flexibility. Notably, in C. elegans parkin, Cys-59 is substituted with a Leu (supplemental Fig. S1). Taken together, the degree of Cys conservation in parkin across different species appears to correlate with their structural importance. Conceivably, modification of any of the numerous highly conserved Cys residues on parkin is likely to influence its structural topology, and thereby, its biochemical properties.

Conventional Cysteine Residues on Parkin Residing Both Within and Outside the RING-IBR-RING Domain Are Important in Maintaining Its Solubility—To examine whether the modification of parkin’s Cys residues would influence its solubility, we generated a large series of parkin Cys→Ala (C→A) point mutants that cover the length of the protein via site-directed mutagenesis and expressed each
of these mutants in SH-SY5Y neuroblastoma cells (Fig. 2A). When cells transfected with these mutants were subjected to sequential detergent extraction, we found that all the C → A mutations occurring on Cys residues that are invariant in parkin across different species, except C431A, show preferential localization to the detergent-insoluble (P) fraction relative to control, wild-type parkin (Fig. 2A). Conversely, Cys residues on parkin such as Cys-59, Cys-95, Cys-268, Cys-323, Cys-431, and Cys-451 that are either not absolutely conserved among different species or otherwise appear structurally unimportant, or both, do not significantly alter parkin solubility when mutated (Fig. 2A). It thus appears that C modification occurring at other regions of parkin result in more significant alterations of the protein (i.e. solubility changes and higher tendency to aggregate) when compared with analogous modification occurring at other regions of the protein.

Susceptibility of Parkin and Other Cysteine-containing Enzymes to Stress-induced Solubility Alterations—Given the demonstrated importance of parkin’s Cys residues in maintaining its structural and biochemical properties, one could appreciate the reported alteration of parkin function via modification of its Cys residues (11). Since parkin and HHARI contain a comparable number of Cys residues (35 and 32, respectively), and the majority of these Cys are found within their RING-IBR-RING domains, it is likely that they share comparable sensitivities to modification by various stress-inducing agents. Thus, we were intrigued by recent findings showing that parkin, but not HHARI, is selectively vulnerable to DA-mediated modification (11). To examine the relative susceptibility of parkin and other cysteine-containing enzymes to stress-induced solubility alterations, we subjected SH-SY5Y cells ectopically expressing parkin, HHARI, c-Cbl (RING domain ubiquitin-protein isopeptide ligase (E3) with 23 Cys residues), CHIP (U box protein with 7 Cys residues), or UCH-L1 (ubiquitin hydrolase with 6 Cys residues) to a variety of stresses, including hydrogen peroxide (H₂O₂), NOC-18 (a nitrogen oxide donor), and DA, all of which have been previously reported to induce parkin insolubility.
Susceptibility of Parkin to Stress-induced Alterations

A

| Enzyme                  | UBL | R1 | IBR | R2 |
|-------------------------|-----|----|-----|----|
| Parkin (35 Cys)         |     |    |     |    |
| HHARI (32 Cys)          |     |    |     |    |
| c-Cbl (23 Cys)          | R1  |    |     |    |
| CHIP (7 Cys)            | R1  |    |     |    |
| UCH-L1 (6 Cys)          | U-BOX |   |    |    |

|                  | H2O2 (mM) | NOC-18 (mM) |
|------------------|-----------|-------------|
|                  | 0 20      | 0 20        |
| anti-FLAG (Parkin) | anti-actin |
| anti-HA (HHARI)   | anti-actin |
| anti-HA (Cbl)     | anti-actin |
| anti-myC (CHIP)   | anti-actin |
| anti-myC (UCH-L1) | anti-actin |

|                  | 0.25 0.5 | 0 0.25 0.5 |
|------------------|---------|------------|
| anti-FLAG (Parkin) | anti-actin |
| anti-HA (HHARI)   | anti-actin |
| anti-HA (Cbl)     | anti-actin |
| anti-myC (CHIP)   | anti-actin |
| anti-myC (UCH-L1) | anti-actin |

B

| DA (mM) | anti-FLAG (Parkin) | anti-actin |
|---------|--------------------|------------|
| 0 0.5 1 | S P                |            |
| anti-HA (Cbl) | anti-myC (CHIP) | anti-actin |

| DA (mM) | anti-FLAG (Parkin) | anti-actin |
|---------|--------------------|------------|
| 0 0.5 1 | S P                |            |
| anti-HA (HHARI) | anti-myC (UCH-L1) | anti-actin |

| DA (mM) | anti-FLAG (Parkin) | anti-actin |
|---------|--------------------|------------|
| 0 0.5 1 | S P                |            |

