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# Parkin Stabilizes Microtubules through Strong Binding Mediated by Three Independent Domains*

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**Mutations of parkin, a protein-ubiquitin isopeptide ligase (E3), appear to be the most frequent cause of familial Parkinson's disease (PD). Our previous studies have demonstrated that parkin binds strongly to α/β tubulin heterodimers and microtubules. Here we show that the strong binding between parkin and tubulin, as well as that between parkin and microtubules, was mediated by three independent domains: linker, RING1, and RING2. These redundant strong interactions made it virtually impossible to separate parkin from microtubules by high concentrations of salt (3.8 M) or urea (0.5 M). Parkin co-purified with tubulin and was found in highly purified tubulin preparation. Expression of either full-length parkin or any of its three microtubule-binding domains significantly attenuated colchicine-induced microtubule depolymerization. The abilities of parkin to bind to and stabilize microtubules were not affected by PD-linked mutations that abrogate its E3 ligase activity. Thus, the tubulin/microtubule-binding activity of parkin and its E3 ligase activity are independent. The strong binding between parkin and tubulin/microtubules through three redundant interaction domains may not only stabilize microtubules but also guarantee the anchorage of this E3 ligase on microtubules. Because many misfolded proteins are transported on microtubules, the localization of parkin on microtubules may provide an important environment for its E3 ligase activity toward misfolded substrates.**

Parkinson's disease (PD)[1] is the most prevalent neurodegenerative movement disorder, affecting about 1–2% of people over 60 years of age (1). Although the pathogenesis of PD remains elusive, a complex interaction between environmental and genetic factors may underlie most sporadic cases (2). Among the PD-linked genes identified so far, parkin is the most prevalent genetic factor causing familial Parkinson's disease; its mutations account for up to 50% of recessive PD cases (3, 4). As a protein-ubiquitin E3 ligase, parkin plays an important role in the ubiquitination and degradation of many substrates (5), such as CDCrel-1 (6), Pael Receptor (7), an *O*-glycosylated α-synuclein (8), synphilin-1 (9), cyclin E (10), α/β tubulin (11), and dopamine transporter (12).

Our previous studies have shown that parkin binds to microtubules and α/β tubulin heterodimers with very high affinity (11). The involvement of tubulin and microtubules in PD has been implicated in many previous reports. Tubulin is a major component of Lewy bodies (13), which are intracytoplasmic inclusions enriched in ubiquitinated proteins (14). It suggests that ubiquitinated tubulin may be present in Lewy bodies. Consistent with this, parkin, ubiquitinated proteins, and acetylated α-tubulin are accumulated in the aggresome in a microtubule-dependent manner when protein degradation through the 26 S proteasome is inhibited (15, 16). On the other hand, 1-methyl-4-phenylpyridinium ($MPP^+$) and rotenone, two neurotoxins known to cause PD-like symptoms in animal models, potently depolymerize microtubules (17–20). Microtubule depolymerization induces tubulin degradation at both protein (21) and RNA levels (22). Because parkin is an E3 ligase of tubulin (11), its ability to ubiquitinate tubulin dissociated from microtubules by these PD toxins may at least be partly responsible for this rapid degradation of tubulin. Parkin exhibits a punctate subcellular localization along microtubules in a variety of cells, including neurons (11). Apart from its E3 ligase activity on tubulin, the strong binding between parkin and microtubules may anchor this E3 ligase on microtubules to serve its functions, as misfolded proteins are known to be transported along microtubules to the aggresome (23).

To understand the molecular determinants of the strong interaction between parkin and tubulin/microtubules, we mapped the domains of parkin that were responsible for this tight binding. Of the five functional domains of parkin, Linker, RING1, or RING2 were able to bind to tubulin and microtubules with high affinity, whereas the ubiquitin-like (Ubl) domain or In-between RING finger (IBR) domain did not have such abilities. Thus, three domains of parkin independently provided tight binding to tubulin and microtubules. Such strong, redundant interactions rendered it almost impossible to separate parkin from microtubules and tubulin. Parkin remained bound to microtubules in the presence of 3.8 M NaCl or 0.5 M urea. It always co-purified with tubulin in ion exchange or gel filtration chromatography and was found in highly purified tubulin preparation (>99% purity).

Expression of the domains that bound to tubulin and microtubules significantly reduced colchicine-induced microtubule depolymerization, suggesting that parkin may stabilize the microtubule network. We also found that PD-linked mutations of parkin (K161N, T240R, and C431F) did not significantly impair the binding of parkin with tubulin heterodimers or microtubules, nor did they affect the ability of parkin to stabilize microtubules against colchicine-induced depolymerization.

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[1] The abbreviations used are: PD, Parkinson's disease; DA, dopamine; ER, endoplasmic reticulum; Ubl, ubiquitin-like; RING, really interesting new gene; IBR, in-between RING; MES, *N*-morpholinoethanesulfonic acid; E3, ubiquitin-protein isopeptide ligase; $MPP^+$, 1-methyl-4-phenylpyridinium; CMV, cytomegalovirus; PIPES, 1,4-piperazinediethanesulfonic acid; MAP, microtuble-associated protein.

Thus, the strong binding of parkin to microtubules may stabilize the microtubule network and anchor this E3 ligase to effectively ubiquitinate misfolded proteins being transported along microtubules.

## EXPERIMENTAL PROCEDURES

*Antibodies and cDNAs*—A polyclonal antibody against parkin was generated previously. It recognizes only the correct band on Western blots (11). Monoclonal antibodies against α-tubulin (DM1A) and FLAG (M2) were purchased from Sigma. Polyclonal FLAG antibody used in the immunocytochemistry was purchased from Cayman Chemical (Ann Arbor, MI). Rhodamine- or fluorescein isothiocyanate-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human parkin cDNA was amplified by reverse transcription PCR from SH-SY5Y cells and was completely sequenced to ensure that no mistake was introduced by PCR. It was subcloned into pCMV-Tag2B (Stratagene, La Jolla, CA), which added a FLAG tag at the N terminus of the protein produced. Point mutants of parkin were generated by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). The mutant constructs were confirmed by DNA sequencing. cDNAs for different domains of parkin were obtained by PCR reactions using the full-length human parkin construct as the template. The cDNA sequences encoding amino acids 1–76 (Ubl), 77–237 (Linker), 217–310 (RING1 or R1), 395–465 (RING2 or R2), 1–237 (Ubl-Linker), 307–465 (IBR-R2), and 217–465 (RIR) of parkin were cloned into pCMV-Tag2B vector between BamHI and SalI sites to generate FLAG-tagged constructs. The cDNA sequences encoding amino acids 307–405 (In-between RING, IBR) and 217–405 (R1-IBR) were cloned into pCMV-Tag2B vector between BamHI and HindIII sites. The cDNAs for these parkin domains tagged with N-terminal FLAG in pCMV-Tag2B vector were further subcloned into pcDNA3.1(+)/hygro vector (Invitrogen) using NotI and ApaI restriction sites. Expression levels of these FLAG-tagged constructs in pcDNA3.1 vector were higher than in pCMV-Tag2B vector and were thus used in all experiments. All constructs in the pcDNA3.1 vector were verified by sequencing.

*Cell Culture, Transfection, Immunoprecipitation, and Western Blot*—HEK293T cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine fetal calf serum and antibiotics. Transient transfections of various constructs were performed by the calcium phosphate method (12). Thirty hours after transfection, cells cultured in 10-cm dishes were lysed on ice in cold lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM EDTA, and 0.1 mM $Na_3VO_4$) for 20 min. Lysates were centrifuged at 16,000 × *g* at 4 °C, and the supernatant fraction was incubated with antibody against α-tubulin for 6 h at 4 °C, followed by incubation with protein A/G plus agarose (Santa Cruz Biotechnology) under the same conditions. Immunoprecipitates were washed three times with the lysis buffer, boiled in 2 × SDS loading buffer for 5 min, followed by separation on SDS-polyacrylamide gel and analysis by Western blots with FLAG antibody. Western blots were carried out using the ECL method according to the manufacturer's protocol (Amersham Biosciences).

