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Parkin Increases Dopamine Uptake by Enhancing the Cell Surface Expression of Dopamine Transporter*

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Mutations of parkin, a protein-ubiquitin E3 ligase, are linked to Parkinson's disease (PD). Although a variety of parkin substrates have been identified, none of these is selectively expressed in dopaminergic neurons, whose degeneration plays a critical role in PD. Here we show that parkin significantly increased dopamine uptake in the human dopaminergic neuroblastoma cell line SH-SY5Y. This effect was accompanied by increased V_{max} of dopamine uptake and unchanged K_m. Consistent with this, increased binding sites for dopamine transporter (DAT) ligand were observed in SH-SY5Y cells overexpressing parkin. The results were confirmed when parkin was transfected in HEK293 cells stably expressing DAT. In these cells, parkin enhanced the ubiquitination and degradation of DAT, increased its cell surface expression, and augmented dopamine uptake. The effects of parkin were significantly abrogated by its PD-causing mutations. Because the cell surface expression of functional DAT requires its oligomerization, misfolded DAT, induced either by the protein glycosylation inhibitor tunicamycin or by its C-terminal truncation, significantly attenuated cell surface expression of native DAT and reduced dopamine uptake. Expression of parkin, but not its T240R mutant, significantly alleviated these detrimental effects of misfolded DAT. Thus, our studies suggest that parkin increases dopamine uptake by enhancing the ubiquitination and degradation of misfolded DAT, so as to prevent it from interfering with the oligomerization and cell surface expression of native DAT. This function of parkin would enhance the precision of dopaminergic transmission, increase the efficiency of dopamine utilization, and reduce dopamine toxicity on neighboring cells.

Parkinson's disease (PD)[1] is characterized by a core set of clinical features that include tremor, rigidity, akinesia, and postural instability. A variety of evidence has strongly demonstrated that these motor symptoms are related to the selective degeneration of dopamine (DA) neurons in substantia nigra (1). Among the several PD-linked genes recently identified, mutations of parkin appear to be the most frequent genetic factor in both familial and sporadic cases (2, 3). Parkin encodes for a protein-ubiquitin E3 ligase (4), which ubiquitinates many substrate proteins to enhance their degradation by the 26 S proteasomes (5). Because parkin mutations abrogate its E3 ligase activity, it is thought that accumulation of parkin substrates in the absence of functional parkin may lead to the selective death of nigral DA neurons. However, none of the parkin substrates identified so far could fully explain the selectivity of cell death, because they are not exclusively expressed in dopamine neurons (6).

In the present study, we found that parkin bound to and ubiquitinated dopamine transporter (DAT), a protein expressed only in dopaminergic neurons within the central nervous system (7). DAT is a 12-transmembrane neurotransmitter transporter responsible for the rapid reuptake of dopamine released by DA neurons (8). It is localized on the plasma membrane of DA neurons at synaptic and extrasynaptic sites (9). The cell surface expression of DAT and its transporter activity are heavily influenced by its *N*-linked glycosylation (10, 11). Inhibition of protein glycosylation with tunicamycin or mutations of the *N*-linked glycosylation sites on DAT significantly reduce its expression on the cell surface and in intracellular membranous compartments (10), suggesting the *N*-glycosylation of DAT is also important for the proper folding and stability of DAT. Recent studies have demonstrated that expression of functional DAT on the plasma membrane requires its oligomerization in the endoplasmic reticulum (ER) (12, 13). C-terminally truncated DAT mutants are localized in the ER and cannot be delivered to the cell surface to transport dopamine (12, 13). Co-expression of these mutants with wild-type DAT retains the full-length DAT in the ER and thus reduces its cell surface expression and transporter activity (13).

Our studies showed that parkin, but not its PD-causing mutants, enhanced the ubiquitination and degradation of DAT. It also increased dopamine uptake and cell surface expression of DAT in a manner dependent on its E3 ligase activity. Parkin significantly enhanced the ubiquitination of misfolded DAT induced by tunicamycin and attenuated the detrimental effects of tunicamycin on dopamine uptake in an E3-dependent manner. Furthermore, parkin, but not its PD-linked T240R mutant, significantly reversed the effect of an ER-retained DAT mutant on wild-type DAT with regard to cell surface expression and transporter activity. These results suggest that parkin increases dopamine uptake through enhancing the cell surface expression of DAT, which is achieved by ubiquitinating and degrading misfolded DAT to prevent it from affecting the oligomerization and surface expression of native DAT molecules. Through this action, parkin may refine the precision of dopaminergic transmission, increase the efficiency of dopamine recycling, and reduce dopamine toxicity on neighboring cells, all of

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[1] The abbreviations used are: PD, Parkinson's disease; DA, dopamine; DAT, dopamine transporter; ER, endoplasmic reticulum; PMA, phorbol 12-myristate 13-acetate; CFT, 2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane; E3, ubiquitin-protein isopeptide ligase; PBS, phosphate-buffered saline.

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which are important to the function and survival of dopaminergic neurons.

EXPERIMENTAL PROCEDURES

Materials—[*N-methyl*-^{3}H]WIN35,428 (60–87 Ci/mmol), and 3,4-[*ring*-2,5,6-^{3}H]dihydroxyphenylethylamine (54.1 Ci/mmol) were purchased from PerkinElmer Life Sciences. [7,8-^{3}H]Dopamine (45.0 Ci/mmol) was purchased from Amersham Biosciences. GBR-12909, nomifensine, dopamine, tunicamycin, anti-FLAG-conjugated (M2)-agarose, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. Monoclonal anti-hemagglutinin and FuGENE 6 were purchased from Roche Applied Science. Anti-c-Myc (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Expression Constructs—Mouse dopamine transporter (DAT) was cloned by PCR amplification of mouse brain cDNA with primers GCA-AGCTTCCCATGAGTAAAAGCAAATGCTCC containing an HindIII site and GCCTCGAGCTTCCACTTTACAGCAACAGCCAA containing an XhoI site. The resultant PCR product was cloned into the HindIII and XhoI sites of pCMV-2B (Stratagene, La Jolla, CA), which contains a FLAG tag at the N terminus. The C-terminally truncated DAT (DATΔC) was generated by removing the last 38 amino acids of the mouse DAT using PCR. The product was subcloned to pCMV-3B (Stratagene, La Jolla, CA), which contains an N-terminal Myc tag. Wild-type and mutant human parkin constructs were generated as described previously (14).