FIGURE 3. Relative susceptibility of parkin and other cysteine-containing enzymes to stress-induced solubility alterations. A, schematic depiction of structural motifs present in the various enzymes examined (left panel). Representative immunoblots of cell extracts sequentially prepared with Triton X-100 (S) and SDS (P)-containing buffer from untreated SH-SY5Y cells transfected with various cysteine-containing enzymes and those treated with various doses of H2O2 (30 min) or NOC-18 (24 h), as indicated (right panel). The blots were stripped and reprobed with anti-actin antibody to reflect loading variations. The experiment was repeated three times with similar results. B, as above but transfected cells treated with different doses of DA (12 h), as indicated. HMW parkin refers to high molecular weight parkin species. Asterisks denotes nonspecific bands, whereas arrows point to bands of interest.

Asterisks denote nonspecific bands, whereas arrows point to bands of interest.

amount of monomeric and high molecular weight (HMW) parkin species in the detergent-insoluble fraction without a corresponding decrease in the levels of soluble parkin (Fig. 3B). Surprisingly, this phenomenon is specific to parkin as all the other Cys-containing enzymes examined, including HHARI, remain unaffected, suggesting that parkin is uniquely sensitive to DA-mediated modification (Fig. 3B). Although our findings on the effect of DA on parkin are consistent with that reported earlier (11), HHARI, despite sharing similar RING-IBR-RING structure and comparable sensitivity to parkin toward the other stress paradigms examined, is spared from DA-mediated effects. Nonetheless, when taken together, our results suggest that RING-IBR-RING proteins are more sensitive than other cysteine-containing enzymes to stress-induced modification but parkin is selectively vulnerable to DA-induced alterations.

DA Modifies Residues Residing on the RING-IBR-RING Motif as well as the Linker Region of Parkin—To map the region on parkin that confers its unique susceptibility to DA-induced modification, we generated deletion mutants of FLAG-tagged parkin devoid either of its linker region (parkin Δ77–237) or its RING-IBR-RING domain (parkin 1–237 and parkin 1–137) (Fig. 4A). We then treated cells expressing these various parkin deletion mutants with the same concentrations of DA as described above. Although DA treatment of cells expressing parkin 1–237 and parkin Δ77–237 promotes an accumulation of detergent-insoluble parkin species in a manner similar to that observed with full-length parkin, parkin 1–137 is apparently spared from DA-mediated modification, even at the higher concentration of DA dose used (Fig. 4B). Thus, the region on parkin stretching from amino acids 138–237 appears to be as susceptible to modification by DA as the RING-IBR-RING domain. Interestingly, this stretch of parkin sequence contains several Cys residues that are not found in HHARI. Given the apparent selective sensitivity of parkin to DA-mediated modification, it is tempting to suggest from our results that DA preferentially modifies Cys residues that are located either at the unique linker region and/or at the C-terminal portion of protein involving residues such as Cys-268, Cys-323, and Cys-451 that are also unique to parkin.

Consistent with their similar structure and abundance of Cys, we found that both parkin and HHARI are more or less equally susceptible to solubility alterations promoted by the treatment of cells with H2O2 and NOC-18 (Fig. 3A). On the other hand, H2O2-mediated stress has no apparent effects on the solubility of c-Cbl, CHIP, and UCH-L1 (Fig. 3A). Although NOC-18 treatment of transfected cells at 0.25 mM markedly altered the solubility of parkin and HHARI, its effects on c-Cbl, CHIP, and UCH-L1 are more apparent only at a higher dose of 0.5 mM (Fig. 3A). For DA-mediated stress, we used a similar treatment paradigm to that reported by LaVoie et al. (11). Interestingly, we found that DA treatment of cells ectopically expressing parkin promotes a significant increase in the
DA Preferentially Modifies Cys-268 and Cys-323 on Parkin—Since DA modification of parkin promotes the formation of insoluble monomeric and HMW parkin species without causing a corresponding decrease in the levels of soluble parkin (Fig. 3B), it is reasonable to assume that DA targets either non-conserved Cys residues or those that are structurally less important. Although four of such Cys residues (Cys-268, Cys-323, Cys-431, and Cys-451) are present at the C-terminal region of parkin, only 1 (Cys-182) is found between amino acids 138 and 237. Accordingly, we repeated the above experiment with a compendium of parkin mutants containing C→A substitution at position Cys-182, Cys-268, Cys-323, Cys-431, or Cys-451. We also included an insoluble mutant, C441A, as a control. Surprisingly, we found that DA modifies the parkin C182A mutant in a similar fashion to that observed with the wild-type protein (Fig. 5A). Parkin C441A, found predominantly in the detergent-insoluble fraction, also appears to be modified by DA protein (Fig. 5A) mutant in a similar fashion to that observed with the wild-type parkin. Notably, the levels of insoluble parkin C323A species generated by DA treatment are so modest that they compare well with untreated wild-type parkin control (Fig. 5A). We next examined the effects of DA on the cellular localization of wild-type, Cys-268, and Cys-323 parkin. Consistent with its susceptibility to DA-mediated modification, we found that cells expressing wild-type parkin exhibit a high tendency to form parkin-positive inclusions following DA treatment. In contrast, such inclusions occur rarely in DA-treated cells expressing C268A and C323A parkin mutant (Fig. 5B). Taken together, our results demonstrate that mutation of Cys-268 and Cys-323, respectively, to alanine render parkin less susceptible to insolubility induced by DA treatment, suggesting that DA modification of parkin’s Cys residues takes place predominantly at these Cys residues.