*Taxol-mediated Microtubule Co-assembly Assay*—Different domains of parkin were transfected in three 10-cm dishes (eight dishes for the R1 domain) of HEK293T cells with the calcium phosphate method. Thirty hours after transfection, each dish of cells was washed twice with PBS and incubated in 0.5 ml of PEM buffer (0.1 M PIPES, 1 mM EGTA, 1 mM $MgSO_4$ with protease inhibitor mixture tablet and 1% phenylmethylsulfonyl fluoride) on ice for 10 min. The cells were homogenized on ice in a tissue grinder (Fisher Scientific). Three-week-old male Sprague-Dawley rats were decapitated after being anesthetized with halothane (Sigma). One half of a rat brain was homogenized for 30 min on ice with the HEK293 cell lysates containing various domains of parkin. After incubation on ice for 15 min, the homogenates were centrifuged at 30,000 × *g* at 4 °C for 15 min, and ultracentrifuged at 180,000 × *g* at 4 °C for 90 min. The supernatant was designated C (cytosol) fraction. Taxol (Sigma) and GTP were added to C fraction to a final concentration of 20 μM and 1 mM, respectively. The solution was incubated at 37 °C for 15 min and centrifuged at 30,000 × *g* through a layer of PEM buffer with 10% sucrose at 37 °C for 30 min. The supernatant was designated $S_1$. The pellet was washed with PEM buffer and resuspended in PEM buffer containing GTP and taxol at 37 °C ($P_1$), which was centrifuged again. The supernatant was designated $S_2$, the pellet was washed with PEM buffer again and resuspended in PEM buffer with GTP but without taxol at 37 °C ($P_2$). $P_2$ was mixed with an appropriate volume of MAP dissociation buffer (PEM buffer with GTP, but without taxol, plus 4 M NaCl) and incubated at 37 °C for 45 min to elute MAPs to the supernatant fraction at 2 or 3.8 M of NaCl. After the mixtures were centrifuged (30,000 × *g*, 30 min, 37 °C), supernatant $S_3$ and pellet $P_3$ were obtained. $P_3$ was rinsed in PEM buffer (without taxol or GTP) at 37 °C and resuspended in PEM buffer at 4 °C to depolymerize microtubules. Equal amount of total proteins (10 μg for parkin blot and 2 μg for tubulin blot) from each fraction were boiled and separated by SDS-PAGE and analyzed by Western blotting with antibodies against FLAG, parkin, or α-tubulin, respectively.

*Chromatographic Purification of Parkin-tubulin Complex*—The microtubule pellet after elution with 2 M NaCl was rinsed and resuspended in PEM buffer without GTP or Taxol ($P_3$), and incubated on ice for 1 h. $P_3$ was then dialyzed (in Slide-A-Lyzer Dialysis Cassette, Pierce) overnight against ion exchange buffer (10 mM NaCl, 25 mM Tris, 1 mM dithiothreitol, 1 mM EDTA, pH 7.5). The dialyzed sample was centrifuged at 16,000 × *g* for 10 min at 4 °C, and the supernatant was injected to an anion exchange chromatographic column (Mono Q, HR 5/5, 1 ml, Amersham Biosciences) at 4 °C. The elution buffer gradient was 10 mM to 1 M NaCl. Equal volume of protein-enriched fractions was loaded for SDS-PAGE and analyzed by Western blotting with antibodies against parkin or α-tubulin. Fractions containing parkin-tubulin complex after Mono Q were collected and applied to a gel filtration column (Superose 12, HR 10/30, 24 ml, Amersham Biosciences) equilibrated in 25 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, pH 7.0, at 4 °C. The fractions were analyzed by Western blotting as described above. Both chromatographic purification steps were run on AKTA fast protein liquid chromatography instrument (Amersham Biosciences).

*Immunocytochemistry*—COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10% bovine fetal calf serum and antibiotics. Transient transfections of various constructs were performed using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. Sixteen hours after transfection, cells were re-plated and grown on coverslips (pre-coated with poly-D-lysine) in 12-well plates for 12–16 h. After the cells were treated with 1 μM colchicine for 12 h, they were fixed and co-stained with anti-FLAG (rabbit polyclonal) and anti-α-tubulin (mouse monoclonal) in methods used previously (11). Images were acquired under a 60× lenses of a Nikon fluorescence microscope with a charge-coupled device camera (Diagnostic Instrument, Sterling Heights, MI) and merged using the SPOT software (Diagnostic Instrument). For quantification of microtubule-containing ($MT^+$) cells, at least 200 transfected cells or 500 untransfected cells from four coverslips were counted in separate experiments. The percentage of cells with at least one visible microtubule was calculated for each condition.

*Measurement of Free Tubulin in the Cell*—Free tubulin was extracted essentially as described previously (24). Briefly, HEK293T cells transfected without or with various constructs were treated without or with colchicine (1 μM) for 40 min at 37 °C in culture media. Cells were washed twice at 37 °C with 1 ml of Buffer A containing 0.1 M MES (pH 6.75), 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA, and 4 M glycerol. After the cultures were incubated at 37 °C for 5 min in free tubulin extraction buffer (Buffer A plus 0.1% Triton X-100 and protease inhibitors), the extracts were centrifuged at 37 °C for 2 min at 16,000 × *g*. The supernatant fractions contained free tubulin extracted from the cytosol. Equal amounts of total proteins from the supernatant fractions were analyzed by Western blotting with anti-α-tubulin (Sigma). The intensity of tubulin bands was quantified from three different experiments with the software NIH imaging. Total cell lysates in a lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 mM EDTA, and 0.1 mM $Na_3VO_4$) were blotted with anti-FLAG or anti-HA to assess expression levels of transfected constructs.

## RESULTS

*Three Domains of Parkin Independently Binds to α/β-Tubulin Heterodimers*—To understand the molecular determinants for the strong interaction between parkin and tubulin (11), we generated a series of constructs expressing various domains of parkin to identify the tubulin-binding region(s). All of these constructs (Fig. 1*A*) were tagged with FLAG at the N terminus and could be efficiently expressed in HEK293T cells, except for the RING1 domain, whose expression level was much lower than that of the others (Fig. 1, *B* and *C*).

We first examined the ability of each individual domain of parkin to co-immunoprecipitate endogenous α/β-tubulin. HEK293 cells transfected without or with these domains or empty vector were lysed at 4 °C in a buffer containing 1%