Generation of Stable Cell Lines and Transient Transfection—HEK293 and SH-SY5Y cells were purchased from ATCC (Manassas, VA). Linearized FLAG-DAT construct was transfected into HEK293 cells with FuGENE 6 (Roche Applied Science) following the manufacturer's protocol. A stable cell line (293/DAT) was selected by limited dilution in the presence of 800 μg/ml Geneticin (Invitrogen) and confirmed by Western blot analysis with anti-FLAG (Sigma). SH-SY5Y cell lines stably expressing parkin or the luciferase control were generated previously (14). All cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). They exhibited similar growth rates and morphological features as the corresponding parental line. HEK293 or 293/DAT cells were grown to 80% confluency and transiently transfected with various constructs by incubation with $Ca_3(PO_4)_2$-DNA complex at 37 °C for 16 h. Subsequent experiments were performed 48–72 h after transfection. SH-SY5Y cells were transiently transfected by electroporation in a buffer containing 20 mM HEPES, pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose, and 0.11 mM 2-mercaptoethanol with a Bio-Rad electroporator set at 230 V/500 microfarads.

[^{3}H]Dopamine Uptake Assay—Dopamine uptake in intact cells was measured as described before (15). Briefly, cells were plated in 0.01% poly-D-lysine-coated 12-well plates. Prior to the experiment, medium was removed, and cells were rinsed with 0.5 ml of prewarmed uptake buffer (10 mM HEPES, 130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, pH 7.4). Cells were incubated in 0.5 ml of uptake buffer containing 5 μM dopamine and 20 nM [^{3}H]dopamine for 10 min at 37 °C. After the assay was terminated by washing the cells thrice with 0.5 ml of ice-cold uptake buffer, cells were lysed in 0.5 ml of 1% SDS for 15 min at room temperature and the entire lysate was measured in a scintillation counter. Nonspecific [^{3}H]dopamine uptake was defined as uptake in the presence of 10 μM GBR12909 or 10 μM nomifensine. There was no significant change in nonspecific dopamine uptake in all conditions tested, and it was subtracted from the total uptake to derive the specific dopamine uptake data shown in this report. Kinetic assays were performed by incubating cells with increasing concentrations of unlabeled dopamine and a constant concentration of [^{3}H]dopamine (20 nM).

[^{3}H]CFT Binding Assay—DAT radioligand binding assay using [^{3}H]CFT was performed as described before (16). Cells were seeded at 4×10^5 cells/well in a 12-well plate. After the cells were washed twice in binding buffer (10 mM phosphate buffer, pH 7.4, 0.32 M sucrose), they were incubated in 0.5 ml of binding buffer with 4 nM [^{3}H]CFT for 10 min at 4 °C. After the assay was terminated by three washes with 0.5 ml of ice-cold binding buffer, cells were lysed in 0.5 ml of 1% SDS for 15 min at room temperature and the entire lysate was measured in a scintillation counter. Nonspecific binding was defined as binding in the presence of 10 μM nomifensine. It was not significantly changed by various conditions tested and was subtracted from total binding to derive the specific [^{3}H]CFT binding data shown.

Cell Surface Biotinylation—Cells were seeded in 6-well plates at 2×10^4 cells/cm^2 and transfected with parkin or its mutants using FuGENE 6 according to the manufacturer's protocol (Roche Applied Science). After 48 h, cells were rinsed four times with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^{2+}) and incubated twice with 1 ml of 1.0 mg/ml NHS-SS-biotin (Pierce) for 20 min each at 4 °C. Excess biotin was quenched by 2×20 min incubations in ice-cold PBS^{2+} containing 0.1 M glycine at 4 °C. After the cells were washed with PBS^{2+}, they were lysed for 1 h at room temperature with gentle shaking in radioimmune precipitation assay buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors. Cleared lysates containing equal amounts of total proteins (50 μg) were incubated with 100 μl of immunopure immobilized streptavidin (Pierce) for 14 h at 4 °C. After centrifugation, biotinylated proteins bound to the streptavidin beads were washed four times in radioimmune precipitation assay buffer, boiled in Laemmli buffer, and separated by SDS-PAGE. Western blots using anti-FLAG were performed to detect biotinylated FLAG-DAT and total FLAG-DAT. In some experiments, cells were stimulated with the PKC activator PMA (5 μM for 30 min at 37 °C).

Immunocytochemistry and Confocal Microscopy—HEK293 cells transfected with FLAG-DAT and pcDNA3.1, parkin, or its mutants were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized in 0.1% Triton X-100 in PBS for 15 min. Immunostaining with anti-FLAG (Sigma) and anti-parkin (17) was performed in the same way as described before (18). Fluorescence images were acquired on a confocal microscope from Bio-Rad. Monochrome images (512×512 pixels) were pseudocolored and merged with the software Image J (National Institutes of Health). For quantification of DAT inclusions, cells were counted under a 40× lenses of a Nikon fluorescence microscope. At least 700–800 DAT-transfected cells were counted for each coverslip, and 3 coverslips were examined for each transfection. All data were expressed as mean ± S.E. Statistical analyses were performed with unpaired t test using the software Origin (Origin Laboratory, Northampton, MA).

RESULTS

Parkin Increases Dopamine Uptake—In our previous study on the protective effect of parkin against dopamine-induced apoptosis, we generated SH-SY5Y cell lines stably expressing parkin (SH/PKN) or the luciferase control (SH/Luc) (14). Overexpression of parkin in the human dopaminergic neuroblastoma cell line significantly increased dopamine uptake (4.55 ± 0.34 pmol/mg/min, $p < 0.01$, $n = 6$) from the basal level in the parental SH-SY5Y cells (0.99 ± 0.08 pmol/mg/min) (Fig. 1*A*). In contrast, dopamine uptake in the control cell line SH/Luc (1.40 ± 0.12 pmol/mg/min) was not significantly different, indicating that the effect was not caused by the process of generating a stable cell line. To confirm this observation, we transiently transfected DAT together with luciferase or parkin into SH-SY5Y cells. As shown in Fig. 1*A*, transient expression of parkin also significantly increased dopamine uptake from 15.7 ± 2.86 pmol/mg/min in the luciferase control to 25.88 ± 2.17 pmol/mg/min ($p < 0.05$, $n = 6$).

