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Parkin Ubiquitinates and Promotes the Degradation of RanBP2*

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Parkinson disease (PD) is a common neurodegenerative disorder, which involves the deterioration of dopaminergic neurons in the pars compacta of the substantia nigra. The etiology of PD is still unknown, but recent identification of mutations in familial cases of PD has advanced the understanding of the molecular mechanisms of this neurological disease. Mutations in the *parkin* gene, which encodes for ubiquitin-protein ligase (E3), have been implicated in autosomal recessive juvenile Parkinsonism, an early onset and common familial form of PD. Here we reported that Parkin selectively binds to RanBP2, which is localized in the cytoplasmic filament of the nuclear pore complex and belongs to the small ubiquitin-related modifier E3 ligase family. We also demonstrated that RanBP2 becomes a target for Parkin E3 ubiquitin-ligase and is processed via Parkin-mediated ubiquitination and subsequent proteasomal degradation. Furthermore, Parkin controls the intracellular levels of sumoylated HDAC4, as a result of the ubiquitination and degradation of RanBP2. Our findings suggested that the intracellular levels of RanBP2 and its functional activity may be modulated by Parkin-mediated ubiquitination and proteasomal pathways.

Parkinson disease (PD)[2] is a major neurodegenerative disease characterized by a distinct set of movement disorders, including muscle rigidity, tremor, and bradykinesia (1). In PD, the level of dopamine is decreased in the striatum, most severely in the putamen. This is largely a result of the deterioration of dopaminergic neurons in the substantia nigra pars compacta (2–4). The etiology of PD remains poorly understood, but several genetic loci have been implicated in the pathogenesis of familial forms of PD. First, two missense mutations of α-synuclein have been linked to a rare dominant form of PD (5). Second, *parkin* has been identified as the causative gene of early onset autosomal recessive juvenile parkinsonism (AR-JP) (6), and a missense mutation in the ubiquitin (Ub) C-terminal hydrolase L1 appears to be responsible for a dominant form of PD (7). Most recently, mutations in *DJ-1* and PTEN-induced putative kinase-1 have been correlated with the incidences of familial PD (8, 9).

The structure of Parkin contains a C-terminal RING-IBR-RING motif and an N-terminal region with homology to ubiquitin (10–12). Proteins destined to degrade in the proteasomes are subject to covalent modification by ubiquitin as a small protein tag. Ubiquitination proceeds through a sequential enzymatic reaction composed of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3) (13). Based on the previous findings that numerous proteins with RING finger motifs have E3 ubiquitin-ligase activity, several studies have revealed that Parkin has an ubiquitin E3 ligase activity. The exquisite specificity for the proteins to be ubiquitinated is usually determined by a diverse family of E3s with a specific E2.

Based on the functional role of Parkin, it can be surmised that the loss of Parkin results in the gradual and abnormal accumulation of Parkin substrates, which might then induce a familial PD syndrome. Several substrates of Parkin have been reported to date, including cell division control-related protein-1 (CDCrel-1; Ref. 13), synphilin-1 (14), Pael-R (10, 15), α-synuclein (16, 17), the p38 subunit of aminoacyl-tRNA synthetase (18), cyclin E (19), α/β-tubulin (20), and polyglutamine protein (21).

Here we attempted to identify the new binding targets of Parkin by using yeast two-hybrid assay. As the result of screening human fetal cDNA library, RanBP2 was identified to bind selectively to Parkin. Using co-immunoprecipitation, *in vitro* GST pull-down assay, and immunocytochemical analysis, we detected the occurrence of specific interactions between Parkin and RanBP2 in mammalian neuronal systems as well as in non-neuronal cells. In addition, RanBP2 was shown to become a substrate of Parkin E3 ubiquitin ligase and to be subsequently processed via the proteasomal machinery.

EXPERIMENTAL PROCEDURES

Materials and cDNA Constructs—Synthetic dropout medium and yeast extract peptone dextrose containing adenine were purchased from Qbiogene (Carlsbad, CA). 3-Amino-1,2,4-triazole was from Sigma, and the human fetal brain cDNA library was from Clontech. Peroxidase-conjugated anti-rabbit and anti-mouse immunoglobulins were from Zymed Laboratories Inc.. Dulbecco's modified Eagle's medium, fetal bovine serum, and cell culture reagents were from Invitrogen. Protein A-Sepharose was from Amersham Biosciences, and enhanced chemiluminescence (ECL) reagents were obtained from PerkinElmer Life Sciences. Polyclonal and monoclonal anti-HA, anti-GFP, anti-GST, and anti-RanBP2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-FLAG antibody was from Sigma, and

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[2] The abbreviations used are: PD, Parkinson disease; AR-JP, autosomal recessive juvenile Parkinsonism; DAT, dopamine transporter; HDAC, histone deacetylase; HEK, human embryonic kidney; lactacystin, clasto-lactacystin β-lactone; SUMO, small ubiquitin-related modifier; Ub, ubiquitin; UPS; ubiquitin-proteasome system; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein ligase; GFP, green fluorescent protein; GST, glutathione *S*-transferase; siRNA, small interfering RNA; ER, endoplasmic reticulum; HA, hemagglutinin.

polyclonal anti-Parkin antibodies were from cell signaling and Chemicon. Clasto-lactacystin β-lactone was purchased from A. G. Scientific (San Diego), and calpeptin was from Calbiochem. Mammalian expression vector encoding for FLAG-tagged Parkin was kindly provided by G. Lee (Ajou University, Suwon, Korea). Plasmids encoding for bacterial recombinant GST fused with wild type and deleted Parkin mutants (77–465 and 1–170) and mammalian expression vectors encoding GFP-tagged wild type Parkin were gifts from H. Rhim (The Catholic University, Seoul, Korea). Plasmids encoding for recombinant GST-tagged deletion mutants of human RanBP2, such as containing the amino acids 2596–2836 (GST-IR) and 2633–2761 (GST-IR1 + 2), and pEGFP-Cl vector for GFP-tagged IR1 + 2 were provided by H. Saitoh (Kyushu University, Fukuoka, Japan). Mammalian expression vectors for HA-tagged SUMO-1 and GFP- and FLAG-tagged HDAC4 were provided by A. Dejean (Institut Pasteur, Paris, France). Plasmid encoding His-tagged Nedd4-2 was provided by P. Snyder (University of Iowa, Iowa City).

Yeast Two-hybrid Assay—The bait vector for yeast two-hybrid assay was constructed by subcloning the wild type Parkin cDNA into the pHybTrp/Zeo vector. The human fetal cDNA library subcloned into the prey vector, pACT2, was purchased from Clontech. The yeast strain L40, harboring the reporter genes *lacZ* and *HIS3* downstream of the *LexA* promoter, was sequentially transformed with bait vector followed by cDNA library vectors and then plated on a synthetic medium containing 5 mM 3-amino-1,2,4-triazole and lacking histidine, leucine, and tryptophan residues. After incubating the plates for 10–14 days at 30 °C, the transformants were tested with a synthetic medium that lacked histidine, leucine, and tryptophan residues and contained 50 μg/ml 5-bromo-4-chloro-3-indoryl-D-galactoside. After incubation for 2–3 days at 30 °C, the blue yeast colonies were selected as positive clones. The positive clone plasmids were extracted from yeast in lysis buffer containing 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, pH 8.0, and 1.0 mM EDTA and then transformed into *E. coli* DH5α via electroporation. Sequences of the inserts in positive library plasmids were analyzed with an automatic DNA sequencer (ALF express, Amersham Biosciences).

