Identification of Far Upstream Element-binding Protein-1 as an Authentic Parkin Substrate*

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Aminoacyl-tRNA synthetase–interacting multifunctional protein type 2 was recently identified as an authentic substrate of the ubiquitin E3 ligase, parkin, a gene associated with autosomal recessive juvenile parkinsonism. Far upstream element-binding protein 1 is known to be degraded in an aminoacyl-tRNA synthetase interacting multifunctional protein type 2 dependent manner, which is crucial for lung cell maturation in early development. Therefore, we wondered whether far upstream element-binding protein 1 levels are altered in the absence of Parkin and in Parkinson disease. We herein report that far upstream element-binding protein 1 accumulates in Parkin knockout mice, patients with autosomal recessive juvenile parkinsonism, sporadic Parkinson disease, and diffuse Lewy body disease as well as the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. Moreover, Parkin interacts with and ubiquinates far upstream element-binding protein 1 facilitating its degradation through the ubiquitin proteasome system. Taken together, these results suggest that far upstream element-binding protein 1 is an authentic substrate of Parkin and that far upstream element-binding protein 1 may play an important role in development of Parkinson disease pathology along with aminoacyl-tRNA synthetase interacting multifunctional protein type 2.

PARKIN disease (PD)1 is the second most common neurodegenerative disease affecting about one percent of the elderly population above the age of 65 (1). Selective loss of dopaminergic neurons in the substantia nigra leads to the major motor symptoms of PD including rest tremor, bradykinesia, and rigidity. Recent genetic advances have provided important clues to understanding the pathogenesis of PD. Several genes are now linked to familial PD, (2), synuclein (3), parkin (4), and TDP-43 (5). Mutations in parkin, DI-1, PINK-1, LRRK2, and α-synuclein (4). Mutations in Parkin are the most common cause of autosomal recessive familial Parkinson disease (3). parkin encodes a ubiquitin E3 ligase that plays important roles in maintaining protein homeostasis by targeting its substrates to the ubiquitin proteasome system.

We and others previously showed that aminoacyl-tRNA synthetase-interacting multifunctional protein type 2 (AIMP2) (also called p58/TTV1) is a potential Parkin substrate (4, 5). Moreover, AIMP2 appears to be an authentic substrate of Parkin (5) as it is selectively increased in the brains of Parkin+/− mice and brains of human patients with autosomal recessive juvenile parkinsonism (AR-JP) due to mutations in parkin, unlike other putative substrates of Parkin (5). In addition, AIMP2 accumulates in sporadic PD patient brains due to S-nitrosylation of Parkin, which inhibits its ubiquitin E3 ligase activity. Thus, selective up-regulation of AIMP2 may play an important and crucial role in the development of PD due to its accumulation in both sporadic PD and AR-JP. Since AIMP2 is known to interact with and promotes the ubiquitination and proteasome-dependent degradation of the far upstream element (FUSE)-binding protein 1 (FBP1), we assessed whether FBP1 protein levels are altered in PD. In this report, we demonstrate that FBP1 is up-regulated in Parkin+/− mice, AR-JP, sporadic PD, diffuse Lewy body (DLB) disease, and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. Moreover, Parkin binds and ubiquitinates FBP1 enhancing its degradation through the ubiquitin proteasome system. Thus, FBP1 is the second authentic Parkin substrate identified to date.

EXPERIMENTAL PROCEDURES
cDNA, Cell Culture, and Antibodies—Full-length hemagglutinin (HA)-tagged human FBP1 and GFP-tagged human FBP1 cDNAs were kindly provided by David Levens (NCI, National Institutes of Health, Bethesda, MD). Human full-length Parkin and ubiquitin cDNAs were cloned into PRKS-myc and pRKS-HA vectors, respectively. Human neuroblastoma SH-SYSY cells were purchased from American Type Culture Collection (Manassas, VA). SH-SYSY cells were transiently transfected with the target vector using the Lipofectamine plus (Invitrogen), according to manufacturer’s instructions.

Immunoprecipitation and Western Blotting—Immunoprecipitation and Western blotting were performed as reported previously (5). Briefly, 48 h after SH-SYSY cells were transfected with 2 μg of each plasmid, cells were harvested and sonicated in lysis buffer. After centrifugal separation, 50 μl of protein G-Sepharose (Amersham Biosciences) preincubated with antibodies against GFP (Abcam Inc., Cambridge, MA) or myc (Roche Applied Science) was added to the supernatants. The precipitate with protein G-Sepharose was pelleted and resolved by SDS-PAGE for Western blot analysis.