Susceptibility of Parkin to Stress-induced Alterations

Distribution of Various Cysteine-containing Enzymes in Normal and PD Human Brains—We and others have previously demonstrated a significant increase in the amount of detergent-insoluble parkin in the caudate region of idiopathic PD brains when compared with those in control brains (10, 11). To examine whether a similar phenomenon occurs with other cysteine-containing enzymes, particularly HHARI, in PD and control brains, we performed anti-HHARI, anti-UCH-L1, anti-c-Cbl, and anti-CHIP immunoblotting on fractionated detergent-insoluble lysates prepared from post-mortem normal and PD brains (Fig. 6A). In contrast to parkin, as reported previously (10, 11), we did not observe an accumulation of any of the enzymes examined in the detergent-insoluble fraction of PD over control brain samples (Fig. 6B). Quantiatively, we only recorded a significant difference in the levels of CHIP between PD and control brains (Fig. 6B). However, the amount of CHIP decreased, instead of increased, in PD brains when compared with controls (Fig. 6B). Taken together, our results demonstrate that unlike parkin, HHARI, UCH-L1, c-Cbl, and CHIP do not accumulate in the detergent-insoluble fractions of PD brains, suggesting that parkin is uniquely sensitive to stress-induced modification in the caudate region of the brain.

DISCUSSION

In this report, we demonstrate that the modification of any of the numerous highly conserved Cys residues on parkin (except Cys-431), both within and outside its RING-IBR-RING motif, leads to alterations of the protein biochemical and cellular properties, and thereby, its function. As several of these structurally important Cys on parkin are not found in other related proteins, our findings suggest an increased vulnerability of parkin to stress-induced modification. Indeed, parkin appears to be more susceptible to oxidative and nitrosative stress and uniquely sensitive to DA-mediated stress. Given parkin’s neuroprotective roles and the oxidative environment of dopaminergic neurons, the enhanced sensitivity of parkin to intracellular stress may underlie the progressive susceptibility of an individual to PD.
of the 20 naturally occurring amino acids found in proteins, cysteines are recognized to be exceptionally susceptible to oxidative modification due to the presence of sulfhydryl groups (21). Sulfhydryl groups are the strongest nucleophile in the cell at physiological pH and thus represent ideal targets for nucleophilic attack by oxidants or nitrosative agents. Accordingly, the abundance of Cys residues on a protein should, in part, contribute to the tendency for the protein to be modified by cellular oxidants. We found that this appears to be the case when we subjected enzymes with different cysteine content to the effects of oxidative/nitrosative stress agents. Although parkin and HHARI, both containing over 30 Cys residues, are comparably susceptible to H$_2$O$_2$- or NOC-18-mediated effects, c-Cbl, CHIP, and UCH-L1, containing 23, 7, and 6 Cys residues, respectively, either remain inert to these stress agents or otherwise require higher concentrations of these agents to produce a similar effect observed with parkin and HHARI.