Cell Culture and Preparation of Cell Lysates—Human embryonic kidney 293 (HEK293) cells and dopaminergic neuroblastoma SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. To prepare cell lysates, the cells were rinsed twice with ice-cold phosphate-buffered saline and solubilized in lysis buffer (20 mM Tris, pH 7.9, containing 1.0% Triton X-100, 1 mM Na_3VO_4, 137 mM NaCl, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 5 mM Na_2EDTA, 10% glycerol, 1 mM β-glycerophosphate, 0.1 g/ml *p*-nitrophenyl phosphate, and 0.2 mM phenylmethylsulfonyl fluoride). The cells were scraped, and the supernatants were collected after 10 min of centrifugation at 14,000 × *g* and 4 °C. Protein concentrations were determined using the detergent-compatible protein assay kit (Bio Rad).

Immunoprecipitation and Western Blot Analysis—One microgram of antibodies was incubated with 1 mg of cell extracts prepared in lysis buffer overnight at 4 °C. Thirty microliters of a 1:1 suspension of protein A-Sepharose beads was added and incubated for 2 h at 4 °C with gentle rotation. The beads were pelleted and washed five times with cell lysis buffer. Bound proteins were dissociated by boiling in SDS-PAGE sample buffer, and whole protein samples were separated on SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Millipore, Japan). The membrane was blocked in TBST buffer containing 20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween 20, and 5% nonfat dry milk for 1 h and then incubated overnight at 4 °C in 3% nonfat dry milk containing antibodies. The membrane was washed several times in TBST, and then incubated with secondary IgG-coupled horseradish peroxidase antibody (Zymed Laboratories Inc.). After 60 min, blots were washed several times with TBST and were visualized by ECL.

RNA Interference—The matched and nonsilencing siRNAs of RanBP2 were generated as described previously (22). The target cells were transfected with the annealed double-strand RNA oligonucleotides (PROLIGO Primer & Probes, Boulder, CO) using Lipofectamine (Invitrogen) at a final concentration of 100 nM.

In Vitro Ubiquitination Assay—Fifty microliters of *in vitro* ubiquitination reaction contained reaction buffer (50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 2 mM dithiothreitol), 10 μg of recombinant ubiquitin, 100 ng of E1 (Calbiochem), 200 ng of UbcH7 (Sigma), and 500 ng of GST-IR as a substrate. The reaction was performed via the addition of either Myc-tagged Parkin or its deleted mutants produced by TNT quick-coupled transcription/translation systems (Promega) for 2 h at 37 °C and terminated by mixing with an equal volume of 2× SDS sample buffer. The reaction products were subjected to Western blot analysis and were analyzed with anti-GST or anti-ubiquitin antibodies (Santa Cruz Biotechnology).

RESULTS

Yeast Two-hybrid Assay for the Identification of New Parkin E3 Ubiquitin Ligase Substrate(s)—To identify the new Parkin-binding partner(s), we performed yeast two-hybrid assay by using full-length wild type Parkin as bait. After screening human fetal brain cDNA library, several previously known and novel proteins were identified to interact with Parkin, including cyclin E, β-tubulin, and RanBP2 (data not shown). Among these, RanBP2 is a component of cytoplasmic filaments in the nuclear pore complex (23). Nuclear pore complex traverses the nuclear envelope, providing a channel through which nucleocytoplasmic transport occurs. RanBP2 is thought to play an important role in CRM1-mediated nuclear protein export (24). In addition, RanBP2 is known to function as a SUMO E3 ligase (25). To determine whether RanBP2 might be a new ubiquitination target of Parkin, and/or whether the potential binding between these two proteins might alter the functional activity of Parkin, RanBP2 was chosen as a target for further analysis.

Parkin Interacts with RanBP2 in Vivo and in Vitro—First, we attempted to determine whether Parkin selectively binds to RanBP2 in mammalian cells. After eukaryotic expression plasmid encoding FLAG-tagged human wild type Parkin was transiently transfected into HEK293 cells, and the cell lysates were prepared, immunoprecipitated with anti-RanBP2 antibodies, and subsequently immunoblotted with anti-FLAG IgG. As shown in Fig. 1*A*, the transfected FLAG-tagged Parkin was able to bind to endogenous RanBP2 in the HEK293 cells. When co-immunoprecipitation was performed in reverse with anti-FLAG antibodies, followed by immunoblot analysis with anti-RanBP2 IgGs, the results was the same. This indicates that FLAG-Parkin binds well to RanBP2 (Fig. 1*A*). We next examined whether this specific association between Parkin and RanBP2 was also present in human dopaminergic neuroblastoma (SH-SY5Y) cell lines. After eukaryotic expression plasmid encoding FLAG-tagged Parkin was transfected into the SH-SY5Y cells (Fig. 1*B*), the immunoprecipitation was performed with either anti-RanBP2 or anti-FLAG antibodies. As shown in Fig. 1*B*, Parkin selectively binds to endogenous RanBP2. When the same co-immunoprecipitation assay was performed in the neuroblastoma SK-N-BE2C cell line, the specific interaction between RanBP2 and Parkin was also observed (data not