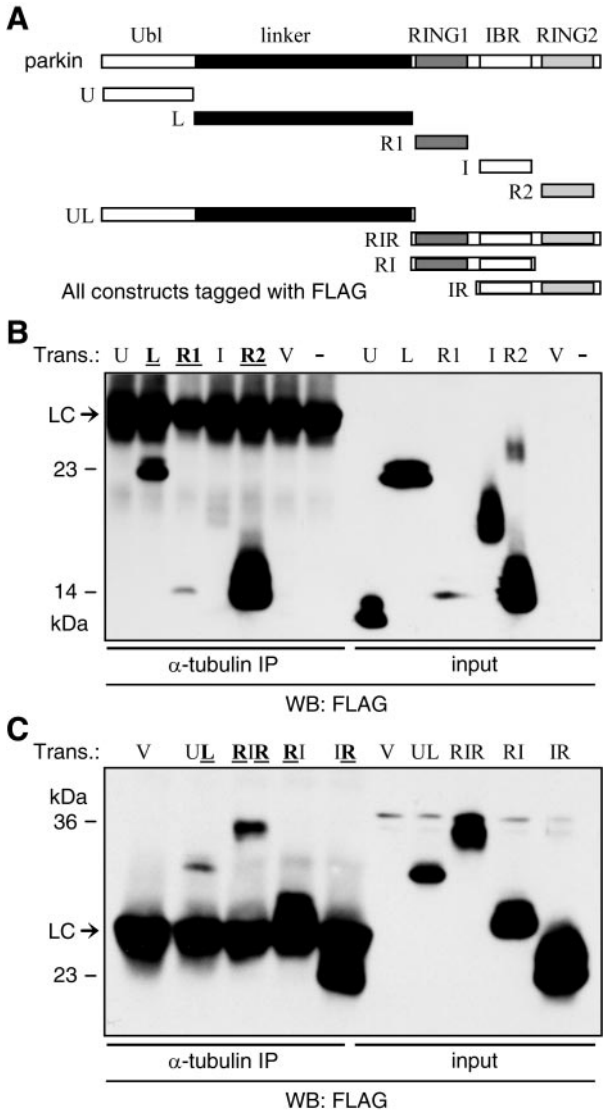


FIG. 1. **Three domains of parkin independently bound to tubulin.** *A*, functional domains of parkin and constructs used in the study. *U*, ubiquitin-like (Ubl) domain; *L*, linker domain; *R1*, the first RING finger; *I*, In-between RING finger (IBR) domain; *R2*, the second RING finger; *UL*, ubiquitin-like domain and linker domain; *RIR*, RING1-IBR-RING2 domains; *RI*, RING1-IBR domains; *IR*, IBR-RING2 domains. *B*, HEK293T cells were transfected without (−) or with empty vector (*V*, pcDNA3.1/hygro+) or FLAG-tagged parkin domains (*U*, *L*, *R1*, *I*, and *R2*). Cleared cell lysates were immunoprecipitated with anti-α-tubulin at 4 °C. Precipitated proteins and 1% of input were analyzed by Western blotting with anti-FLAG. Linker, R1 and R2, but not Ubl or IBR domain, were co-immunoprecipitated tubulin. Although expression level of R1 was significantly lower than other domains, its co-immunoprecipitation with tubulin was very strong compared with the input. *LC*, IgG light chain. *C*, HEK293 cells were transfected with vector (*V*) or FLAG-tagged combinations of parkin domains (*UL*, *RIR*, *RI*, and *IR*). All parkin domain combinations were co-immunoprecipitated with tubulin. Experiments in *B* and *C* were repeated at least five times with the same results. Tubulin-binding domains are *underlined* and marked in *bold*.

Triton X-100. After centrifugation, cleared cell lysates were immunoprecipitated with an antibody against α-tubulin at 4 °C. Anti-FLAG blot of α-tubulin immunoprecipitates and 1% of the input lysates showed that the Linker (L), RING1 (R1), or RING2 (R2) domain was strongly co-immunoprecipitated with tubulin. In contrast, the ubiquitin-like (Ubl or U) domain or In-between RING finger (IBR or I) domain was not co-immunoprecipitated with α-tubulin (Fig. 1*B*). Similar results were obtained when we perform the same experiments in the presence of 25 μM colchicine (data not shown). This is consistent with our previous result that the binding between parkin and tubulin α/β heterodimer is not affected by colchicine (11) and suggests that each of the three domains (Linker, RING1, and RING2) binds to tubulin heterodimer at 4 °C. Although the RING1 domain was expressed at a much lower level, its ability to co-immunoprecipitate with tubulin was commensurate with its expression.

To confirm these observations, we expressed combinations of various parkin domains in HEK293T cells and performed the same co-immunoprecipitation assays with anti-α-tubulin. As shown in Fig. 1*C*, the UL constructs, which covered Ubl and Linker domains, was strongly co-immunoprecipitated with tubulin. So were the RIR (RING1-IBR-RING2), RI (RING1-IBR), or IR (IBR-RING2) constructs. As long as a construct included one of the tubulin-binding domains (Linker, RING1, or RING2), it exhibited strong co-immunoprecipitation with α/β-tubulin. Thus, the Ubl or IBR domain did not interfere with the tubulin-binding activity of Linker, RING1, or RING2.

*The Three Tubulin-binding Domains of Parkin Also Strongly Associate with Microtubules*—Because our previous study has shown the strong binding between parkin and microtubules in taxol-mediate microtubule co-assembly assays (11), we wondered whether the interaction was mediated by the three tubulin-binding domains of parkin. To test this, we combined lysates of HEK293T cells expressing individual parkin domains with rat brain homogenates, which were used as a rich source of tubulin. After ultracentrifugation of the mixed lysates at 4 °C, the supernatant fraction was used in taxol-mediated microtubule co-assembly experiments. The pellet fraction after two cycles of co-assembly ($P_2$) was incubated in MAP dissociation buffer containing 2 M NaCl to assess the binding affinity between parkin domains and microtubules.

As shown in Fig. 2, α-tubulin was highly enriched in the pellet fractions containing microtubules ($P_1$, $P_2$, and $P_3$), compared with the soluble fractions ($S_1$, $S_2$, and $S_3$). Consistent with our previous result, endogenous parkin from rat brain (and negligible amount of endogenous parkin from HEK293T cells) was found exclusively in the pellet fractions (11). Under the same condition, we found that Linker or RING1 (R1) domain was always in the pellet fraction with microtubules, even in the presence of 2 M NaCl. The RING2 (R2) domain exhibited partial co-assembly with microtubules. In contrast, the Ubl or IBR domain was in the $S_1$ fraction, unable to co-assemble with microtubules. We also tested constructs that expressed combinations of various parkin domains. IBR-R2, Ubl-Linker, or R1-IBR was able to strongly co-assemble with microtubules. Thus, as long as the combined construct contained one of the tubulin-binding domains (Linker, R1, or R2), it co-assembled with microtubules. Because IBR itself did not co-assemble with microtubules at all, the ability of IBR-R2 to bind to microtubules most likely comes from R2. It seems that there might be some structural hindrance that affects the efficient binding of R2 with microtubules, which appears to be removed when R2 is in the context of IBR-R2, as in the native sequence of parkin. Together, these results suggest that the three tubulin-binding domains of parkin also mediate its strong association with microtubules.

*Parkin Cannot Be Dissociated from Microtubules with High Concentrations of NaCl or Urea*—To assess the affinity between parkin and microtubules, we performed the taxol-mediated microtubule co-assembly assay and incubated microtubules in the $P_2$ pellet in MAP dissociation buffer (PEM buffer plus GTP, but no taxol) containing 2 or 3.8 M of NaCl for 45 min at 37 °C. After centrifugation, the supernatant and pellet fractions were designated as $S_3$ and $P_3$ (for 2 M NaCl wash) and $S_4$ and $P_4$ (for 3.8 M NaCl wash), respectively. As shown in Fig. 3*A*,

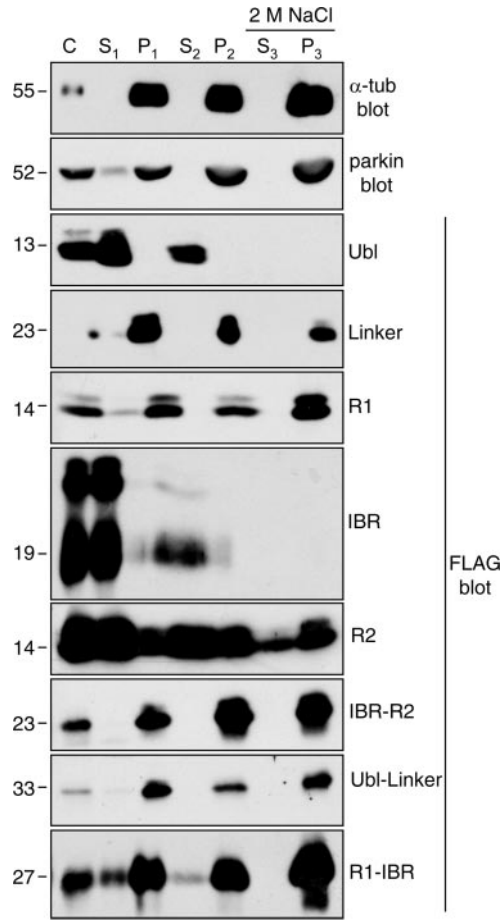


FIG. 2. **Strong binding between three domains of parkin and microtubules.** HEK293T cells overexpressing different domains of parkin were homogenized in PEM buffer. Rat brain were added to the cell lysates and homogenized further with the HEK293T cell lysate. The cytosolic fraction (*C*) from ultracentrifuged homogenates was subjected to two cycles of taxol-mediated microtubule assembly assays. The third cycle was done in the presence of 2 M NaCl without taxol. Supernatant and pellet fractions from each cycle were designated as $S_1$, $P_1$, $S_2$, $P_2$, $S_3$, and $P_3$, respectively. Equal amounts of total proteins from each fraction (2 $\mu$g for $\alpha$-tubulin blot, 10 $\mu$g for the rest) were analyzed by Western blotting with antibodies against $\alpha$-tubulin, parkin, or FLAG. Endogenous parkin and tubulin were always in microtubule fraction in the pellet. Exogenously expressed Linker, R1, IBR-R2, Ubl-Linker, and R1-IBR constructs strongly co-assembled with microtubules, whereas the R2 domain co-assembled with microtubules to a lesser degree. Ubl or IBR domains did not co-assemble with microtubules. All experiments were repeated at least three times with similar results.