In Vivo Ubiquitination Assay—For in vivo ubiquitination assays, SH-SYSY cells were transfected with 1.5 μg of pRKS-myc-tagged Parkin or myc-tagged Parkin mutants, pCMV-GFP-FBP1, and 1.5 μg of pMti123-HA-ubiquitin for 48 h. Total cell lysates were subjected to immunoprecipitation conducted according to the method described previously (5). The antibodies used were anti-HA and anti-GFP.

Animals and MPTP Injections—All procedures involving animals were approved by and conformed to the guidelines of the Institutional Animal Care Committee of Johns Hopkins University. 8-Week-old male C57/bl mice (Charles River Laboratories, Wilmington, MA) (n = 5 per group) received four intraperitoneal injections of MPTP-HCl (20 mg/kg free base, Research Biochemicals) in saline or saline alone at 2-h intervals. Animals were decapitated 2, 4, 24, 48, and 96 h and 7 days after the last MPTP injection, and their ventral midbrain and striata were quickly dissected out and processed for Western blot analysis.

Human Tissues—Human brain tissue was obtained through the brain donation program of the Morris K. Udall Parkinson’s Disease Research Center at Johns Hopkins Medical Institutions in compliance with Health Insurance Portability and Accountability Act regulations. Preparation of Human and Mouse Brain Tissue—Frontal caudate (n = 3) and cingulate (n = 2) tissues from control brains and eight PD and DLB brains with high Lewy body burden (Table 1) were used to analyze Parkin substrates in PD and DLB brains. AR-JP brains described previously (6) were used to compare Parkin substrate levels. Parkinson+/− mice were generated as originally described (7). Triton-soluble and -insoluble fractions were separated from the brain homogenates. The homogenization procedure was accomplished in accordance with published methods (5). Each fraction was analyzed through Western blotting against anti-FBP1 (BD Biosciences, Franklin Lakes, NJ) antibody.

RESULTS

The steady-state level of FBP1 Is Up-regulated in the Brain Stem of Parkin+/− Mice—We examined the levels of FBP1 in cortex, cerebellum, brain stem, ventral midbrain, and striatum of Parkin+/− mice versus age-matched wild type controls (Fig. 1). Actin was used as a loading control. We observed a significant increase in levels of FBP1 in the brain stem (Fig. 1A) and cortex (Fig. 1B) compared with wild type controls. We failed to observed a significant...
up-regulation of FBP1 as assessed by Western blot analysis and quantification in the ventral midbrain, cerebellum, and striatum (Fig. 1).

**FBP1 Accumulates in Human AR-JP, PD, and DLB Samples and MPTP-treated Mice**—To ascertain whether the up-regulation of FBP1 in Parkin/H11002/H11002 mice has pathophysiological relevance, Western blot analysis was performed on lysates from cortex of patient with AR-JP and age-matched controls. A 14% increase in FBP1 levels was detected in AR-JP brains when compared with controls in the soluble fraction, and we observed a 3-fold increase in FBP1 levels in the insoluble fraction (Fig. 2A). Recent observations suggest that S-nitrosylation of Parkin inhibits its ubiquitin E3 ligase activity, which could lead to accumulation of Parkin substrates in PD and DLB brains (8). Accordingly, we assessed the levels of FBP1 in sporadic PD and DLB. A more than 9-fold increase in FBP1 was observed in PD/DLB brains compared with controls (Fig. 2B). Previously, we had shown that MPTP leads to the S-nitrosylation of Parkin in a biphasic manner (8). Mice were injected with MPTP (20 mg/kg body weight) every 2 h for a total of 4 doses. This regimen causes a reduction of more than 90% of striatal dopamine and its metabolites and a loss of dopamine neurons (9). Under these conditions, we found that FBP1 was up-regulated in a biphasic manner at 2 h and 48 h post-MPTP treatment similar to the S-nitrosylation of Parkin (8). The up-regulation was sustained until about 7 days post-MPTP injection (Fig. 2C).