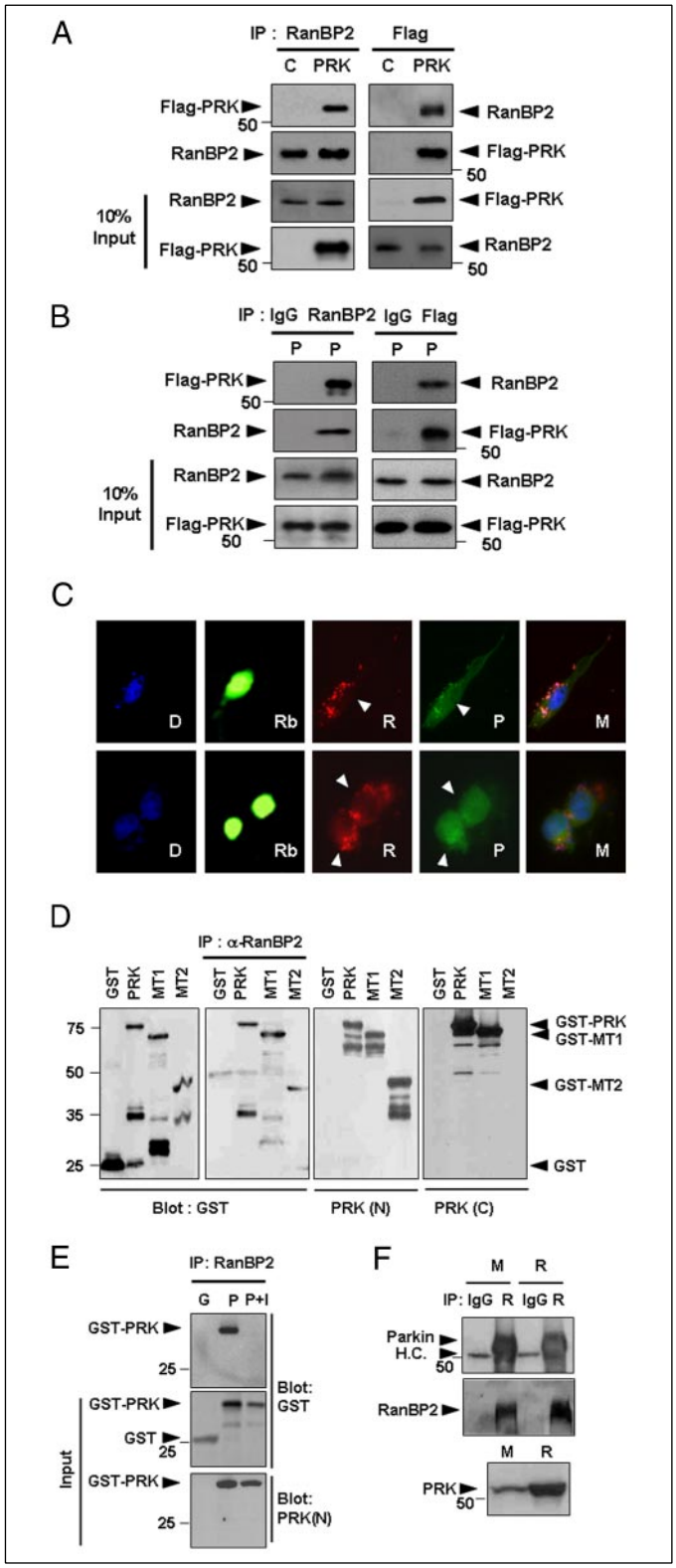

FIGURE 1. **Parkin interacts with endogenous RanBP2 both *in vivo* and *in vitro*.** *A*, where specified, HEK293 cell lines were mock-transfected (*C*) or transfected with plasmids for FLAG-tagged wild type Parkin (*PRK*) in a transient manner. After 24 h, immunoprecipitation (*IP*) was performed with either anti-RanBP2 or anti-FLAG antibodies, as indicated. The immunocomplexes were then resolved by SDS-PAGE and analyzed by Western blotting with anti-FLAG or anti-RanBP2 antibodies. The proper expression of transient-transfected and endogenous proteins in cell lysates was identified with Western blot analysis with anti-FLAG or anti-RanBP2 antibodies. *B*, where specified, SH-SY5Y cells were transfected with 3 μg of plasmid encoding FLAG-tagged wild type Parkin (*PRK* or *P*). The cells were immunoprecipitated with preimmune IgG, anti-RanBP2, or anti-FLAG antibodies, followed by immunoblot analysis with anti-FLAG IgG, as indicated. The proper expression of transient-transfected and endogenous proteins in cell lysates was also identified, as described in *A*. *C*, SH-SY5Y cells were transfected with plasmids for GFP-tagged Parkin (*P*). After 24 h of incubation, the cells were fixed, permeabilized, and labeled with anti-RanBP2 antibodies (*R*), followed by staining with nuclear marker, anti-Rb (*Rb*) IgG, or rhodamine-attached secondary antibodies, and stained with DAPI (*D*). Immunostained preparations were examined with confocal microscopy. *Arrowheads* indicate the expression of RanBP2 and Parkin. *M* indicates the "merge" of the *P* and *R* images. *D*, in the *left* and *right panels*, immunoblots with either anti-GST or anti-Parkin (two antibodies specific to the N-terminal or C-terminal region, respectively) antibodies validated the expression of recombinant GST-Parkin and its deleted mutants constructs used for the *in vitro* binding assay. In the *middle panel*, after the HEK293 cell extract was incubated with the purified recombinant GST-fused wild type Parkin (*PRK*) or its deleted mutants (*MT1*, residues 77–465; *MT2*, residues 1–170) or GST as a control, immunoprecipitation was performed with anti-RanBP2 antibodies, and the precipitated protein complexes were analyzed by Western blotting with anti-GST antibodies. *E*, HEK293 cell extracts were incubated with the recombinant GST-fused Parkin peptide, which spans 78–180 amino acids (*P*) or GST alone (*G*) as a control, in the absence or presence of 100 nM blocking peptide (*I*); immunoprecipitation was performed with anti-RanBP2, followed by Western blotting with anti-GST antibodies. The presence of GST-Parkin or GST in the complexes was examined by using anti-GST or anti-Parkin antibodies, as indicated. *F*, after the mouse (*M*) and rat (*R*) brain cortex were isolated, cell lysates were prepared with gentle homogenization. Where indicated, the immunoprecipitation of the cortical cell lysates was done with either preimmune IgG or anti-RanBP2 antibodies (*R*) and immunoprecipitates were analyzed via Western blot analysis with anti-RanBP2 or anti-Parkin antibodies (*PRK*). The proper expression of Parkin in cortical cell extracts was identified by the immunoblotting with anti-Parkin antibodies (*right panel*).

shown). These results suggest that the specific binding of Parkin to RanBP2 occurs in mammalian cells.

To characterize further the interactions between Parkin and RanBP2, SH-SY5Y cells were cultured on glass coverslips and transfected with GFP-Parkin. The expression of GFP-Parkin proteins was visualized by using a confocal microscope. Immunofluorescence analysis of endogenous RanBP2 was carried out with anti-RanBP2 IgG and then rhodamine-conjugated secondary antibodies. As shown in Fig. 2*C*, the co-localization of RanBP2 and GFP-Parkin was observed (Fig. 1*C*).

To confirm the specific interaction between Parkin and RanBP2 and to identify the interaction site, an *in vitro* GST pull-down assay was performed by using recombinant GST-Parkin fusion protein (Fig. 1*D*). To confirm the proper expression and purification of GST-Parkin and its mutants, Western blotting was performed with anti-GST IgG and two anti-Parkin antibodies; one is specific for amino acid residues 83–97 of the human Parkin N terminus, and the other is specific for several residues around the C-terminal 400th amino acid of Parkin. After HEK293 cell extracts were incubated with GST fused either with wild type Parkin or its deleted mutants (amino acids 77–465 or 1–170), immunoprecipitation was carried out with anti-RanBP2 antibodies, followed by immunoblot analysis with either anti-GST antibodies. As shown in Fig. 1*D*, both wild type and deleted mutant Parkin fused with GST were co-immunoprecipitated with endogenous RanBP2. When used as a negative control, GST alone was determined not to interact with RanBP2. The Parkin fragment spanning its 78–170th amino acid fused with GST was shown to interact with RanBP2 (Fig. 1*E*). In addition, an excess amount of GST-Parkin peptide (amino acids 78–170) blocked the binding of RanBP2 to Parkin (Fig. 1*E*). These data indicated that Parkin directly binds to RanBP2 *in vitro* as well as *in vivo*, and the region spanning amino acid residues 78–170 appears to be important for its binding to RanBP2.

We next attempted to determine whether the specific association between Parkin and RanBP2, which had been encountered in both *in vitro* and in cell-based assays, was also present in mammalian neural tissues of the central nervous system. After the rat and mouse brain cortex were isolated and homogenized, Western blot analysis revealed that significant levels of Parkin are present (Fig. 1*F*, *left*). The cell extracts were then immunoprecipitated with anti-RanBP2 antibodies, followed by the immunoblot analysis with either anti-Parkin or anti-RanBP2 antibodies. As shown in Fig. 1*F*, Parkin selectively associated