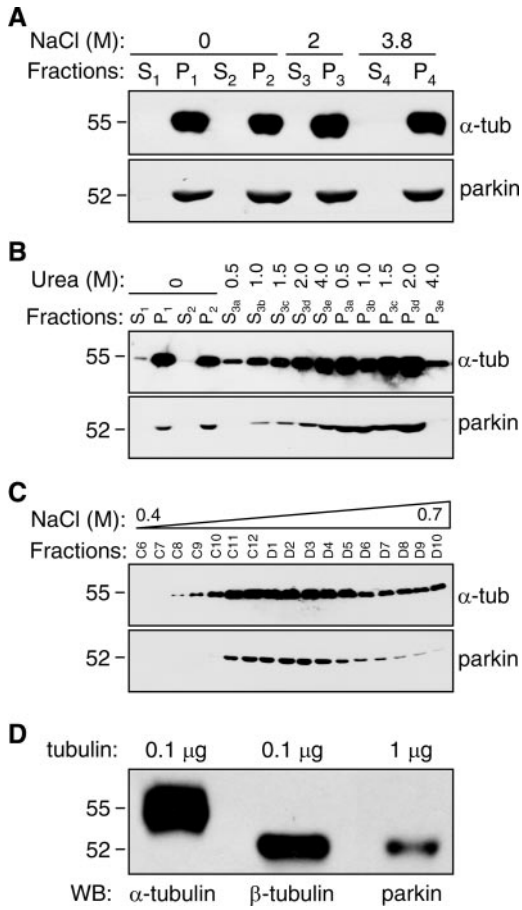


FIG. 3. **Extremely tight binding between parkin and microtubules.** *A*, after two cycles of taxol-mediated microtubule assembly, which produced fractions $S_1$, $P_1$, $S_2$, and $P_2$, microtubules in the pellet fraction $P_2$ were incubated with 2 or 3.8 M NaCl in PEM buffer at 37 °C for 45 min. After centrifugation, supernatant fractions were designated as $S_3$ and $S_4$, whereas pellet fractions ($P_3$ and $P_4$) were resuspended in PEM buffer on ice for 30 min to dissociate microtubules. Western blotting of equal amounts of total proteins from each fraction (2 $\mu$g for tubulin blot and 10 $\mu$g for parkin blot) showed that parkin remained in the pellet fraction with microtubules even in the presence of 3.8 M NaCl. *B*, the pellet fraction $P_2$ was resuspended and separated into five equal parts, each incubated at 37 °C for 45 min in PEM buffer containing 0.5, 1, 1.5, 2, or 4 M urea, respectively. Western blotting of equal amounts of total proteins from each fraction (2 $\mu$g for tubulin blot and 10 $\mu$g for parkin blot) showed that parkin was eluted from the pellet into the supernatant fraction with urea at or above 1 M. *C*, microtubules in the pellet fraction $P_3$ from *A* were dialyzed overnight at 4 °C against ion exchange purification buffer (10 mM NaCl, 20 mM sodium phosphate, 1 mM dithiothreitol, 1 mM EDTA, pH 7.5) and centrifuged to obtain clear supernatant, which were separated by ion exchange chromatography with a NaCl gradient from 10 mM to 1 M at 4 °C. Western blotting of equal fractions of the eluted samples with antibodies against parkin or $\alpha$-tubulin showed that parkin co-purified with tubulin. *D*, purified tubulin (>99% purity) from a commercial source (Cytoskeleton, Inc., Denver, CO) was analyzed by Western blotting with antibodies against $\alpha$-tubulin, $\beta$-tubulin, or parkin, respectively. The amounts of total protein loaded on the gel were 0.1, 0.1, or 1 $\mu$g for each blot, respectively. Parkin was detected in purified tubulin. All experiments were repeated for at least three times with similar results.

parkin still remained in the pellet fraction with microtubules even in the presence of 3.8 M NaCl.

To separate parkin from microtubules, we resorted to urea and incubated microtubules in the $P_2$ pellet with MAP dissociation buffer containing 0.5–4 M urea for 45 min at 37 °C. After centrifugation, the supernatant and pellet fractions were designated as $S_{3a}$ and $P_{3a}$ (for 0.5 M urea wash), $S_{3b}$ and $P_{3b}$ (for 1.0 M urea wash), $S_{3c}$ and $P_{3c}$ (for 1.5 M urea wash), $S_{3d}$ and $P_{3d}$ (for 2.0 M urea wash), and $S_{3e}$ and $P_{3e}$ (for 4.0 M urea wash). As shown in Fig. 3*B*, no significant amount of parkin was eluted from microtubules in the pellet ($P_{3a}$) with 0.5 M urea. Only when urea concentrations were increased to 1 M or above did we see parkin in the supernatant fractions ($S_{3b}$ to $S_{3e}$). However, increasing amount of tubulin was observed in these supernatant fractions, suggesting that microtubules were dissociated by urea at 0.5 M or above. Thus, parkin in the supernatant fractions may still bind to tubulin dissociated from microtubules by urea. It appears that we cannot separate parkin from microtubules unless we dissociate microtubules.

*Co-purification of Parkin and α/β-Tubulin*—Having failed to separate parkin from microtubules, we tried to isolate parkin from tubulin. As parkin was highly enriched in the microtubule pellet after 2 M NaCl wash ($P_3$), we used this preparation from rat brains as the starting material to purify parkin by ion-exchange and gel-filtration chromatographies. The $P_3$ pellet was first resuspended in PEM buffer without GTP or taxol at 4 °C to dissociate microtubules into tubulin $\alpha/\beta$ heterodimers. After overnight dialysis at 4 °C in the

sample buffer for ion-exchange chromatography, cleared supernatant, which was enriched for tubulin and parkin, was applied to a 1-ml Mono Q HR 5/5 ion-exchange chromatography column on a fast protein liquid chromatography system. Proteins were eluted with a linear NaCl gradient from 0.01 to 1 M. Western blotting of protein-containing fractions with antibodies against α-tubulin or parkin showed that parkin co-purified with tubulin (Fig. 3*C*). We also tried gel-filtration chromatography using either pooled fractions after ion-exchange chromatography purification or tubulin dissociated from $P_3$ microtubule pellet. Western blotting of fractions after gel-filtration chromatography showed that parkin was still in the same fractions as tubulin (data not shown). This led us to directly examine highly purified tubulin (>99% pure) from a commercial source (Cytoskeleton, Inc.) by Western blotting with anti-parkin. As shown in Fig. 3*D*, parkin was clearly seen in this purified tubulin preparation when we ran different amounts of tubulin (0.1 or 1 μg) side by side and blotted each lane separately with antibodies against α-tubulin, β-tubulin, or parkin, respectively. As the amount of parkin in this preparation appears to be much lower than those of tubulins, this minor "contaminant" is masked by the overabundance of tubulins, which migrate very closely to parkin on SDS-PAGE. These independent lines of evidence suggest that parkin cannot be easily separated from α/β-tubulin by conventional methods and may naturally exist in a complex with tubulin.