**Parkin Interacts with and Ubiquitinates FBP1 Leading to Its Degradation**—The accumulation of FBP1 in Parkin/H11002/H11002 brain stem and cortex, AR-JP brains, PD/DLB brains, and MPTP-treated mice prompted us to determine whether

| Subjects     | Brain region | Diagnosis                        | Age | PMD |
|--------------|--------------|----------------------------------|-----|-----|
| Control 1    | Caudate      |                                  | 83  | 5   |
| Control 2    | Caudate      |                                  | 79  | 10  |
| Control 3    | Caudate      |                                  | 81  | 20  |
| Control 4    | Cingulate    |                                  | 83  | 5   |
| Control 5    | Cingulate    |                                  | 92  | 19  |
| Patient 1    | Caudate      | PD/LB CHG NEOCORTICAL            | 80  | 19  |
| Patient 2    | Caudate      | PD/LB CHG                        | 84  | 17.5|
| Patient 3    | Caudate      | PD/LB CHG LIMBIC                 | 83  | 14  |
| Patient 4    | Caudate      | PD/DLB                           | 77  | 5   |
| Patient 5    | Caudate      | PD/LB CHG NEOCORTICAL            | 84  | 13  |
| Patient 6    | Caudate      | PD/LB CHG                        | 88  | 19.5|
| Patient 7    | Caudate      | PD                               | 73  | 6   |
| Patient 8    | Caudate      | PD W/DEMENTIA, LBD NEOCORTICAL   | 84  | 5   |

**TABLE 1**

**Brain region, diagnosis, age, and post mortem delay (PMD) of subjects that were used in this study**

**FIGURE 1.** FBP1 is unregulated in brain stem and cortex of Parkin/H11546/H11546 mice. FBP1 levels in brain stem (A), cortex (B), cerebellum, ventral midbrain, and striatum (C) of 18-month-old wild type (n = 4) and Parkin/H11002/H11002 mice (n = 5) were assessed and compared with actin control by Western blots. Quantification shows a significant increase in FBP1 level in brain stem (121.9 ± 23.66, A) and cortex (117.1 ± 14.96, B) compared with wild type controls (61.4 ± 10.59 and 73.79 ± 23.78, respectively). Densitometric analyses of band intensities normalized to actin are presented as mean ± S.E. (B). *, p < 0.05, Student’s t test.
Parkin interacts with ubiquitins, and degrades FBP1. Co-transfection experiments with myc-tagged Parkin and GFP-tagged FBP1 using human SH-SYSY cells were performed followed by immunoprecipitation using anti-GFP antibody. Parkin co-immunoprecipitates with FBP1 (Fig. 3A). Next, SH-SYSY cells were co-transfected with GFP-tagged FBP1, myc-tagged Parkin as well as HA-tagged ubiquitin, and total cell lysates were immunoprecipitated with an anti-GFP antibody. Increased anti-HA immunoreactivity in the form of a high molecular weight smear characteristic of polyubiquitinated protein on GFP-FBP1 in the presence of wild type Parkin indicates that Parkin ubiquitinates FBP1 (Fig. 3B). The failure of the familial linked T240R mutant Parkin to affect Parkin-mediated ubiquitination of GFP-FBP1 indicates that the ubiquitination of GFP-FBP1 is specific to wild type Parkin. We next evaluated whether the ubiquitination of Parkin of FBP1 facilitates its degradation by the 26 S proteasome. SH-SYSY cells were treated with cycloheximide (100 μg/ml) with or without Parkin. Total cell lysates were obtained at various time points after cyclohexamide treatment, and endogenous FBP1 levels were assessed and compared with actin. The decrease in the steady-state levels of FBP1 was accelerated in the presence of wild type Parkin compared with cells transfected with empty myc vector or familial linked T240R mutant Parkin (Fig. 3C). Taken together these results indicate that Parkin binds to FBP1 and targets it for proteosomal degradation through ubiquitination.

**DISCUSSION**

The major finding of the current study is that FBP1 is an authentic Parkin substrate. FBP1 interacts with Parkin via co-immunoprecipitation, and this interaction leads to its ubiquitination, which is impaired by the catalytically inactive T240R familial associated mutant. Overexpression of wild type Parkin, but not T240R mutant Parkin, accelerates the degradation of FBP1. Although, we were not able to directly show that Parkin interacts with and ubiquitinates FBP1, the accumulation of FBP1 in Parkin−/− brains and AR-JP brains that are deficient in Parkin supports the notion that FBP1 is an authentic Parkin substrate. Moreover, the up-regulation of FBP1 in MPTP-treated mice and in patients with PD or DLB, which have inactivated Parkin due to 5-nitrosoylation is consistent with the idea that FBP1 may be an authentic Parkin substrate.