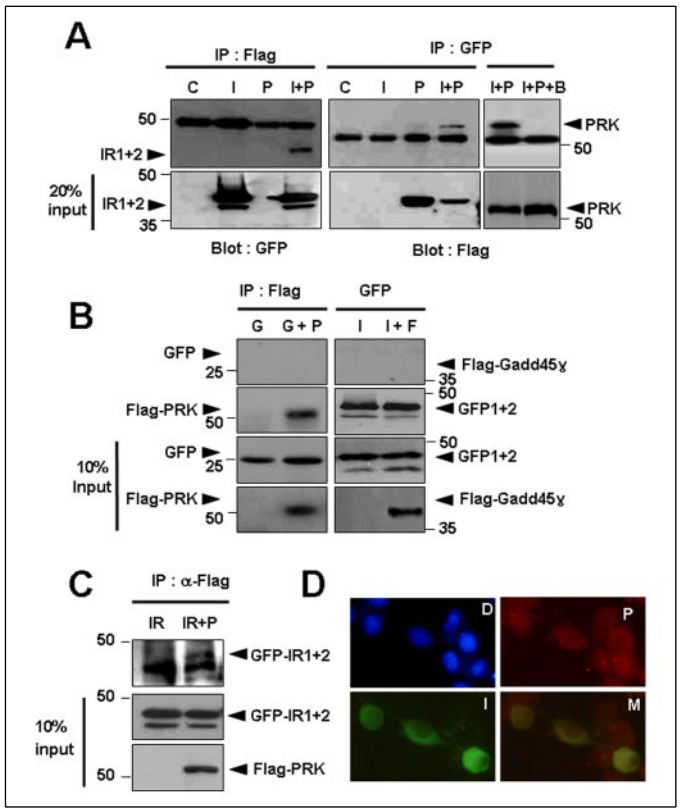

FIGURE 2. **Parkin interacts with IR1 + 2, the deletion mutant of RanBP2.** *A,* where indicated, 3 μg of plasmid encoding for either FLAG-tagged whole Parkin (*P*) or GFP-tagged deleted RanBP2 mutants (IR1 + 2 or denoted as *I*) were transfected, or two plasmids were co-transfected into HEK293 cells (*I*+*P*) in the absence or presence of 100 nM recombinant GST-IR1 + 2 (*B*) as a blocking peptide. Immunoprecipitation (*IP*) was performed with either anti-FLAG or anti-GFP antibodies, and the immunocomplexes (*upper panel*) and cell lysates (*lower panel*) were analyzed by Western blotting with anti-GFP or anti-FLAG antibodies, as indicated. *B,* where specified, the cells were transfected with GFP alone (*G*), GFP plus FLAG-Parkin (*G* + *P*; *left panel*), GFP-IR1 + 2 (*I*), or GFP-IP1 + 2 plus FLAG-Gadd45γ (*I* + *F*; *right panel*). The cells were immunoprecipitated with anti-FLAG or anti-GFP antibodies, followed by the immunoblot analysis with anti-GFP or anti-FLAG antibodies, as indicated. The proper expression of transiently transfected proteins in cell lysates was analyzed with anti-GFP or anti-FLAG antibodies. *C,* where specified, dopaminergic MN9D cells were transfected with 3 μg of mammalian plasmid encoding GFP-tagged IR1 + 2 mutant of RanBP2 (*IR*) or co-transfected with FLAG-Parkin plus GFP-IR1 + 2 (*IR*+*P*), as indicated. The cells were immunoprecipitated with anti-FLAG antibodies, followed by immunoblot analysis with anti-GFP IgG. The proper expression of transiently transfected proteins in cell extracts was examined with Western blot analysis with either anti-FLAG or anti-GFP antibodies. *D,* where specified, SH-SY5Y cells were co-transfected with plasmids encoding for FLAG-tagged Parkin (*P*) plus GFP-tagged IR1 + 2 (*I*). After 24 h of incubation, the cells were fixed, permeabilized, and labeled with anti-Parkin antibodies, followed by staining with rhodamine-attached secondary antibodies, and stained with DAPI (*D*). *M* indicates the "merge" of the *P* and *I* images. Immunostained preparations were examined with fluorescence microscopy.

with RanBP2. As a control, the analysis of immunocomplexes prepared with preimmune IgG revealed that there is no obvious Parkin band for interaction with RanBP2 (Fig. 1*F*). The same interaction between Parkin and RanBP2 was also observed in the extracts from other brain tissues, such as the hippocampus and midbrain (data not shown).

Parkin Interacts with the IR1 + 2 Domain of RanBP2—To verify further this binding and to map the binding domain within the RanBP2, plasmids encoding for FLAG-tagged Parkin were co-transfected into HEK293 cells with a construct designed for the expression of GFP-tagged RanBP2 deletion mutant (IR1 + 2), spanning amino acids 2633–2761 of RanBP2, followed by immunoprecipitation with either anti-FLAG or anti-GFP antibodies, respectively. The IR1 + 2 region was shown previously to bind to the SUMO-conjugating E2 enzyme, Ubc9, and to possess the SUMO-E3 ligase activity of RanBP2 (25–27). As shown in Fig. 2*A*, Western blot analysis of the immunocomplexes with either anti-GFP or anti-FLAG antibodies demonstrated that Parkin binds to IR1 + 2. In addition, the incubation of excess recombinant IR1 + 2 proteins, as a blocking peptide, resulted in the inhibition of Parkin binding to IR1 + 2 (Fig. 2*A*). Furthermore, when the plasmid encoding unrelated FLAG-GAdd45γ or intact GFP was used as a negative control, they did not bind to GFP-IR1 + 2 or FLAG-Parkin, respectively (Fig. 2*B*). The binding between Parkin and IR1 + 2 domain was also observed in murine dopaminergic MN9D cells (Fig. 2*C*). In addition, the immunohistochemical analysis was performed to examine whether Parkin is co-localized with IR1 + 2. After SH-SY5Y cells were co-transfected with plasmids encoding for Myc-tagged Parkin plus GFP-tagged IR1 + 2, the cells were labeled with anti-Parkin antibodies, followed by staining with rhodamine-attached secondary antibodies. As shown in Fig. 2*C*, fluorescence microscopic observation indicated that the localization of IR1 + 2 expression is coincident with that of Parkin within the nucleus. These data indicate that IR1 + 2 domain of RanBP2 specifically interacts with Parkin.

Parkin Promotes the Ubiquitination of RanBP2 and Its Degradation via Ubiquitin-Proteasome System (UPS) Pathway—To determine the functional relevance of the mutual interaction between Parkin and RanBP2, we attempted to ascertain whether RanBP2 could function as a substrate of Parkin E3 ubiquitin ligase. To evaluate whether Parkin could mediate the degradation of RanBP2 via the UPS, we first measured the effects of Parkin overexpression on the endogenous levels of RanBP2. After the transient transfection of plasmid encoding for FLAG-Parkin into HEK293 cells, cell lysates were prepared, and Western blot analysis was performed with anti-RanBP2 or anti-FLAG antibodies. As shown in Fig. 3*A*, Parkin was properly expressed inside the cells after its transient transfection, and a significant decrease in RanBP2 protein levels was detected after 48 h in the cells exhibiting Parkin overexpression, as compared with control cells transfected with vehicle only (Fig. 3*A*, *right*). These findings suggest that Parkin may either promote the degradation of and/or block the synthesis of RanBP2. To ensure that this Parkin-mediated reduction in endogenous RanBP2 levels was not an event specific to the HEK293 cells, the same experiment was conducted in SH-SY5Y cells and H19-7 cells. Just as seen in the HEK293 cells, the endogenous RanBP2 protein levels also decreased after 36 and 48 h of post-transfection with Parkin plasmid in the SH-SY5Y cells (Fig. 3*A*, *left*) and H19-7 cells (data not shown), indicating that Parkin overexpression results in the down-regulation of intracellular RanBP2.

In addition, we tested whether the dominant-negative Parkin mutant containing the 77–465-amino acid domain affects the Parkin-mediated reduction in RanBP2 levels. When the plasmid encoding the functionally defective Parkin mutant was transfected into SH-SY5Y cells, the endogenous RanBP2 levels were not significantly changed, as compared with mock-transfected cells (Fig. 3*B*). Therefore, this result indicated that the E3 ubiquitin ligase activity of Parkin was required for the reduction of endogenous RanBP2 levels.

To determine whether Parkin-mediated RanBP2 degradation occurred via the UPS pathway, the endogenous levels of RanBP2 in the cells transiently transfected with FLAG-Parkin were measured and compared in the absence or presence of clasto-lactacystin β-lactone (lactacystin), the highly specific proteasomal proteolysis inhibitor. Based on our previous report (17), in which Parkin was shown to cleave intracellular α-synuclein inclusions via calpain activation, we also tested the effect of the calpain inhibitor, calpeptin, to confirm the involvement of calpain in the Parkin-mediated degradation of RanBP2. When SH-SY5Y cells were transiently transfected with Parkin, followed by the stimulation of lactacystin, the reduction of endogenous RanBP2 levels was remarkably blocked, as compared with the control cells that were