The extent of a protein’s alteration via its Cys modification is obviously also related to the importance of the targeted Cys residue to the overall tertiary structure of the protein. We have demonstrated that the large majority of Cys residues residing on parkin (23 out of 28 examined), both within and outside the RING-IBR-RING domain, are important in maintaining its solubility. With the notable exception of Cys-431, all the Cys residues of parkin found to be invariant across diverse species resulted in parkin insolubility when they are mutated to alanine, suggesting their importance in fulfilling critical structural roles. Although the Zn$^{2+}$-coordinating Cys residues in RING1 and RING2 are obviously structurally important, it is interesting that almost all the Cys residues located at the IBR, a domain whose function remains unclear, appears to be critically important for the native folding of parkin. On the other hand, despite being an absolutely conserved residue, Cys-431 does not appear to play an important role in maintaining parkin’s RING2 structure, which probably explains its lack of effects on parkin solubility when mutated. Nonetheless, our result with the C431A mutant in this study appears to contradict our previous finding that the C431F mutant promotes parkin insolubility and intracellular aggregation (18). In the latter case, it is conceivable that the substitution of Cys with the bulky Phe could result in marked steric hindrance that might indirectly affect parkin’s RING2 structure. Accordingly, we generated a homology model of parkin’s RING2 structure with Phe substituting for Cys at position 431 and found that the benzene ring of Phe may interfere sterically with the RING structure (supplemental Fig. S2B).

We speculated that substitution of Cys-431 with the equally bulky Tyr residue would bring about similar effects to that observed with the C431F mutant. Using both homology modeling and in vitro experiments similar to the ones described above, we were able to confirm our speculation with the C431Y mutant (supplemental Fig. S2, B and C). Thus, parkin solubility alteration mediated by C431F mutation is likely to arise from steric rather than overt structural aberrations.

Interestingly, all the invariant Cys residues residing within the parkin RING-IBR-RING motif are also conserved among members of the RING-IBR-RING family (19). This feature probably explains the comparable susceptibility of HHARI and parkin to H$_2$O$_2$ or NOC-18-mediated solubility alterations, and at the same time, suggests the vulnerability of other RING-IBR-RING members to stress-induced modifications. The implication of this is that oxidative stress occurring in the brain, particularly in dopaminergic neurons, could promote the dysfunction of not just parkin but potentially all RING-IBR-RING family members as well. It is well known that dopaminergic neurons in the brain are particularly exposed to oxidative stress because the metabolism of DA produces various reactive oxygen species (peroxide, superoxide, and hydroxyl radicals) (21, 22). If not handled properly, the reactive oxygen species generated could create a considerable damaging environment. Further, DA could auto-oxidize to DA-quinone, a reactive species that has been demonstrated to covalently modify cellular
Susceptibility of Parkin to Stress-induced Alterations

macromolecules, including parkin, and contribute to DA-induced neurotoxicity (11, 21, 22). However, unlike our previous observation with parkin (10), we did not observe an accumulation of HHARI in the detergent-insoluble fraction from the caudate region of PD brains relative to controls. It would appear that parkin is uniquely vulnerable in this region of the brain, at least when compared with HHARI, to PD-linked stress. Conceivably, the modification of parkin in this case must involve regions or residues of the protein that are distinct from HHARI. Interestingly, DA modification of parkin appears to fit this requirement nicely, as suggested by our following findings in this study. 1) DA-mediated promotion of insoluble parkin species occurs without apparent depletion of corresponding detergent-soluble parkin species, a milder effect when compared with the solubility alterations of parkin produced by H$_2$O$_2$ or NOC-18 as described above or by various other stressors that we have previously reported (10). Consistent with this, both C268A and C323A fail to protect parkin against H$_2$O$_2$-induced solubility alterations (data not shown). Given the selective vulnerability of brain parkin to stress-induced modifications, as shown in this study, it is tempting to suggest that the principal culprit promoting brain parkin insolubility is likely DA, DA-related, or otherwise one that influences parkin in a similar manner to that brought about by DA. Whether and how detergent-insoluble monomeric and HMW parkin species influence soluble parkin function and cellular survivability remain to be characterized. However, their association with PD brains (10, 11) would suggest pathogenic roles.

In conclusion, our results here offer significant insights into the components important for parkin solubility, and at the same time, provide some structural basis for the solubility alterations of the protein produced by cellular stress. Further, we have mapped 2 Cys residues on parkin that render the protein less susceptible to insolubility induced by DA treatment, thus potentially representing the major target sites of DA-mediated modification. Notably, both these residues are unique to parkin and might therefore explain the selective vulnerability of parkin to DA-induced changes. Future experiments should clarify the potential pathogenicity of the insoluble parkin species promoted by DA modification.

Acknowledgments—We thank Michelle Ho for technical assistance. We thank Juan Troncoso and Olga Pletinkova and the Morris K. Udall Parkinson’s Disease Center Neuropathology Core for the human postmortem brain material.