*Parkin Attenuates Colchicine-induced Microtubule Depolymerization through Its Three Tubulin/Microtubule-binding Domains*—The strong binding between parkin and microtubules suggests that parkin may stabilize microtubule networks in the cell. To test this, we transfected COS-7 cells with various FLAG-tagged parkin constructs and treated the cultures with the microtubule-depolymerizing agent colchicine (1 μM for 12 h). COS-7 cells were used because of their flat shape, which enabled easy observation of microtubules. Fixed cultures were co-stained with anti-FLAG (*red*) and anti-α-tubulin (*green*) to observe microtubules in transfected and untransfected cells. Although the rabbit polyclonal antibody against FLAG had some cross-reactivity with other cellular proteins in Western blotting and immunostaining (data not shown), it was sufficient to distinguish transfected and untransfected cells. As shown in Fig. 4, *A* and *B*, microtubules in untransfected COS-7 cells were totally depolymerized by the colchicine treatment. In contrast, expression of the full-length wild-type parkin significantly reduced colchicine-induced microtubule depolymerization (Fig. 4, *C* and *D*). Although expression of the Linker (Fig. 4, *G* and *H*), R1-IBR (Fig. 4, *I* and *J*), or R2 (Fig. 4, *M* and *N*) construct had an effect similar to that of the wild-type parkin, transfection of the Ubl (Fig. 4, *E* and *F*) or IBR (Fig. 4, *K* and *L*) domain had no such an effect. Because the expression level of the R1 construct was much lower than the other constructs (Fig. 1*B*), we observed very few cells that were visibly transfected with the R1 construct. Thus, we used the R1-IBR construct instead of the R1 construct. The R1-IBR construct behaved in the same way as the R1 domain and oppositely to the IBR domain in co-immunoprecipitation with tubulin (Fig. 1) and co-assembly with microtubules (Fig. 2). Consequently, it is reasonable to assume that the behavior of R1-IBR following colchicine treatment would represent R1, instead of IBR. Indeed, the effect of R1-IBR on colchicine-induced microtubule depolymerization was similar to other microtubule-binding domains and different to constructs that did not bind to microtubules (Fig. 4).

To quantify the effects of these constructs, we counted at least 200 transfected cells (for each condition in Fig. 4, *C–N*) or 500 untransfected cells (for each condition in Fig. 4, *A* and *B*) from four coverslips in separate experiments. The percentage of transfected cells with at least one obvious microtubule after colchicine treatment was calculated for each construct. As shown in Fig. 4*O*, 36.8 ± 1.8% of cells transfected with wild-type parkin still had at least one obvious microtubule after the colchicine treatment. It is significantly different from the situation in untransfected cells (2.7 ± 0.4%). The effects of Linker (32.8 ± 2.1%), R1-IBR (33.7 ± 2.7%), and R2 (31.4 ± 2.9%) were similar to that of the wild-type and significantly different from that of the untransfected ($p < 0.001$). In contrast, the effects of Ubl (17.6 ± 1.4%) and IBR (14.3 ± 0.8%) were significantly smaller than that of the wild-type ($p < 0.001$). We also transfected COS-7 cells with unrelated constructs (β3 or β2 subunits of $Na^+/K^+$-ATPase) to see whether the effect of Ubl or IBR was nonspecific. Expression of β3 (16.3 ± 0.9%) or β2 (data not shown), which has no known connections to microtubules, attenuated colchicine-induced microtubule depolymerization to a similar extent as that caused by Ubl or IBR ($p > 0.05$ among the three constructs). Thus, these relatively small effects appear to be nonspecific and are quite different from that of wild-type parkin.

To further substantiate our findings, we directly measured the amount of free tubulin in the cell by gently lysing the cells in a low concentration of detergent (0.1% Triton X-100) at 37 °C without disturbing polymerized tubulin in microtubules. The amount of tubulin in the extract represents tubulin originally existed as free α/β heterodimers in the cell (24). HEK293T cells transfected without or with various FLAG-tagged parkin constructs or HA-β3 were treated without or with 1 μM colchicine for 40 min. As expected, colchicine treatment greatly increased the amount of free tubulin in the cell by depolymerizing microtubules (Fig. 4*P*, *upper panel*, *lanes 1 versus 2*). Expression of wild-type parkin (WT), Linker, R1, R1-IBR, or R2 domain markedly attenuated colchicine-induced increase in the amount of free tubulin. In contrast, Ubl or IBR domain did not have such an effect and behaved just like untransfected or the control construct HA-β3 (Fig. 4*P*, *upper panel*). Quantification of results from three independent experiments showed that the amount of free tubulin after colchicine treatment in cells transfected with WT (157 ± 12% of untransfected, untreated controls, $p < 0.001$), Linker (209 ± 19%, $p < 0.05$), R1 (187 ± 11%, $p < 0.01$), R1-IBR (178 ± 11%, $p < 0.01$), or R2 (185 ± 9%, $p < 0.01$) was significantly less than that in untransfected cells (269 ± 12%). The effect of Ubl (274 ± 13%, $p > 0.75$), IBR (269 ± 13%, $p > 0.95$), or HA-β3 (272 ± 14%, $p > 0.85$) was not significantly different from that of untransfected (269 ± 12%). Thus, like the wild-type, any parkin construct containing one of its three tubulin/microtubule-binding domains greatly reduced colchicine-induced microtubule depolymerization. The two domains that did not bind to tubulin and microtubules (Ubl and IBR) also did not attenuate microtubule depolymerization induced by colchicine. The expression level of each FLAG-tagged parkin construct or HA-tagged β3 is shown in the *lower panel* of Fig. 4*P*. The β3 subunit of $Na^+/K^+$-ATPase is a heavily glycosylated transmembrane protein and migrates as multiple bands on SDS-PAGE.

Together, these morphological and biochemical results suggest that microtubule-binding domains of parkin, like the full-length, stabilize microtubule networks against colchicine-induced microtubule depolymerization. In contrast, parkin domains that did not bind to microtubules had no such an effect.

*PD-linked Mutations of Parkin Do Not Disrupt the Binding between Parkin and α/β-Tubulin*—To test whether the strong binding between parkin and tubulin is affected by mutations of parkin that cause Parkinson's disease, we transfected FLAG-tagged wild-type parkin or its PD-linked mutants (K161N, T240R, or C431F) in HEK293T cells and examined the co-