To substantiate our findings, we utilized the HEK293 cell line, which lacks endogenous DAT and is frequently used to study molecular mechanisms involved in DAT regulation (13). HEK293 cells were transiently transfected with DAT and luciferase or DAT and parkin. As shown in Fig. 1*B*, parkin significantly enhanced dopamine uptake through DAT in HEK293 cells from 32.46 ± 1.6 pmol/mg/min to 48.78 ± 2.8 pmol/mg/min ($p < 0.01$, $n = 6$). In untransfected HEK293 cells, there was virtually no dopamine uptake. In a HEK293 cell line stably expressing FLAG-tagged mouse DAT (293/DAT cells), transient transfection of parkin also significantly increased dopamine uptake (276.1 ± 13.1 pmol/mg/min), compared with transfection of the empty vector (213.1 ± 10.6 pmol/mg/min, $p < 0.01$, $n = 6$). Expression of parkin in 293/DAT cells did not change the expression level of FLAG-DAT, which existed as heavily glycosylated species around 98 kDa and a very minor unglycosylated band at 57 kDa (Fig. 1*C*). Immunostaining with anti-FLAG showed that most of the FLAG-DAT expressed in 293/DAT cells was localized on the plasma membrane (Fig. 1*D*). Thus, our results above showed that parkin increased dopa-

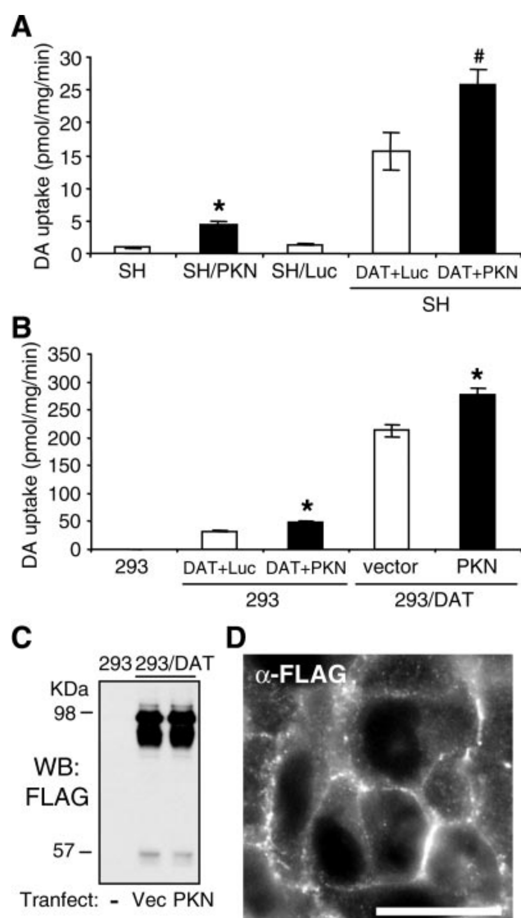

FIG. 1. **Parkin increases dopamine uptake through the dopamine transporter.** *A*, DA uptake in human dopaminergic neuroblastoma SH-SY5Y cells (*SH*), SH cells stably expressing parkin (*SH/PKN*) or luciferase (*SH/Luc*), as well as SH cells transiently transfected with DAT plus luciferase (*Luc*) or DAT plus parkin (*PKN*). Expression of parkin, either stably or transiently, increased dopamine uptake through DAT significantly. DA uptake data was expressed in picomoles of dopamine taken up by the cell per milligram of total cellular proteins per min. *, $p < 0.01$ *versus* SH; #, $p < 0.05$ *versus* DAT+Luc; $n = 6$ experiments. *B*, DA uptake was significantly increased in HEK293 cells transiently transfected with DAT plus parkin (*PKN*), compared with DAT plus luciferase (*Luc*). *, $p < 0.01$ *versus* DAT+Luc; $n = 6$ experiments. Transient transfection of parkin (*PKN*), compared with empty vector, significantly increased DA uptake in a HEK293 cell line stably expressing DAT (293/DAT). *, $p < 0.01$ *versus* vector; $n = 6$ experiments. *C*, expression of FLAG-DAT in 293/DAT cells transfected with empty vector (*Vec*) or parkin (*PKN*). The ~98 kDa bands represent glycosylated FLAG-DAT, whereas the 57-kDa band is the unglycosylated version. *D*, anti-FLAG staining of 293/DAT cells showed that most of FLAG-DAT was localized on the plasma membrane. *Bar*, 10 μm.

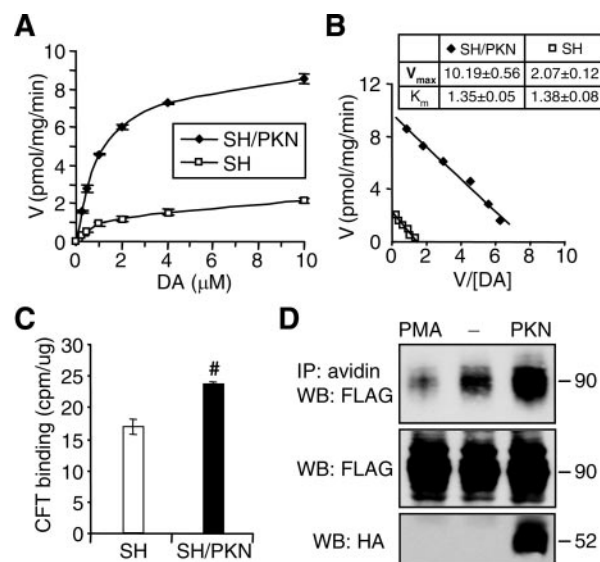

FIG. 2. **Parkin enhances cell surface expression of DAT.** *A*, kinetics of DA uptake in SH-SY5Y cells (*SH*) and SH-SY5Y cells stably expressing parkin (*SH/PKN*). *B*, Scatchard analysis of the kinetics data in *A* showed that stable expression of parkin in SH-SY5Y cells increased the V_{max}, but not the K_m of DA uptake. *C*, expression of parkin in SH/PKN cells significantly increased CFT-binding sites, compared with those in SH-SY5Y cells (*SH*). *, $p < 0.05$, $n = 6$ experiments. *D*, cell surface proteins of 293/DAT cells were labeled with biotin and immunoprecipitated with streptavidin. Anti-FLAG blot of the precipitated proteins showed that transfection of HA-parkin (*PKN*) increased the surface expression of DAT, while stimulation with the phorbol ester PMA reduced it (*upper panel*). Total cell lysates were blotted with anti-FLAG (*middle panel*) or anti-HA (*bottom panel*) to show the expression level of FLAG-DAT or HA-parkin, respectively.

mine uptake through DAT in SH-SY5Y cells and HEK293 cells.