REFERENCES

1. Savitt, J. M., Dawson, V. L., and Dawson, T. M. (2006) J. Clin. Investig. 116, 1744–1754
2. Lim, K. L., Dawson, V. L., and Dawson, T. M. (2003) Ann. N. Y. Acad. Sci. 991, 80–92
3. Klein, C., Hedrich, K., Wellenbrock, C., Kann, M., Harris, J., Marder, K., Lang, A. E., Schwingers, E., Ozelius, L. J., Vieregge, P., Pramstaller, P. P., and Kramer, P. L. (2003) Ann. Neurol. 54, 415–417
4. Lucking, C. B., Durr, A., Bonifati, V., Vaughan, J., De Michele, G., Gasser, T., Harhangi, B. S., Meco, G., Denele, P., Wood, N. W., Agid, Y., and Brice, A. (2000) N. Engl. J. Med. 342, 1560–1567
5. Oliveira, S. A., Scott, W. K., Martin, E. R., Nance, M. A., Watts, R. L., Hubble, J. P., Koller, W. C., Pahwa, R., Stern, M. B., Hiner, B. C., Ondo, W. G., Allen, F. H., Jr., Scott, B. L., Goetz, C. G., Small, G. W., Mastaglia, F., Stajich, J. M., Zhang, F., Booze, M. W., Winn, M. P., Middleton, L. T., Haines, J. L., Pericak-Vance, M. A., and Vance, J. M. (2003) Ann. Neurol. 53, 624–629
6. 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., and Farrer, M. (2002) Am. J.

FIGURE 6. Distribution of various cysteine-containing enzymes in normal and PD brains. A, detergent-insoluble fractions of brain lysates from caudate (lanes 1–3) and cingulate cortex (lanes 4 and 5) of control and caudate (lanes 1–9) of sporadic PD brains were subjected to SDS-PAGE and immunoblotted with anti-HHARI, anti-UCH-L1, anti-CHIP, or anti-Cbl antibodies. B, histograms showing the levels of HHARI, UCH-L1, CHIP, and c-Cbl in control and PD brains after normalization to their respective actin levels. (*, p < 0.05, n.s. = not significant, Student’s t test).
Susceptibility of Parkin to Stress-induced Alterations

7. West, A. B., Maraganore, D., Crook, J., Lesnick, T., Lockhart, P. J., Wilkes, K. M., Kapatos, G., Hardy, J. A., and Farrer, M. J. (2002) *Hum. Mol. Genet.* **11**, 2787–2792

8. Cookson, M. R. (2003) *Curr. Biol.* **13**, R522–524

9. Feany, M. B., and Pallanck, L. J. (2003) *Neuron* **38**, 13–16

10. Wang, C., Ko, H. S., Thomas, B., Tsang, F., Chew, K. C., Tay, S. P., Ho, M. W., Lim, T. M., Soong, T. W., Pletnikova, O., Troncoso, J., Dawson, V. L., Dawson, T. M., and Lim, K. L. (2005) *Hum. Mol. Genet.* **14**, 3885–3897

11. LaVoie, M. J., Ostaszewski, B. L., Weihofen, A., Schlossmacher, M. G., and Selkoe, D. J. (2005) *Nat. Med.* **11**, 1214–1221

12. Tonks, N. K. (2005) *Cell* **121**, 667–670

13. Capili, A. D., Edghill, E. L., Wu, K., and Borden, K. L. (2004) *J. Mol. Biol.* **340**, 1117–1129

14. Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000) *Cell* **102**, 533–539

15. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22**, 4673–4680

16. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) *Nucleic Acids Res.* **31**, 3381–3385

17. Guex, N., Diemand, A., and Peitsch, M. C. (1999) *Trends Biochem. Sci.* **24**, 364–367

18. Wang, C., Tan, J. M., Ho, M. W., Zaiden, N., Wong, S. H., Chew, C. L., Eng, P. W., Lim, T. M., Dawson, T. M., and Lim, K. L. (2005) *J. Neurochem.* **93**, 422–431

19. Morett, E., and Bork, P. (1999) *Trends Biochem. Sci.* **24**, 229–231

20. Sakata, E., Yamaguchi, Y., Kurimoto, E., Kikuchi, J., Yokoyama, S., Yamada, S., Kawahara, H., Yokosawa, H., Hattori, N., Mizuno, Y., Tanaka, K., and Kato, K. (2003) *EMBO Rep.* **4**, 301–306

21. Stokes, A. H., Hastings, T. G., and Vrana, K. E. (1999) *J. Neurosci. Res.* **55**, 659–665

22. Lotharius, J., and Brundin, P. (2002) *Nat. Rev. Neurosci.* **3**, 932–942