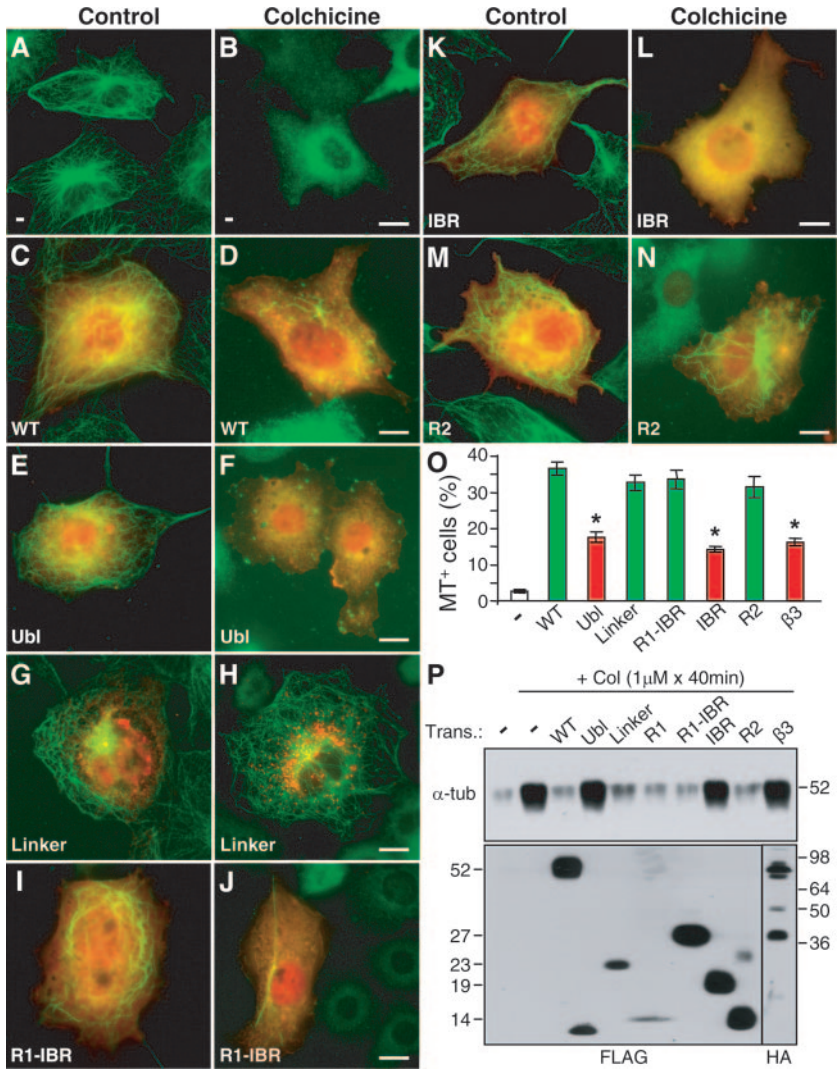


FIG. 4. **Expression of microtubule-binding domains of parkin attenuated colchicine-induced microtubule depolymerization.** *A–N*, COS-7 cells were transfected with vector (*A* and *B*), wild-type parkin (*C* and *D*), Ubl domain (*E* and *F*), Linker domain (*G* and *H*), R1-IBR domain (*I* and *J*), IBR domain (*K* and *L*) or R2 domain (*M* and *N*), and treated with vehicle control (*A*, *C*, *E*, *G*, *I*, *K*, and *M*) or 1 μM colchicine (*B*, *D*, *F*, *H*, *J*, *L*, and *N*) for 12 h. All constructs were tagged with FLAG at the N terminus. Fixed cells were co-stained with antibodies against α-tubulin (*green*) and FLAG (*red*). Wild-type parkin or microtubule-binding domains of parkin (Linker, R1-IBR, and R1) reduced colchicine-induced microtubule depolymerization, whereas parkin domains that did not bind to microtubules (Ubl and IBR) did not have such effect. *Bars*, 10 μm. *O*, percentages of cells with at least one obvious microtubule after colchicine treatment were calculated for all the constructs used in *A–N*, as well as a control construct expressing the β3 subunit of $Na^+/K^+$-ATPase. At least 200 transfected cells (for each condition in *C–N*) and 500 untransfected cells (for *A* and *B*) were counted from four coverslips in separate experiments for each construct. *, $p < 0.001$ *versus* wild-type parkin. No significant differences were found between full-length parkin, Linker, R1-IBR, and R2 ($p > 0.1$). There were also no significant differences between Ubl, IBR, and β3 ($p > 0.05$). All the transfected cells showed significant difference compared with non-transfected cells ($p < 0.001$). *P*, HEK293T cells were transfected without or with various FLAG-tagged parkin constructs or HA-tagged β3 subunit of $Na^+/K^+$-ATPase and treated without or with colchicine (1 μM) for 40 min. Free tubulin was extracted from the cells and analyzed by Western blotting with anti-α-tubulin (*upper panel*). Expression of wild-type parkin and constructs containing at least one of its tubulin-binding domains significantly reduced colchicine-induced microtubule depolymerization. Total cell lysates were blotted with anti-FLAG or anti-HA to show expression levels of FLAG-tagged parkin constructs or HA-β3 (*lower panel*).

immunoprecipitation of these parkin constructs with endogenous α/β-tubulin. As shown in the *top panel* of Fig. 5*A*, all three point mutants and wild-type parkin were found at comparable levels in α-tubulin immunoprecipitates, indicating that the binding between parkin and tubulin was not disrupted by any of the three PD-causing mutations. Western blotting of the total lysates showed that parkin and its mutants were expressed at similar levels (Fig. 5*A*, *bottom panel*).

Each of the three point mutations that we tested is in one of the tubulin-binding domains: K161N in the Linker, T240R in RING1, and C431F in RING2. As each mutation would only affect one tubulin-binding domain, the lack of difference between wild-type parkin and these point mutants could be simply due to the fact that the other two tubulin-binding domains can still compensate for the interaction. To test this, we introduced these point mutations to their corresponding tubulin-binding domains and examine whether the co-immunoprecipitation between these parkin domains and tubulin is affected or not. As shown in Fig. 5*B*, each of the three constructs L/K161N (Linker with K161N mutation), R1/T240R (RING1 with T240R mutation), and R2/C431F (RING2 with C431F mutation) was expressed in HEK293T cells and was found in α-tubulin immunoprecipitates. Thus, none of the three PD-linked mutations disrupted the binding between its corresponding domains and tubulin. Expression level of R1/T240R was much lower than the other two constructs (Fig. 5*B*). When we examined the expression levels of L *versus* L/K161N; R1 *versus* R1/T240R; and R2 *versus* R2/C431F side by side on the same gel, we found

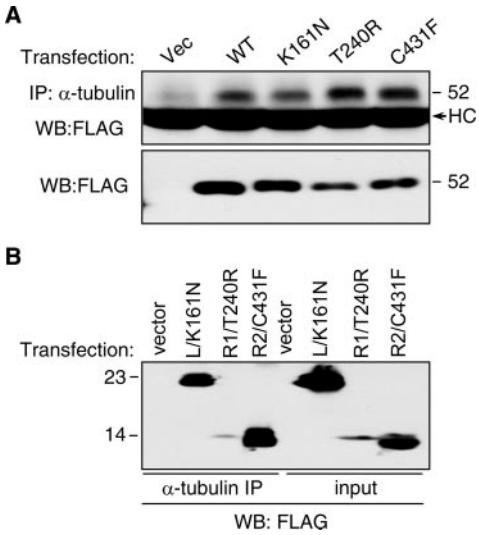


FIG. 5. **PD-linked mutations did not affect the binding between parkin and tubulin.** *A*, HEK293T cells were transfected with vector, FLAG-tagged wide-type parkin or its PD-linked point mutants (K161N, T240R, and C431F). Cleared cell lysates were immunoprecipitated with anti-α-tubulin at 4 °C. Precipitated proteins (*upper panel*) and 1% of input (*lower panel*) were analyzed by Western blotting with anti-FLAG. The three PD-linked mutations of parkin did not disrupt the co-immunoprecipitation between parkin and tubulin. *B*, HEK293T cells were transfected with vector or FLAG-tagged domains of parkin with the corresponding point mutations (Linker/K161N, R1/T240R, and R2/C431F). Co-immunoprecipitation assay with anti-α-tubulin showed that the three point mutations did not disrupt the binding between the corresponding parkin domains and tubulin. The expression level of R1/T240R was much lower than other constructs. Experiments were repeated at least three times with the same results.