Parkin Enhances the Cell Surface Expression of DAT—An increase in dopamine uptake could be due to elevated transporter activity or more DAT molecules expressed on the cell surface. To differentiate the two possibilities, we compared the kinetics of dopamine uptake between SH-SY5Y cells and SH/PKN cells. Dopamine uptake assays were performed on the two cell lines at various concentrations of dopamine. The speed of dopamine uptake at all concentrations tested was significantly greater in SH-SY5Y cells expressing parkin (Fig. 2*A*). When we performed a Scatchard analysis of the kinetics data (Fig. 2*B*), it was clear that the K_m value for dopamine uptake was virtually unchanged by parkin expression (1.35 ± 0.05 μM for SH/PKN cells *versus* 1.38 ± 0.08 μM for SH-SY5Y cells) and was comparable to the K_m values measured in previous studies (15, 19). In contrast, the maximum velocity (V_{max}) of dopamine uptake was significantly increased by parkin from 2.07 ± 0.12 pmol/mg/min to 10.19 ± 0.56 pmol/mg/min ($p < 0.01$, $n = 5$). These results suggest that parkin does not affect the affinity of DAT toward dopamine.

To test whether parkin enhances DA uptake by increasing the total number of DAT molecules on the cell, we compared the amount of DAT binding sites on SH and SH/PKN cells with [^{3}H]CFT (2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane), a potent cocaine analog that specifically binds to DAT. As shown in Fig. 2*C*, CFT binding sites were significantly increased from 16.8 ± 1.15 cpm/μg of protein in SH cells to 23.6 ± 0.4 cpm/μg of protein in SH/PKN cells ($p < 0.05$, $n = 6$). Thus, the result suggests that parkin increases the amount of DAT on the cell surface. Due to our lack of a good DAT antibody and the low expression level of DAT in SH-SY5Y cells, we used 293/DAT cells instead to examine the effect of parkin on the cell surface expression of DAT. 293/DAT cells were transfected without or with HA-tagged parkin. As a control, untransfected cells were stimulated with the protein kinase C activator PMA (5 μM for 30 min at 37 °C), which is known to reduce the cell surface expression of DAT (20). After cell surface proteins were biotinylated, cleared total cell lysates were incubated with streptavidin beads to pull down biotinylated proteins originally localized on the cell surface. Western blotting of these proteins with anti-FLAG showed that the amount of FLAG-DAT on the surface of 293/DAT cells was greatly increased by parkin expression, whereas activation of PKC by the phorbol ester PMA significantly decreased surface expression of DAT (Fig. 2*D*). In contrast, the amount of FLAG-DAT in the total cell lysates was not changed by PMA treatment or parkin transfection (Fig. 2*D*, *middle panel*). These results suggest that parkin increases dopamine uptake by enhancing the cell surface expression of DAT. The effect is in agreement with the data that overexpression of parkin increased [^{3}H]CFT-binding sites on SH-SY5Y cells (Fig. 2*C*).