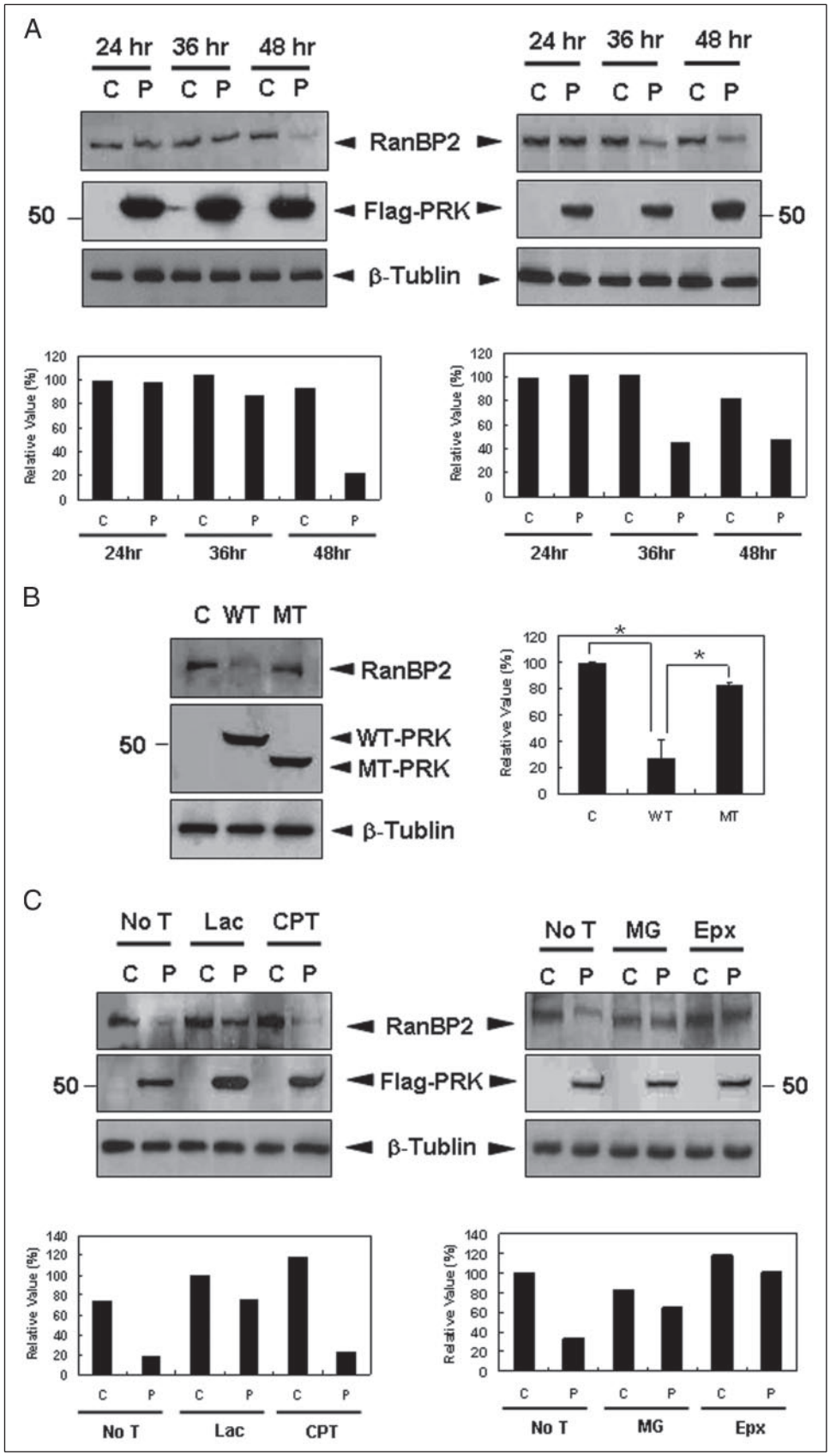

FIGURE 3. **Parkin-induced degradation of RanBP2 occurs through UPS system.** *A,* after either HEK293 (*left*) or SH-SY5Y cells (*right*) were mock-transfected (*C*) or transiently transfected with 3 μg of plasmid encoding for FLAG-tagged wild type Parkin (*P*) for the indicated times, the cells were lysed, and the cell extracts were resolved by SDS-PAGE and then analyzed by Western blotting with either anti-RanBP2 or anti-FLAG antibodies, as indicated. The graphs indicate the intensity of the bands using the densitometric analysis (Image 3.1 program from the National Institutes of Health). To confirm the equal loading of protein, the level of β-tubulin in each lane was determined as a control. *B,* after the SH-SY5Y cells were mock-transfected (*C*) or transfected with plasmids encoding for Myc-tagged wild type Parkin (*WT*) or its deleted mutant (amino acids 77–465; *MT*) for 48 h, the cell extracts were resolved by SDS-PAGE and then analyzed by Western blotting with either anti-RanBP2 or anti-Myc antibodies (*WT-PRK* or *MT-PRK*), as indicated. The graph indicating the intensity of the bands is expressed as in Fig. 3*A*. Data are presented as means $\pm$ S.E. of three independent experiments conducted in triplicate. *, $p < 0.05$. *C,* after the SH-SY5Y cells were mock-transfected (*C*) or transfected with 3 μg of plasmid encoding for FLAG-tagged Parkin, the cells were untreated (*No T*) or treated with either 10 μM proteasome inhibitor, clasto-lactacystin β-lactone (*Lac*), 50 μM calpeptin (*CPT*), 10 μM MG132 (*MG*), or 200 nM epoxomicin (*Epx*) for 6 h, as indicated. Total cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with anti-RanBP2 or anti-FLAG antibodies, as indicated. The graphs indicated the intensity of the bands, quantitated as in *A*.

not treated with lactacystin. In contrast, we witnessed no significant effects on the recovery of the reduced RanBP2 levels as a result of pretreatment with calpeptin (Fig. 3*C*). These results indicated that Parkin promotes RanBP2 degradation, which appears to be mediated somewhat by the activation of proteasome-dependent proteolytic machinery, but not through calpain activation. Moreover, the same results were

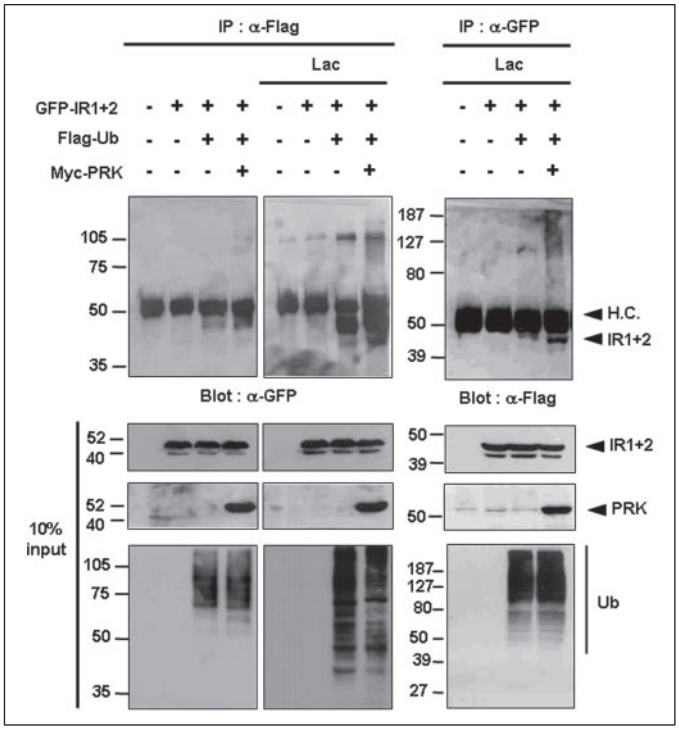

FIGURE 4. **Parkin promotes the ubiquitination of IR1 + 2-RanBP2 *in vivo*.** After HEK293 cells were transfected with the plasmids for either FLAG-tagged ubiquitin (*FLAG-Ub*) and GFP-tagged IR1 + 2 or together with Myc-tagged Parkin (*Myc-PRK*), the cells were incubated in the absence or presence of 10 μM clasto-lactacystin β-lactone (*Lac*) for 6 h. The cell lysates were immunoprecipitated (*IP*) with anti-FLAG or anti-GFP antibodies, followed by immunoblot analysis with anti-GFP or anti-FLAG antibodies. To determine the proper expression of GFP-IR1 + 2, Myc-Parkin and FLAG-ubiquitin, Western blot analysis of the cell lysates was performed with anti-GFP, anti-Myc, or anti-FLAG antibodies, as indicated. The intact IR1 + 2, Myc-Parkin (*PRK*), FLAG-ubiquitin (*Ub*), and immunoglobulin heavy chain (*H.C.*) are denoted by *arrowheads*.