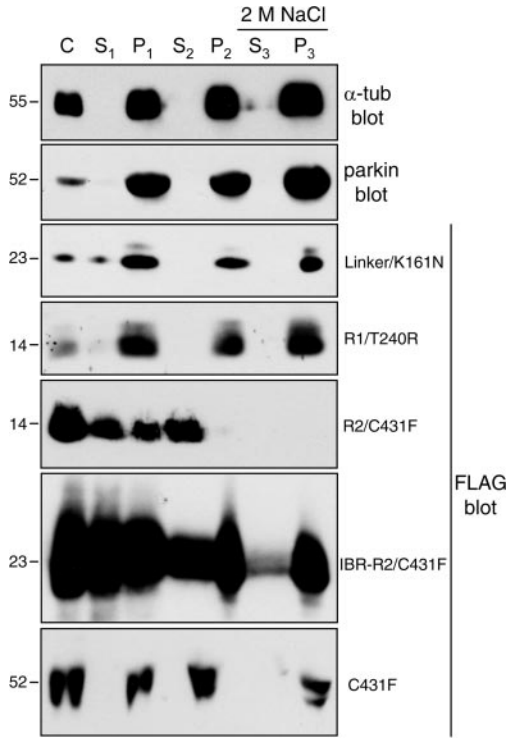


FIG. 6. **PD-linked mutations did not disrupt the binding between parkin and microtubules.** HEK293T cells overexpressing various constructs indicated were homogenized in PEM buffer. Rat brain was added to the cell lysates and homogenized further with the HEK293T cell lysate. The cytosolic fraction (*C*) from ultracentrifuged homogenates was subjected to two cycles of taxol-mediated microtubule assembly assays. The third cycle was done in the presence of 2 M NaCl without taxol. Supernatant and pellet fractions from each cycle were designated as $S_1$, $P_1$, $S_2$, $P_2$, $S_3$, and $P_3$ respectively. Equal amounts of total proteins from each fraction (2 μg for α-tubulin blot, 10 μg for the rest) were analyzed by Western blotting with antibodies against α-tubulin, parkin, or FLAG. Endogenous parkin and tubulin were always in the microtubule fraction in the pellet. Exogenously expressed Linker/K161N (Linker with the K161N mutation) or R1/T240R strongly co-assembled with microtubules, whereas R2/C431F did not. However, the C431F mutation on the backbone of IBR-R2 (IBR-R2/C431F) or full-length parkin (C431F) did not significantly affect the co-assembly with microtubules. All experiments were repeated at least three times with similar results.

that the mutations did not significantly affect the expression of the corresponding parkin domains (data not shown).

*Co-assembly of PD-linked Parkin Mutants with Microtubules*—Because the three PD-linked mutations of parkin did not disrupt the binding between parkin and tubulin, we tested whether they affected the ability of parkin to co-assemble with microtubules. HEK293T cells transfected with Linker/K161N, R1/T240R, or R2/C431F were homogenized with rat brain. Microtubule co-assembly assays were performed in the same way as in Fig. 2. As shown in Fig. 6, endogenous parkin was always in the pellet fractions with microtubules, even in the presence of 2 M NaCl ($S_3$ *versus* $P_3$). Linker/K161N and R1/T240R strongly co-assembled with microtubules and could not be eluted from the microtubule pellet with 2 M NaCl (Fig. 6). In contrast, R2/C431F did not co-assemble with microtubules effectively (Fig. 6). Because the ability of R2 to co-assemble with microtubules was greatly enhanced by adding the upstream IBR domain (Fig. 2, R2 *versus* IBR-R2), we tested the IBR-R2/C431F construct and found that it co-assembled with microtubules much better than R2/C431F did (Fig. 6). The C431F mutant on the backbone of full-length parkin strongly co-assembled with microtubules (Fig. 6, *last panel*). These results suggest that none of the three PD-linked mutations disrupts the strong association between parkin and microtubules, because each of these point mutations would at most affect only one of the three microtubule-binding domains.

*PD-linked Mutations Do Not Affect the Ability of Parkin to Attenuate Colchicine-induced Microtubule Depolymerization*—Because the three PD-linked mutations did not disrupt the binding between parkin and microtubules, they should not significantly affect the ability of parkin to stabilize microtubules against colchicine-induced depolymerization. To test this, COS7 cells were transfected with wild-type or mutant parkin and treated without or with colchicine (1 μM) for 12 h. We co-stained fixed cultures with anti-FLAG (*red*) and anti-α-tubulin (*green*) to compare microtubule networks in transfected *versus* untransfected cells. Almost no microtubule was visible after colchicine treatment in untransfected cells (Fig. 4, *A versus B*). In many cells transfected with wild-type parkin or its PD-linked mutants (K161N, T240R, and C431F), microtubules were still quite visible after the colchicine treatment. After counting at least 200 transfected cells (for each condition in Fig. 7, *C–H*) and 500 untransfected cells (for each condition in Fig. 7, *A* and *B*) from four coverslips in separate experiments for each construct, we found that parkin or its mutants significantly increased the percentage of cells with at least one visible microtubule after colchicine treatment, in comparison to untransfected cells ($p < 0.001$).

To further substantiate these results, we measured the amount of free tubulin in HEK293T cells transfected with parkin or its mutants and treated without or with 1 μM colchicine for 40 min. Expression of wild-type or mutant parkin greatly reduced the amount of free tubulin induced by colchicine treatment (Fig. 7*J*, *upper panel*). Quantification of results from three different experiments showed that the amount of free tubulin after colchicine treatment in cells transfected with WT (157 ± 12% of untransfected, untreated controls, $p < 0.01$),

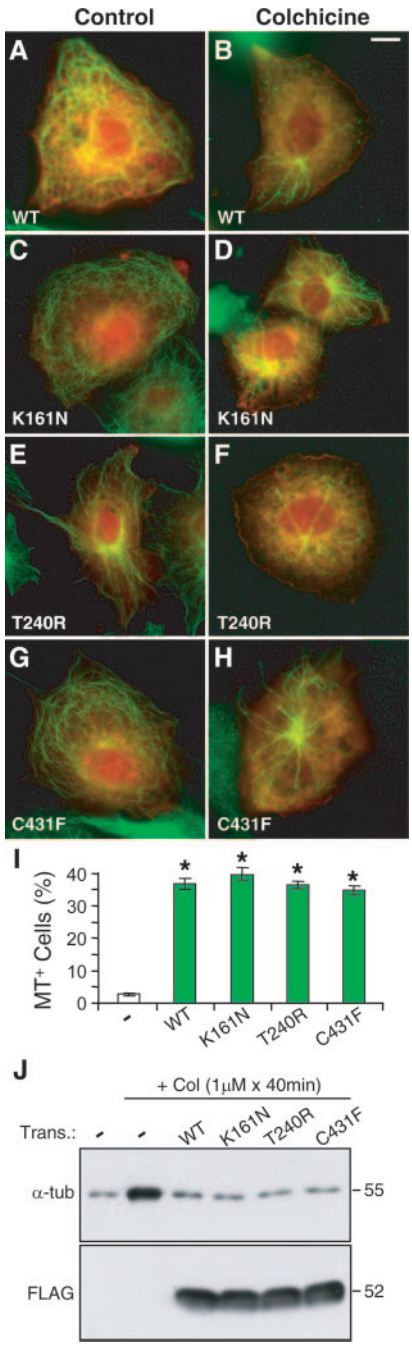


FIG. 7. **PD-linked mutations did not abrogate the microtubule-stabilizing effect of parkin against colchicine.** *A–H*, COS-7 cells were transfected with FLAG-tagged wild-type parkin (*A* and *B*) or its PD-linked mutants K161N (*C* and *D*), T240R (*E* and *F*), or C431F (*G* and *H*). After treatment with vehicle control (*A*, *C*, *E*, and *G*) or 1 $\mu$M colchicine (*B*, *D*, *F*, and *H*) for 12 h, cells were co-stained with anti-FLAG (*red*) and anti-$\alpha$-tubulin (*green*). Each of the four constructs greatly attenuated colchicine-induced microtubule depolymerization. *Bar*, 10 $\mu$m. *I*, percentages of cells with at least one obvious microtubule after colchicine treatment were calculated for all the constructs used in *A–H*. At least 200 transfected cells (for each condition in *C–H*) and 500 untransfected cells (for each condition in *A* and *B*) were counted from four coverslips in separate experiments for each construct. *, $p < 0.001$ *versus* untransfected controls. No significant differences were found between parkin and its mutants ($p > 0.1$). *J*, HEK293T cells were transfected without or with various FLAG-tagged parkin constructs and treated without or with colchicine (1 $\mu$M) for 40 min. Free tubulin was extracted from the cells and analyzed by Western blotting with anti-$\alpha$-tubulin (*upper panel*). Expression of wild-type parkin or its mutants significantly reduced colchicine-induced microtubule depolymerization. Total cell lysates were blotted with anti-FLAG to show expression levels of FLAG-tagged parkin constructs (*lower panel*).

K161N (166 ± 12%, $p < 0.01$), T240R (152 ± 11%, $p < 0.01$), or C431F (167 ± 10%, $p < 0.01$) was significantly less than that in untransfected cells (269 ± 12%). Expression levels of FLAG-tagged parkin or its mutants were very similar (Fig. 7*J*, *lower panel*). Thus, the above results from two different approaches strongly suggest that the ability of parkin to stabilize microtubules against colchicine-induced depolymerization is not affected by PD-linked point mutations of parkin.