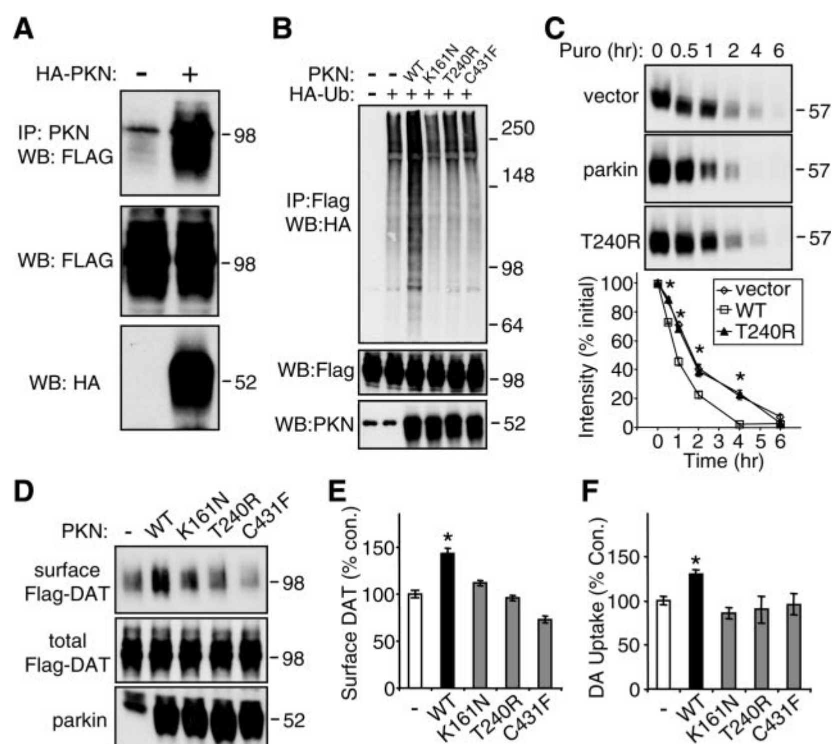

FIG. 3. **Parkin enhances cell surface expression of DAT through its E3 ligase activity on DAT.** *A*, co-immunoprecipitation of parkin and DAT in 293/DAT cells. Anti-parkin immunoprecipitates from 293/DAT cells transfected with or without HA-parkin were blotted with anti-FLAG (*top panel*). Total cell lysates were blotted with anti-FLAG (*middle panel*) or anti-HA (*bottom panel*) to show expression levels of FLAG-DAT and HA-parkin, respectively. *B*, parkin, but not its PD-linked mutants, increased the ubiquitination of DAT. 293/DAT cells were transfected without or with HA-tagged ubiquitin (*HA-Ub*) and wild-type (*WT*) or mutant parkin. Anti-FLAG immunoprecipitates from the cell lysates were blotted with anti-HA to show the ubiquitination of FLAG-DAT (*top panel*). Total cell lysates were blotted with anti-FLAG (*middle panel*) or anti-parkin (*PKN*, *bottom panel*) to show expression levels of FLAG-DAT and parkin, respectively. *C*, parkin, but not its T240R mutant, accelerated the degradation of DAT. 293/DAT cells were transfected with empty vector, parkin, or its T240R mutant, and treated with 100 μM puromycin for various durations. Western blotting of total cell lysates with anti-FLAG showed the degradation rates of unglycosylated FLAG-DAT (57 kDa) in the presence of vector, parkin, or its T240R mutant, respectively (*top three panels*). Results from four separate experiments were quantified in the *bottom panel*. *, $p < 0.05$, WT *versus* vector or T240R. *D* and *E*, parkin, but not its PD-linked mutants, enhanced the cell surface expression of DAT. Cell surface proteins on 293/DAT cells transfected with wild-type parkin (*WT*) or one of the three mutants (K161N, T240R, or C431F) were biotinylated. Streptavidin immunoprecipitates were blotted with anti-FLAG to detect biotinylated FLAG-DAT on cell surface (*top panel*). Total cell lysates were blotted with anti-FLAG (*middle panel*) or anti-parkin (*PKN*, *bottom panel*) to show expression levels of FLAG-DAT and parkin, respectively. Results from four separate experiments were quantified in *E*; *, $p < 0.01$ *versus* untransfected 293/DAT cells. *F*, parkin, but not its PD-linked mutants, increased dopamine uptake in 293/DAT cells. *, $p < 0.01$ *versus* untransfected 293/DAT cells.

Parkin Enhances Cell Surface Expression of DAT through Its Ability to Ubiquitinate DAT—Because parkin is a protein-ubiquitin E3 ligase, we examined whether the effect of parkin on DAT was mediated by its E3 ligase activity. First, we tested whether parkin was bound to DAT. After 293/DAT cells were transfected without or with HA-parkin, cleared total cell lysates were immunoprecipitated with the parkin antibody (17). Anti-FLAG Western blot of the parkin immunoprecipitates showed that FLAG-DAT was co-immunoprecipitated with parkin (Fig. 3*A*, *top panel*), which suggests that parkin binds to DAT. We then examined whether parkin ubiquitinated DAT by transfecting 293/DAT cells with wild-type parkin or its PD-linked mutants, along with HA-tagged ubiquitin. Cleared total cell lysates were immunoprecipitated with anti-FLAG to pull down FLAG-DAT. Western blotting of the immunoprecipitates with anti-HA showed that wild-type parkin significantly increased the ubiquitination of FLAG-DAT, compared with the situation without parkin transfection. In contrast, none of the three PD-linked mutants had any significant effect over the basal ubiquitination level of DAT revealed by the expression of HA-ubiquitin alone (Fig. 3*B*, *top panel*). The amount of FLAG-DAT in the total cell lysates was not changed by these parkin constructs, which were expressed at similar levels (Fig. 3*B*, *middle* and *bottom panels*).

If parkin ubiquitinates DAT as these results suggest, then expression of parkin, but not its PD-linked mutants, should accelerate the degradation of DAT. To test this, we transfected 293/DAT cells with parkin, its PD-linked T240R mutant, or the empty vector. Cells were treated with the protein synthesis inhibitor puromycin (100 μM) for various durations. As new protein synthesis was blocked, levels of FLAG-DAT revealed by anti-FLAG immunoblot would indicate the degradation rate of DAT. It is known that glycosylated misfolded proteins in the ER must be deglycosylated and retrotranslocated back to the cytosol for ubiquitination and degradation (21). Consistent with this, the glycosylated DAT species around ~98 kDa were very stable and did not show significant degradation even after the cells were treated with puromycin for 48 h (data not shown). In contrast, unglycosylated DAT at 57 kDa was degraded rapidly. As shown in the *top panel* of Fig. 3*C*, the level of unglycosylated FLAG-DAT (57 kDa) decreased as the incubation with puromycin continued. Transfection of parkin, but not its T240R mutant, markedly accelerated the degradation rate of FLAG-DAT. By 4 h of treatment, there was virtually no FLAG-DAT in 293/DAT cells transfected with parkin, but a small amount of DAT remained in vector- or T240R-transfected cells even at 6 h. Quantification of the data from four separate experiments confirmed that wild-type parkin significantly enhanced the degradation of unglycosylated DAT, compared with the PD-causing T240R mutant or the vector control (Fig. 3*C*, *bottom panel*).

If the ability of parkin to ubiquitinate DAT is involved in

facilitating the cell surface expression of DAT, then the three PD-linked mutants of parkin, which lost their E3 ligase activity on DAT (Fig. 3*B*) and other substrates (17, 22), should not be able to increase the expression of DAT on the plasma membrane. To test this, we transfected wild-type parkin or its PD-linked mutants into 293/DAT cells and examined the cell surface expression of DAT. Unlike wild-type parkin, none of the three point mutants could increase the expression level of FLAG-DAT on the plasma membrane (Fig. 3*D*, *top panel*). The amount of FLAG-DAT in the total cell lysates was not affected by the different parkin proteins, which were expressed at comparable levels (Fig. 3*D*, *middle* and *bottom panel*). Quantification of cell surface DAT levels from four independent experiments showed that only parkin, but not its PD-linked mutants, significantly increased DAT expression on the plasma membrane (Fig. 3*E*). Consistent with this result, we found that only wild-type parkin, but none of the three mutants, significantly increased dopamine uptake in 293/DAT cells (Fig. 3*F*). Thus, the above results suggest that parkin facilitates the cell surface expression of DAT through its ability to ubiquitinate DAT.

Parkin Ubiquitinates Misfolded DAT to Attenuate Its Detrimental Effects on Native DAT in Cell Surface Expression and Dopamine Uptake—Previous studies have shown that oligomerization of DAT is required for its proper post-translational processing through the secretory pathway and correct targeting to the plasma membrane (12, 13). Co-expression of ER-retained mutant DAT with wild-type DAT causes the retention of the wild-type in intracellular compartments (13). Because DAT has 12 transmembrane regions and is heavily glycosylated (8), misfolded DAT may be produced during its synthesis and post-translational processing. It may oligomerize with native DAT proteins and interfere with their targeting to the plasma membrane. By ubiquitinating misfolded DAT, parkin could facilitate its degradation so that it would not impede the cell surface expression and normal function of native DAT.