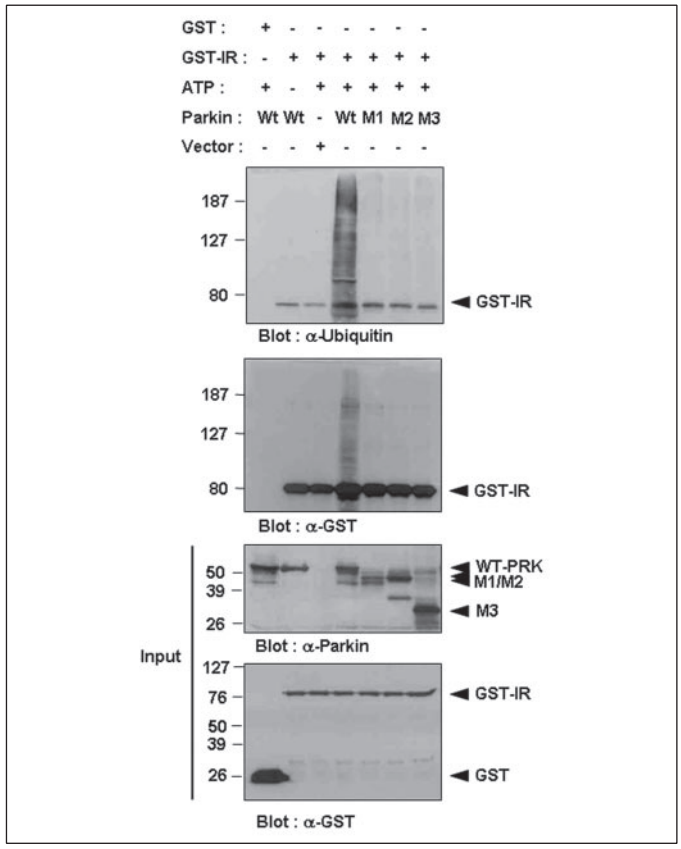

FIGURE 5. **Parkin ubiquitinates RanBP2 *in vitro*.** Where indicated, either recombinant deleted IR-RanBP2 protein fused with GST (*GST-IR*) or GST as a control was incubated with an *in vitro* ubiquitination system, which contains recombinant Ub, E1, and E2. As the source of E3 ubiquitin ligase, wild type Parkin (*Wt*) or its deleted mutants (*M1*, 77–465; *M2*, 1–415; and *M3*, 217–465) produced using TNT *in vitro* transcription/translation kit was added to the reaction assay. The formation of ubiquitinated reaction products was evaluated by Western blot analysis with either anti-ubiquitin or anti-GST antibodies. To confirm the proper expression and purification of Parkin and its mutants and GST-IR, Western blot analysis was performed with anti-Parkin antibodies or anti-GST antibodies.

observed in the cells treated with other proteasome inhibitors, such as MG132 and epoxomycin (Fig. 3*C*).

The observation that RanBP2 expressed in cultured cells is liable to be digested through the proteasomal system and its degradation is facilitated by Parkin prompted us to examine whether RanBP2 can be covalently modified by ubiquitin via the action of Parkin. After HEK293 cells were transfected with eukaryotic expression plasmids for either FLAG-tagged ubiquitin plus GFP-tagged IR1 + 2 alone or together with Myc-Parkin as indicated, the cells were untreated or treated with lactacystin. The cell lysates were immunoprecipitated with anti-FLAG or anti-GFP antibodies, followed by immunoblot analysis with anti-GFP or anti-FLAG IgG. As shown in Fig. 4, the pretreatment of lactacystin accelerated the ubiquitination of IR1 + 2 in the cells transfected with Parkin, compared with control cells. These results indicated that RanBP2 becomes a target of Parkin E3 ubiquitin ligase, and its levels are regulated by Parkin and the proteasomal complex system.

Parkin Ubiquitinates RanBP2 in Vitro—Using *in vitro* ubiquitination assay, we attempted to determine whether Parkin could directly ubiquitinate RanBP2. To reconstitute the ubiquitin-conjugation-ligation reaction, *in vitro* translated wild type Parkin or its deleted mutants (amino acids 77–465, 1–415, and 217–465) were added to reaction mixtures containing recombinant ubiquitin, E1, and E2. It has been reported that the E3 ubiquitin ligase activity of these mutants is absent (11). Because the intact RanBP2 is too large (~358 kDa) to detect further processing, such as into polyubiquitination, the recombinant-deletion RanBP2 mutant fused with GST (GST-RanBP2-IR), spanning amino acids 2596–2836 of RanBP2 and possessing the full SUMO-E3 ligase activity (26, 27), was used as a potential substrate in an *in vitro* ubiquitination assay. Western blot analysis with anti-Parkin antibodies demonstrated that the synthesis of Parkin proteins and its mutants properly occurs by TNT *in vitro* translation (Fig. 5).

As shown in Fig. 5, in order to ascertain whether wild type Parkin promotes the ubiquitination of RanBP2-IR, the reaction samples were immunoanalyzed with anti-ubiquitin antibodies. When compared with the samples prepared in the absence of Parkin or in the presence of its deleted mutant, the samples added with wild type Parkin exhibited significant increases in ubiquitinated protein bands, thereby indicating that Parkin functions as an E3 ubiquitin ligase and enhances protein ubiquitination. The analysis of reaction samples with anti-GST antibodies confirmed that the addition of *in vitro* translated wild type Parkin proteins promotes the ubiquitination of RanBP2 more profoundly than does the fraction containing *in vitro* translated control vector or *in vitro* translated Parkin mutants (Fig. 5). These data strongly suggest that the high molecular weight ubiquitinated proteins represent the ubiquitinated RanBP2-IR, and Parkin is able to ubiquitinate RanBP2 directly *in vitro*.

Parkin Controls the Intracellular Levels of Sumoylated HDAC4 as a Result of the Ubiquitination and Degradation of RanBP2—Next we tried to determine how Parkin functionally affects intracellular metabolic activities as a result of RanBP2-ubiquitination and its consequent degradation. Based on the previous findings that two target proteins, SP100 (27) and HDAC4 (26), are known to be sumoylated by RanBP2, it was