## DISCUSSION

Our previous study has demonstrated that parkin binds to microtubules with very high affinity; the interaction cannot be disrupted even in the presence of 2 M NaCl (11). The goal of the present study is to understand the molecular mechanism of this strong binding. Our results identified three separate domains of parkin, Linker, RING1, and RING2, which provided strong, independent binding to both tubulin and microtubules. The other two domains of parkin, Ubl and IBR, exhibited no significant interaction with tubulin or microtubules. Thus, the full-length parkin interacts with tubulin heterodimers and microtubules through three of the five functional domains, which may explain why parkin binds to tubulin and microtubules so tightly.

This strong interaction means that there is very little chance for parkin to separate from $\alpha/\beta$-tubulin heterodimers and microtubules (*i.e.* polymerized tubulin heterodimers). In fact, we were not able to separate parkin from microtubules with 3.8 M NaCl (Fig. 3*A*) or 0.5 M urea (Fig. 3*B*). The binding between parkin and microtubules did not rely on taxol, because parkin strongly co-assembled with microtubules in temperature-mediated microtubule assembly assays in the absence of taxol (data not shown). Furthermore, parkin did not dissociate from microtubules in MAP dissociation buffer containing high concentrations of NaCl, but no taxol (Figs. 2, 3*A*, and 6). The strong association between parkin and tubulin heterodimers led to their co-purification in ion-exchange chromatography (Fig. 3*C*) and gel-filtration chromatography (data not shown). We actually found parkin by Western blot analysis of highly purified bovine tubulin (>99% purity) from a widely used commercial source (Fig. 3*D*). It is difficult for us to determine the relative amount of parkin in this tubulin preparation, because both parkin and tubulin migrated very closely on SDS-PAGE. Even with two-dimensional gel electrophoresis, it is unlikely to separate tubulin and parkin, because the first dimension for isoelectric focusing is done in native condition, in which parkin and tubulin would be in a complex. In any case, these results confirm the tight binding of parkin to tubulin and microtubules and suggest that parkin is very likely to be always associated with tubulin or microtubules in the cell under the normal situation.

Such inference is corroborated by our previous study, which has shown that taxol treatment of cells shifts parkin from the soluble fraction to the pellet fraction enriched with bundled microtubules (15). Consistent with this idea, we found in the present study that overexpression of parkin or any one of its tubulin/microtubule-binding domains significantly stabilized microtubules against colchicine-induced depolymerization. In contrast, the parkin domains that did not bind to tubulin or microtubules failed to confer such an effect (Fig. 4). Thus, one of the physiological consequences of a tight binding between parkin and microtubules seems to be the stabilization of microtubule networks in the cell. This may have many implications, because microtubules play essential roles in diverse cellular functions, such as intracellular transport, structural support, motility, etc. However, the ability of parkin to stabilize microtubules against colchicine-induced depolymerization was independent of its E3 ligase activity. We tested three PD-linked

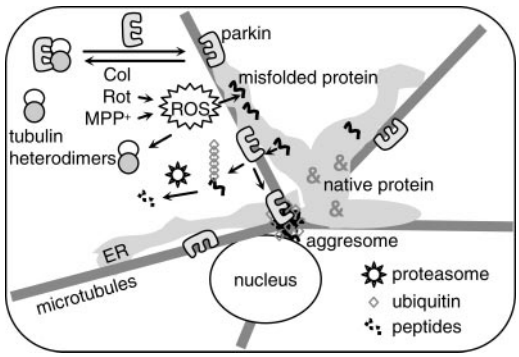


FIG. 8. **A model for the interaction of parkin with tubulin and microtubules.** Through three strong, redundant interaction domains, parkin binds to $\alpha/\beta$-tubulin heterodimers and microtubules very tightly. It stabilizes microtubules against depolymerizing agents such as colchicine. Some parkin substrates are transmembrane proteins that are prone to misfold in the ER. As the ER is attached to microtubules, the association of parkin with microtubules provides an ideal location for the efficient ubiquitination of misfolded substrates that are retrotranslocated from the ER. Ubiquitinated substrates are degraded by the 26 S proteasome or transported along microtubules to the centrosome area to form the aggresome when the proteasome is overwhelmed by misfolded proteins. PD toxins such as rotenone and $MPP^+$ depolymerize microtubules and inhibit complex I of the mitochondrial respiratory chain. The former activity detaches the ER from microtubules, whereas the latter one produces reactive oxygen species (*ROS*) that cause protein oxidation and misfolding. Under the dual insults, ER-associated degradation would be greatly compromised without the abilities of parkin both to stabilize microtubules and to ubiquitinate misfolded transmembrane proteins. The combination of the two independent properties in parkin seems to make it uniquely suited to protect against these PD toxins.

point mutations of parkin, which abrogate its E3 ligase activity toward many substrates (6–12). None of these mutations significantly disrupted the binding of parkin to tubulin (Fig. 5) and microtubules (Fig. 6), nor did they affect the ability of parkin to attenuate colchicine-induced microtubule depolymerization (Fig. 7). These results suggest that the binding of parkin to tubulin and microtubules serves to anchor this E3 ligase; its enzymatic activity does not affect the anchorage.

This idea is consistent with our previous results that parkin exhibits punctate localization along microtubules (11). As many misfolded proteins are transported along microtubules to the centrosome area to form a single large inclusion termed the "aggresome" (23), the localization of parkin along microtubules would greatly facilitate the ubiquitination of its substrates and their subsequent transport on microtubules to the aggresome (see the *model* in Fig. 8). Previous studies have shown that parkin and its substrates, such as CDCrel-1 (6), Pael-R (7), and dopamine transporter (12), are accumulated in the centrosome along with many ubiquitinated proteins when protein degradation through the 26 S proteasome is inhibited (12, 15, 25). Acetylated $\alpha$-tubulin, a potential substrate of parkin (11), has also been found in the aggresome under similar conditions (16). Accumulation of these proteins in the centrosome is a microtubule-dependent process, because disruption or overt stabilization of microtubules abolishes this phenomenon (15). Thus, parkin anchored on microtubules may serve the function of sentinels to efficiently ubiquitinate misfolded proteins for their destruction by the 26 S proteasome or transportation along microtubules to the aggresome, should proteasomes be overwhelmed by misfolded proteins.

We noticed that many parkin substrates, such as Pael-R (7), dopamine transporter (12), synaptotagmin XI (26), CDCrel-1 (6), are transmembrane proteins or membrane-associated proteins. At least some of these proteins are prone to misfolding in the endoplasmic reticulum (ER) (7, 12), which causes unfolded protein stress if left unchecked (27). Previous studies have demonstrated that misfolded membrane proteins are reversely translocated from the ER to the cytosol, where they must be immediately ubiquitinated to avoid aggregation due to the abundance of hydrophobic residues left exposed by the disordered polypeptide chain (27). Under normal situations, the ER is attached to microtubules to maintain its morphology and stability (28–30). The proximity of the ER to parkin, which is anchored on microtubules, would make parkin ideally suited to ubiquitinate misfolded substrates as they are retrotranslocated from the ER (Fig. 8). It is also interesting to note that microtubule depolymerization causes the retraction of the ER toward the cell center (28). By stabilizing microtubules against depolymerization and thus keeping the ER attached to microtubules, parkin may also maintain ER-associated degradation in the face of depolymerizing agents. It has been shown that PD toxins such as $MPP^+$ and rotenone have strong microtubule-depolymerizing activity (17–20), in addition to their complex I-inhibiting activity (31, 32). The latter action generates reactive oxygen species (33, 34), which oxidize proteins and thereby promote their misfolding (Fig. 8). The combination of microtubule depolymerization and misfolded proteins would make ER-associated degradation particularly vulnerable. The ability of parkin to stabilize microtubules and the anchorage of this E3 ligase on microtubules seem to make it strategically located to meet this challenge (Fig. 8).

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