The identification of both FBP1 and AIMP2 as authentic Parkin substrates provides additional potential insight into the pathogenesis of Parkinson disease due to the absence or inactivation of Parkin function. FBP1 and AIMP2 are likely to function in a common pathogenic pathway as they are interacting proteins and

**FIGURE 2.** FBP1 accumulates in human AR-JP, PD, and DLB samples and MPTP-treated mice brains. A, homogenates of human frontal cortex from control (n = 4) and AR-JP (n = 4) brains were immunoblotted with anti-FBP1 antibody. The blot was stripped and reprobed with anti-actin antibody to confirm equivalent loading in all lanes. Lower panel, quantification indicates that FBP1 level is significantly increased in homogenates from AR-JP (130.16 ± 8.62) compared with control (114.12 ± 15.38) in the soluble fraction and in AR-JP (64.02 ± 4.26) compared with control (16.78 ± 18.2) in the insoluble fraction. B, human brain tissues from control (cortex: n = 3; caudate: n = 2) and PD/DLB cases (caudate: n = 8) were subjected to Western blot analyses with antibody against FBP1. In PD/DLB cases, profound augmentation in FBP1 level (94.42 ± 66.40) is observed compared with age-matched control (10.1 ± 6.48). (The lower panel represents the quantification. C, brain lysates were prepared after the last injection of MPTP at indicated time points to test whether the FBP1 level was altered as detected by Western blot. Densitometric analyses of band intensities normalized to actin levels are presented as mean ± S.E. * p < 0.05, Student's t test.

**FIGURE 3.** Parkin interacts with and ubiquitinates FBP1. A, Parkin and FBP1 interact in SH-SYSY cells. Lysates prepared from SH-SYSY cells transfected with GFP-tagged FBP1 and myc-tagged Parkin were subjected to immunoprecipitation (IP) with anti-GFP, followed by anti-myc immunoblotting. The blot was stripped and reprobed with anti-GFP antibody (bottom) to show the equivalent amount of immunoprecipitated FBP1. B, Parkin ubiquitinates FBP1 and familial associated mutation in Parkin disrupts this ubiquitination. Lysates prepared from SH-SYSY cells transfected with myc-tagged wild type or T240R mutant Parkin, HA-tagged ubiquitin, and GFP-tagged FBP1 were subjected to IP with anti-GFP, followed by immunoblotting against anti-HA (middle) and anti-GFP (bottom). Lysates were also probed with an anti-myc antibody to show Parkin expression (data not shown). The arrow indicates immunoprecipitated FBP1, and brackets indicate ubiquitinated FBP1. C, wild type Parkin accelerates the degradation of FBP1. SH-SYSY cells were transiently transfected with mock, wild type, or T240R mutant Parkin and treated with cycloheximide (100 μg/ml) for the indicated time durations. Total cell lysates were Western-blotted with anti-FBP1 antibody (top). The blot was stripped and reprobed with anti-actin to confirm equivalent loading (bottom).
AIMP2 promotes the ubiquitination and proteasome-dependent degradation of FBP1 through an as yet unidentified ubiquitin E3 ligase (10). Since Parkin interacts with and ubiquitinates both AIMP2 and FBP1, it is conceivable that it is the E3 ligase that mediates AIMP2-dependent ubiquitination and degradation of FBP1. Thus, the absence of Parkin potentially removes a negative feed back loop that controls FBP1 levels when AIMP2 levels are elevated. This coupled with the observation that FBP1 up-regulates AIMP2 (10) could set in motion a feed forward mechanism, leading to the sustained up-regulation of AIMP2 and FBP1 in the absence of Parkin, which seems to be an essential E3 ligase for both proteins. Preliminary results suggest that increased level of FBP1 itself is not toxic (data not shown), but AIMP2 is selectively toxic to dopaminergic neurons (5). Thus, the up-regulation of FBP1 and AIMP2 could contribute to a progressive degenerative loss of dopaminergic neurons due to Parkin deficiency in AR-JP or Parkin inactivation in sporadic PD.

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