To test this hypothesis, we examined the effect of parkin on the ubiquitination of FLAG-DAT when 293/DAT cells were treated with the protein glycosylation inhibitor, tunicamycin, which increases misfolding of DAT (10). As shown in the *upper panel* of Fig. 4*A*, 293/DAT cells were transfected with various combinations of constructs and treated without or with tunicamycin (10 μg/ml for 24 h) and the 26 S proteasome inhibitor lactacystin (10 μM for 12 h). Application of tunicamycin alone increased the ubiquitination of FLAG-DAT (*lanes 3 versus 2*, Fig. 4*A*). Co-application of lactacystin (with an overlap of the last 12 h) further elevated the amount of ubiquitinated DAT (*lanes 4 versus 3*, Fig. 4*A*), because the drug blocks the degradation of ubiquitinated proteins by the 26 S proteasome (23). When parkin was overexpressed, it raised the level of ubiquitinated DAT even further (*lanes 5 versus 4*). Without tunicamycin, DAT ubiquitination was tremendously reduced (*lanes 6 versus 5*), suggesting that the bulk of ubiquitinated DAT proteins were those misfolded by tunicamycin. Application of tunicamycin greatly decreased the amount of glycosylated FLAG-DAT as shown in the immunoblot of total FLAG-DAT (Fig. 4*A*, *middle panel*). Expression levels of endogenous or transfected parkin were not significantly affected by tunicamycin or lactacystin treatment (Fig. 4*A*, *bottom panel*).

Because parkin ubiquitinated misfolded DAT induced by tunicamycin, it would prevent misfolded DAT from interfering with the normal oligomerization and function of native DAT. This may rescue the detrimental effect of tunicamycin on dopamine uptake. To test this, we performed dopamine uptake assays on 293/DAT cells transfected with wild-type parkin, its T240R mutant or the empty vector. Cells were treated without or with 10 μg/ml tunicamycin for 24 h. As shown in Fig. 4*B*,

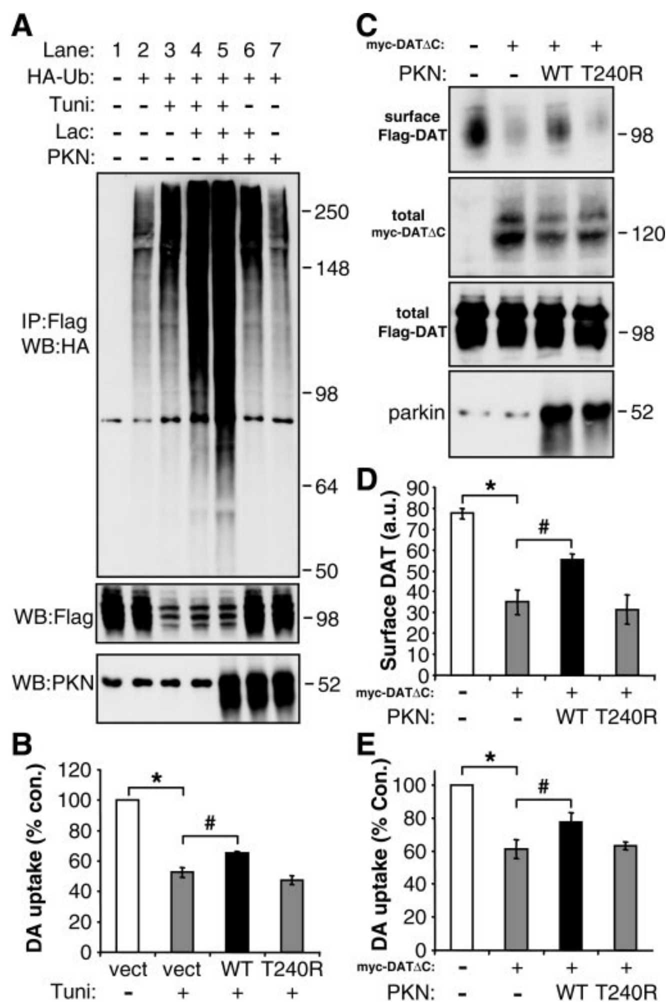

FIG. 4. **Parkin ubiquitinates misfolded DAT to increase the surface expression of native DAT and dopamine uptake.** *A*, parkin increases the ubiquitination of misfolded DAT induced by tunicamycin. 293/DAT cells transfected with various combinations of HA-tagged ubiquitin (*HA-Ub*) and parkin were treated with or without the protein glycosylation inhibitor tunicamycin (10 μg/ml for 24 h) and the proteasome inhibitor lactacystin (10 μM for 12 h). FLAG immunoprecipitates from cleared cell lysates were blotted with anti-HA to show the ubiquitination of FLAG-DAT (*top panel*). Total cell lysates were blotted with anti-FLAG (*middle panel*) or anti-parkin (*PKN*, *bottom panel*) to show expression levels of FLAG-DAT and parkin, respectively. *B*, parkin, but not its T240R mutant, significantly rescued tunicamycin-induced decrease in dopamine uptake. 293/DAT cells transfected with empty vector, parkin or its T240R mutant were treated without or with tunicamycin (10 μg/ml for 24 h). Dopamine uptake in these cells was quantified from 6 different experiments. *, $p < 0.01$ between indicated conditions. #, $p < 0.05$ between indicated conditions. *C–E*, parkin, but not its T240R mutant, significantly reversed the detrimental effects of ER-retained DAT mutant on DAT surface expression and DA uptake. 293/DAT cells were transfected without or with myc-tagged C-terminally truncated DAT (myc-DATΔ C) and parkin (*WT*) or its T240R mutant (T240R). Surface expression of FLAG-DAT (*first panel*), as well as the levels of total myc-DATΔ C (*second panel*), total FLAG-DAT (*third panel*) or parkin (*last panel*) were examined (*C*). Results from three separate experiments were quantified in *D*. Dopamine uptake in these cells were also quantified from five separate experiments (*E*). *, $p < 0.01$ between indicated conditions. #, $p < 0.05$ between indicated conditions (*D* and *E*).

tunicamycin treatment of vector-transfected 293/DAT cells greatly reduced dopamine uptake to 52.5 ± 3% of control ($p < 0.01$, $n = 6$). Transfection of wild-type parkin significantly attenuated the effect of tunicamycin (65.5 ± 0.8%, $p < 0.05$ *versus* vector transfected, $n = 6$). In contrast, the T240R mutant parkin did not significantly change the effect of tunicamycin (47.2 ± 2.9%, $p > 0.2$ *versus* vector transfected, $n = 6$).