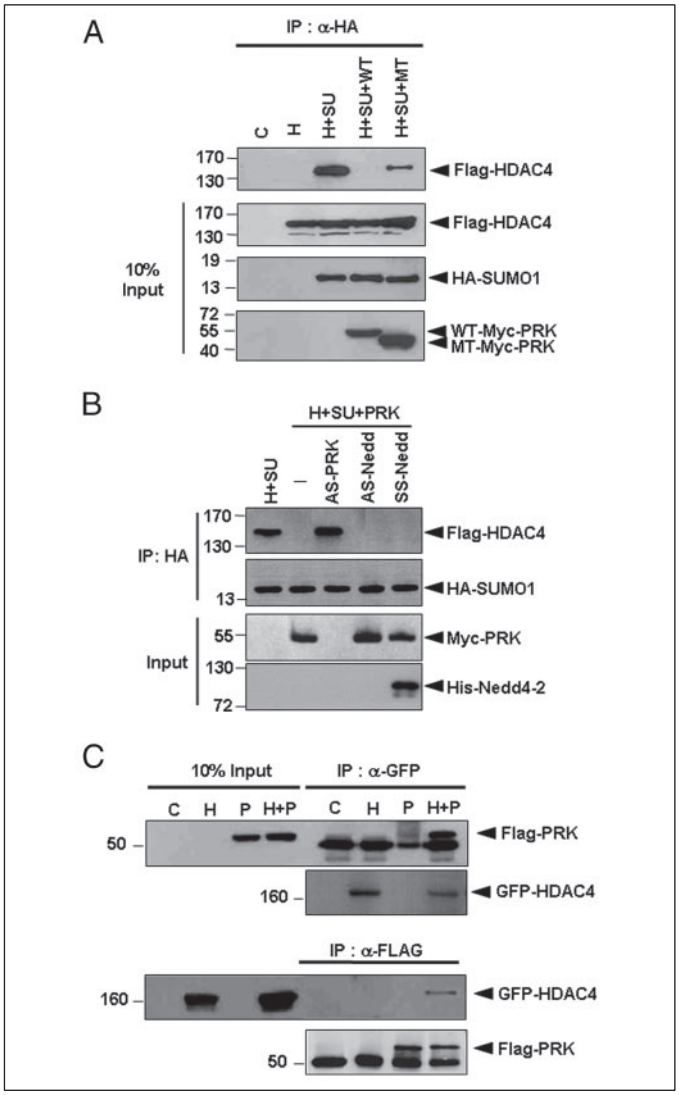

FIGURE 6. **Parkin controls intracellular levels of sumoylated HDAC4 through the indirect degradation of RanBP2.** *A,* where specified HEK293 cells were co-transfected with two plasmids of HA-tagged SUMO-1 (*SU*) and FLAG-tagged HDAC4 (*H*) and/or with either Myc-tagged wild type Parkin vector (*WT*) or its deleted mutant (amino acids 77–465; *MT*), followed by immunoprecipitation (*IP*) with anti-HA IgG. Then the immunocomplexes were resolved by SDS-PAGE and analyzed using anti-FLAG IgG. The expression of transfected target proteins in the cell lysates was identified with immunoblotting with anti-HA, anti-FLAG, or anti-Myc antibodies. *B,* HEK293 cells were co-transfected with HA-SUMO-1 (*SU*) and FLAG-HDAC4 (*H*) and/or with Myc-tagged wild type Parkin (*PRK*), as indicated. Where specified, the cells were co-transfected together with the plasmid encoding antisense Parkin (*AS-PRK*), sense (*SS-Nedd*), or antisense His-tagged Nedd4–2 (*AS-Nedd*), followed by immunoprecipitation with anti-HA IgG. Then the immunocomplexes were resolved by SDS-PAGE and analyzed using anti-FLAG IgG. The expression of transfected target proteins in the cell lysates was identified with immunoblotting with anti-Myc or anti-His antibodies. *C,* as indicated, 3 μg of plasmid encoding for GFP-tagged HDAC4 (*H*) were co-transfected with FLAG-tagged Parkin plasmid (*P*) into HEK293 cells. Immunoprecipitation (*IP*) was performed with anti-FLAG or anti-GFP antibodies, and immunoprecipitates were analyzed by Western blotting with anti-GFP or anti-FLAG antibodies, as indicated. "*C*" in *A* and *C* indicates "control."

examined whether Parkin was able to modulate intracellular levels of sumoylated HDAC4 in either a direct or an indirect way. HDACs modulate cell growth and differentiation by governing chromatin structure and repressing the activity of specific transcription factors. First, to verify the SUMO modification of HDAC4, the HEK293 cells were transiently transfected with plasmids encoding for either FLAG-tagged HDAC4 alone or together with SUMO-1 construct. As shown in Fig. 6*A*, the FLAG-tagged HDAC4 proteins were significantly sumoylated in the presence of SUMO-1. Second, we attempted to determine whether the amount of sumoylated HDAC4 could be altered by Parkin overexpression. After the HEK293 cells were transiently transfected with either HA-tagged SUMO-1 plus FLAG-tagged HDAC4 or together with wild type Parkin plasmid or the deleted form of Parkin (amino acids 77–465), the sumoylated HDAC4 levels were compared by the immunoprecipitation with anti-HA, followed by immunoblotting with anti-FLAG antibodies. As shown in Fig. 6*A*, the levels of sumoylated HDAC4 were diminished completely in the cells transfected with wild type Parkin, but there was a marked enrichment of sumoylated HDAC4 levels in the cells transfected with the deleted Parkin mutant. In addition, when the plasmid encoding antisense Parkin was co-transfected into the cells to block the Parkin expression (50), the reduced level of sumoylated HDAC4 was also remarkably enhanced (Fig. 6*B*). However, the co-transfection of Parkin with either sense or antisense Nedd4-2, as a control of unrelated protein, did not affect the Parkin-mediated inhibition of the mutual interaction between HDAC4 and SUMO-1 (Fig. 6*B*). To determine whether Parkin directly interacts with HDAC4, a eukaryotic expression plasmid for GFP-tagged HDAC4 was transiently co-transfected into HEK293 cells with FLAG-Parkin, followed by immunoprecipitation with either anti-GFP or anti-FLAG antibodies (Fig. 6*C*). When the immunocomplexes were resolved by SDS-PAGE, and analyzed using anti-FLAG or anti-GFP antibodies, respectively, Parkin was demonstrated to bind to HDAC4. This Parkin/HDAC4 interaction might occur via direct mutual interaction and/or indirect interaction via RanBP2 linking. To distinguish between these two possibilities, *in vitro* binding assays were conducted followed by Western blot analysis or autoradiography using recombinant GST-Parkin and *in vitro* translated HDAC4 proteins. As shown in Fig. 7*A*, wild type Parkin did not interact directly with HDAC4 *in vitro*. To confirm this finding further, the siRNA of RanBP2 was transfected into the cells to reduce intracellular RanBP2 levels, and it was then determined whether the binding between Parkin and HDAC4 still occurred. As shown in Fig. 7*B*, the knock down of endogenous RanBP2 levels, which was induced by its siRNA, resulted in the abrogation of Parkin binding to HDAC4, whereas there was no effect by using the nonsilencing mismatched siRNAs (Fig. 7*C*). These data indicated that the interaction between Parkin and HDAC4 in the Parkin immunocomplexes might be indirectly possible through RanBP2 linking. Taken together, our data suggest that, as a result of RanBP2 ubiquitination, Parkin could also control the intracellular levels of sumoylated HDAC4, the sumoylation target of RanBP2.

DISCUSSION

Parkin is the causative gene of early onset AR-JP (6). As the Parkin gene product functions as an E3 ubiquitin ligase (10, 11, 13), it has been hypothesized that the functional loss of Parkin because of the mutations or deletions at the gene level results in a gradual accumulation of its substrates, eventually leading to the cell death within the substantia nigral neurons. In this study, we performed a yeast two-hybrid assay to identify additional potential factors or substrates that are involved in PD. As a result of screening, RanBP2 was observed to interact specifically with Parkin. As RanBP2 has been reported to play a role as an E3 SUMO ligase, it could be speculated that the SUMO-E3 ligase activity of RanBP2 might be modulated by Parkin and/or Parkin might be sumoylated by RanBP2.