These data suggest that parkin ubiquitinates and degrades misfolded DAT to enhance dopamine uptake by native DAT.

To further demonstrate this point, we generated a myc-tagged C-terminally truncated DAT (myc-DATΔC) by removing the last 38 amino acids of the mouse DAT. This mutant was similar to the human DAT C-terminal truncation construct S582*, which is known to be retained in the ER and does not have significant transporter activity (13). Expression of myc-DATΔC in 293/DAT cells greatly reduced cell surface expression of FLAG-DAT (Fig. 4*C*, *top panel*). It suggests that the ER-retained truncated DAT oligomerizes with the full-length FLAG-DAT to reduce its surface expression as previous studies have demonstrated (12, 13). Expression of parkin, but not its T240R mutant, significantly attenuated the effect of myc-DATΔC on the surface expression of FLAG-DAT (Fig. 4*C*, *top panel*). The myc-DATΔC mutant migrated at an abnormally high molecular mass of ~120 kDa but was expressed to a similar level in transfected cells (Fig. 4*C*, *second panel*). The amount of FLAG-DAT in the total cell lysates was not significantly affected by myc-DATΔC or parkin constructs (Fig. 4*C*, *third panel*). Quantification of results from three separate experiments showed that myc-DATΔC significantly reduced the cell surface expression of FLAG-DAT from 77.45 ± 2.32 to 34.98 ± 5.64 ($p < 0.01$) (Fig. 4*D*). Expression of parkin significantly restored the surface expression of DAT to 55.4 ± 2.9 ($p < 0.05$, *versus* no parkin transfection), while the effect of the T240R mutant (31.47 ± 6.93) was not significantly different from the control without parkin ($p > 0.1$) (Fig. 4*D*). Consistent with this, dopamine uptake was significantly decreased by myc-DATΔC ($p < 0.01$, $n = 5$, Fig. 4*E*). Expression of parkin, but not its T240R mutant, significantly mitigated the effect of myc-DATΔC ($p < 0.05$, $n = 5$, Fig. 4*E*). Taken together, these results suggest that parkin, by ubiquitinating and degrading misfolded DAT, attenuates the detrimental effects of misfolded DAT on the cell surface expression of native DAT and dopamine uptake.

Parkin, but Not Its PD-linked Mutants, Significantly Attenuates the Accumulation of DAT in the Perinuclear Region of the ER—Overexpression of many transmembrane proteins in the cell leads to their accumulation in the perinuclear region (24). We transfected HEK293 cells with FLAG-DAT plus empty vector (pcDNA3.1), wild-type parkin, or its PD-linked mutants to examine whether overexpression of FLAG-DAT produces intracellular inclusions and whether parkin or its mutants affect it. In HEK293 cells transfected with FLAG-DAT and pcDNA3.1 empty vector, DAT was markedly accumulated in the perinuclear region of some cells (Fig. 5*A*). Closer examination of the inclusion-like structure with confocal microscopy showed that FLAG-DAT was highly enriched in the perinuclear compartment of the endoplasmic reticulum (inset of Fig. 5*A*). This effect was greatly reduced in cells co-transfected with wild-type parkin (Fig. 5*B*). Co-expression of parkin rendered FLAG-DAT evenly distributed in the ER throughout the cell (*inset* of Fig. 5*B*). In contrast, co-expression of PD-linked parkin mutants, such as K161N (Fig. 5*C*), T240R (Fig. 5*D*) or C431F (Fig. 5*E*), did not have any obvious effect on the accumulation of FLAG-DAT in the perinuclear region of the ER. Results from three independent experiments were quantified and summarized in Fig. 5*F*. In HEK293 cells co-transfected with FLAG-DAT and empty vector, 8.66 ± 0.78% of DAT-transfected cells contained perinuclear inclusions. The effect was significantly attenuated by co-expression of wild-type parkin (5.99 ± 0.66%, $p < 0.05$), but not its PD-linked mutants, such as K161N (7.40 ± 0.67%), T240R (7.59 ± 0.53%), or C431F (7.94 ± 0.52%). Together, these results suggest that parkin facilitates the removal misfolded DAT through its E3 ligase activity, thereby decreasing the retention of misfolded DAT in the perinuclear compartment of the endoplasmic reticulum.