Additional co-immunoprecipitation binding assays confirmed that RanBP2 physically interacts with Parkin in mammalian cells. In addition, it was found that Parkin ubiquitinates RanBP2 *in vitro* and promotes the degradation of RanBP2 via UPS in HEK293 and SH-SY5Y cells. Our current findings suggest that the functional activity of RanBP2

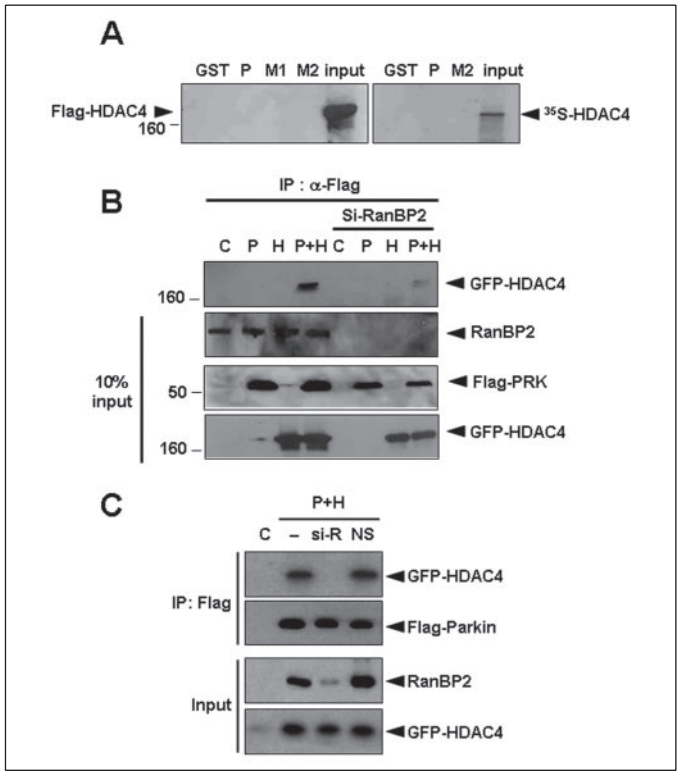

FIGURE 7. **Parkin did not bind directly to HDAC4 *in vitro*.** *A,* after *in vitro* translated FLAG-tagged HDAC4 using the TnT transcription/translation kit was incubated with the indicated recombinant GST fused with either wild type Parkin (*P*) or its deleted mutants (*M1*, 77–465; *M2*, 1–170), or GST alone as a control, we performed a GST pull-down assay. The precipitated protein complexes were then analyzed by Western blotting with anti-FLAG antibodies (*left*) or by autoradiography (*right*). The *last lane* in each panel shows the proper expression and purification of FLAG-HDAC4 as an *input*. *B,* as indicated, HEK293 cells were mock-transfected (*C*) or transfected with plasmid encoding FLAG-Parkin (*P*) or GFP-HDAC (*H*), respectively. Where specified, the cells were co-transfected with GFP-HDAC4 and FLAG-Parkin (*P* + *H*) in the absence or presence of siRNA of RanBP2 (100 nM). After 72 h, immunoprecipitation (*IP*) was performed with anti-FLAG antibodies, followed by Western blot analysis with anti-GFP IgG. Western blot analysis was carried out with anti-RanBP2, anti-FLAG, or anti-GFP antibodies to identify the proper expression of transfected or endogenous RanBP2 in the cell lysates. *C,* in the same way as *B*, HEK293 cells were transfected with suitable plasmids, as indicated. Where specified, the cells were co-transfected with the 100 nM siRNA-RanBP2 (*si-R*) or its mismatched RNA (*NS*). After 72 h, immunoprecipitation was performed with anti-FLAG antibodies, followed by Western blot analysis with anti-GFP or FLAG IgGs. Western blot analysis was carried out with anti-RanBP2 or anti-GFP antibodies to identify the proper expression of RanBP2 or GFP-HDAC4 in the cell lysates.

SUMO-E3 ligase may be regulated by Parkin-mediated ubiquitination and subsequent proteasomal degradation.

Among several Parkin substrates are transmembrane proteins or membrane-associated proteins. They are ubiquitinated by Parkin and subsequently degraded through the proteasome, which finally leads to the down-regulation of their activities. For instance, newly synthesized Pael-R is folded into the endoplasmic reticulum (ER) and then transported to the cell membrane. Misfolded Pael-R is normally translocated across the ER membrane into the cytosol, ubiquitinated by Parkin, and then degraded through the proteasome pathway (15). However, excessive amounts of unfolded Pael-R in the ER cause ER stress and finally leads to cell death. Parkin inhibits ER stress-mediated cell death by ubiquitinating and enhancing the degradation of unfolded Pael-R (10, 15). In addition, Parkin ubiquitinates and promotes the degradation of misfolded dopamine transporter (DAT). Parkin increases dopamine uptake by enhancing the ubiquitination and degradation of misfolded DAT, in order to prevent it from interfering with the oligomerization and cell surface expression of native DAT (28). Furthermore, Parkin promotes the degradation of synaptic-vesicle-associated CDCrel-1, which is localized mainly to presynaptic axon terminals of inhibitory neurons (13). This study reveals that the nuclear membrane-associated RanBP2 is subject to the ubiquitination and subsequent degradation via Parkin and proteasomal machinery, and Parkin could affect the nucleocytoplasmic protein transport in some way.

To date, two major forms of multiubiquitin chains are known to be formed through distinct types of linkages, in which the isopeptide bond linkages involve Lys-48 or Lys-63. Lys-48-linked ubiquitin tagging is mostly used to target proteins for degradation by the proteasome, whereas Lys-63-linked ubiquitination has been linked to numerous cellular events that do not rely on the degradative signaling via the proteasome. Of these, the endocytosis of membrane proteins (41–44), protein sorting and trafficking (45–47), and post-replicative DNA repair (48) are well characterized. Doss-Pepe *et al.* (49) have reported recently that Parkin has dual specificity for the assembly of ubiquitin chains through two different linkages to the substrate (Ub-Lys-48 and Ub-Lys-63). Based on the current finding that Parkin promotes the degradation of RanBP2 via UPS, Ub-Lys-48 is thought to be involved in its linkage to RanBP2.

Histone acetylation promotes gene transcription by relaxing chromatin structure, thereby facilitating access of the transcriptional machinery to DNA target sequences. The transcription-activating effect of histone acetylation is counterbalanced by histone deacetylation, which favors chromatin condensation and transcriptional repression. There are two major classes of HDACs (29). The class I HDACs (HDAC1–3 and 8) are widely expressed and consist mainly of a catalytic domain. In contrast, the class II HDACs (HDAC4, -5, -7, and -9) display cell type-restricted patterns of expression and contain an N-terminal extension that links them to specific transcription factors and confers responsiveness to a variety of signal transduction pathways, thereby connecting the genome with the extracellular environment (30). Class II HDACs intervene in cell differentiation, particularly in muscle (31–33). Most interestingly, all known class II proteins can shuttle from the cytoplasm to the nucleus, whereas most class I HDACs are constitutively present within the nucleus (34).

HDAC4 shows the highest expression in heart, skeletal muscle, and brain (35–37). HDAC4 is also known to interact with the MEF2 family, well known transcriptional activators playing roles in myogenesis and inhibit muscle differentiation (31, 37–39). Most interestingly, this transcription-repressing ability and histone deacetylase activity of the sumoylation-deficient HDAC4 mutant was impaired when compared with that of the wild-type HDAC4 protein (26). Although HDAC4 is part of large multiprotein complexes that mediate its recruitment to specific promoters (30), little is known about how its expression and stability are regulated. Recently, caspases are reported to modulate specifically gene repression and apoptosis through the proteolytic processing of HDAC4 (40). The current finding shows that Parkin could modulate the intracellular levels of sumoylated HDAC4 which is known to be modified by RanBP2 (26). This might have an implication that Parkin could play a role during the process of muscle differentiation. Furthermore, it may be predicted that the abrogation of Parkin function caused by gene level mutations, and seen in AR-JP, negatively affects proper RanBP2 degradation, and the abnormal accumulation of RanBP2 might consequently contribute to the formation of abnormal protein inclusions and the pathogenesis of PD. It will be also very interesting to find out whether Lewy bodies, which are typically found in the dopaminergic neurons of the substantia nigra in PD patients, also include RanBP2.

In conclusion, this study has provided valuable evidence that RanBP2 is a new substrate of Parkin, and Parkin can modulate the enzymatic activity of RanBP2 by ubiquitination and subsequent degradation. Our

study also suggests that the abnormal accumulation or turnover of RanBP2 and its substrates, including HDAC4, may contribute to neuronal cell death in PD. Further studies will be required to determine whether, and in what manner, abnormal RanBP2 processing plays a role in PD pathogenesis.

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