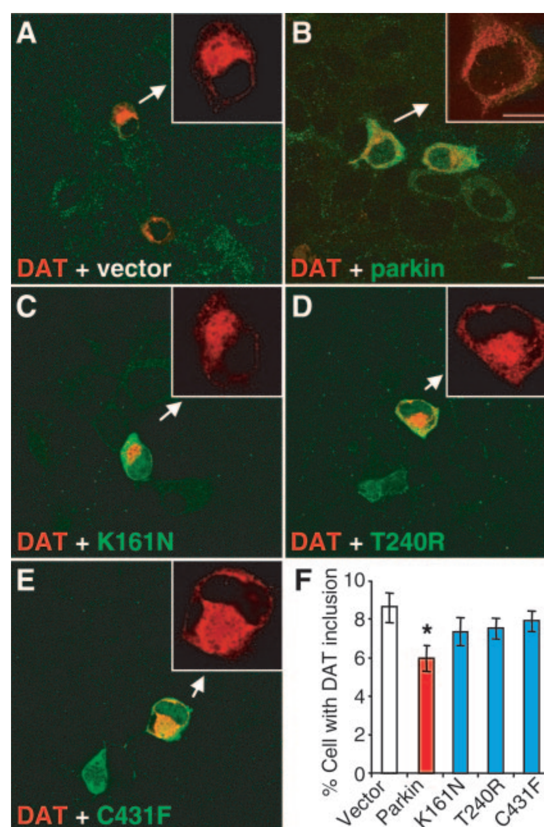

FIG. 5. **Parkin, but not its PD-linked mutants, significantly decreases the accumulation of DAT in the perinuclear region of the endoplasmic reticulum.** After HEK293 cells were co-transfected with FLAG-DAT and empty vector, parkin, or its PD-linked mutants (K161N, T240R, or C431F), they were co-stained with antibodies against FLAG (*red*) and parkin (*green*). *Weak* or *strong green signals* were from endogenous or transfected parkin, respectively. Merged confocal images showed that FLAG-DAT accumulated in the perinuclear region of the ER (*A*). This effect was greatly reduced by the co-expression of parkin (*B*), but not parkin mutants K161N (*C*), T240R (*D*), or C431F (*E*). *Insets*, enlarged portion of the indicated cells showing the localization of FLAG-DAT (*red* signal only). *Bars*, 10 μ m. *F*, percentages of DAT-transfected cells with inclusions were calculated from three separate experiments. *, $p < 0.05$ *versus* vector.

DISCUSSION

Mutations of parkin have been clearly linked to the selective degeneration of nigral DA neurons and Parkinson's disease (25). Because most of these mutations abrogate the protein-ubiquitin E3 ligase activity of parkin (4, 17, 22), it is generally thought that accumulation of parkin substrates in the absence of functional parkin proteins may lead to specific degeneration of dopaminergic neurons. In this study, we identified dopamine transporter (DAT) as a novel substrate of parkin. In the central nervous system, DAT is exclusively expressed in dopaminergic neurons and is responsible for the rapid reuptake of released dopamine back to the DA neuron (26). We showed that only wild-type parkin, but not its PD-causing mutants, could ubiquitinate DAT to accelerate its degradation (Fig. 3, *B* and *C*). Experiments with tunicamycin, a protein glycosylation inhibitor that causes misfolding of many glycosylated proteins including DAT (10), suggest that the bulk of DAT that parkin ubiquitinates is misfolded. Thus, when parkin is mutated, misfolded DAT may not be efficiently ubiquitinated and degraded. Because misfolded DAT would oligomerize with properly folded DAT to affect its correct targeting to the plasma membrane (12, 13), it would reduce the cell surface expression of native DAT.

If this is true, loss-of-function mutations of parkin would hamper DAT functions. Indeed, in parkin knockout mice, expression of DAT in the striatum (presumably on the surface of terminals where most DAT proteins are enriched) is significantly reduced (27). Consistent with this, dopamine uptake in midbrain neuronal cultures from parkin-deficient mice is significantly decreased, compared with that in wild-type cultures (27). Furthermore, the amount of dopamine is increased significantly in limbic system (27). Extracellular dopamine concentration in striatum of another line of parkin knockout mice is also significantly increased (28). Thus, both *in vivo* studies have provided independent evidence that loss of parkin significantly decreases normal DAT function. This is consistent with our results that wild-type parkin, but not its PD-linked mutants, augmented dopamine uptake by enhancing the cell surface expression of DAT.

The ability of parkin to ubiquitinate misfolded DAT to accelerate their degradation may serve as a quality control mechanism for the proper folding and assembly of DAT in the ER. Previous studies have shown that parkin ubiquitinates Pael-R, a transmembrane protein homologous to the endothelin receptor type B (29). Through this action, parkin reduces the toxicity caused by Pael-R-induced unfolded protein stress (29). Recent studies have increasingly demonstrated the importance of ER-associated degradation in the quality control of proteins in the secretory pathway (30). Misfolded transmembrane proteins are reverse translocated to the cytosol where they are immediately ubiquitinated and degraded by the 26 S proteasome (21). The hydrophobic residues in the transmembrane regions of these proteins make it very easy to form inclusions, which very often coalesce into a single large aggregate in the centrosome region of the cell (24). Previous studies by our group and many others have shown that parkin is recruited to this "aggresome" (18, 31–33), perhaps in an effort to maximize the ubiquitination of misfolded proteins in it. We found that transient overexpression of DAT in HEK293 cells also caused its perinuclear accumulation (Fig. 5*A*), which appears to be similar to the situation for other membrane protein substrates of parkin, such as Pael-R (34) or CDCrel-1 (18). Closer examination showed that these accumulations were in fact strong enrichments of DAT in the perinuclear region of the endoplasmic reticulum (Fig. 5*A*, *inset*). Parkin, but not its PD-causing mutants, reduced the accumulation and rendered DAT more evenly distributed in the ER throughout the cell (Fig. 5*B*, *inset*). Thus, the E3 ligase activity of parkin toward misfolded DAT appears to decrease its retention in the perinuclear compartment of the ER, presumably by enhancing its degradation.

By increasing the cell surface expression of DAT, parkin may facilitate dopaminergic transmission by increasing dopamine reuptake. Without this function, the reuptake of dopamine could be significantly slowed down, which would affect the precision of dopamine signaling. On the other hand, without the efficient reuptake of released dopamine, there would be increased burden of DA neurons in the *de novo* synthesis of dopamine. Higher extracellular dopamine concentration would also elevate the production of reactive oxygen species through the autooxidation and enzymatic degradation of dopamine. These harmful effects could be mitigated by parkin through its action on DAT. However, this function of parkin also increases the intracellular concentration of dopamine, which is a key factor in the degeneration of dopaminergic neurons (35). Our recent studies have shown that overexpression of parkin protects SH-SY5Y cells against dopamine-induced apoptosis (14). The protection may seem counter-intuitive, as more dopamine is taken up by cells overexpressing parkin. Our ongoing studies suggest that other mechanisms afforded by parkin are at work to offset the impact of increased cytosolic dopamine concentration. The ability of parkin to reduce ER stress induced by misfolded DAT may contribute to the protective effect of parkin.

In summary, this study provided evidence that parkin increased dopamine uptake by enhancing the expression of dopamine transporter on the plasma membrane. The underlying mechanism was that parkin ubiquitinated and degraded misfolded DAT to prevent it from interfering with the oligomerization and cell surface targeting of native DAT. This novel function of parkin impacts on DAT, a protein that is unique to dopaminergic neurons and plays a critical role in dopaminergic transmission and the survival of DA neurons.

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