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Parkin Suppresses the Expression of Monoamine Oxidases*

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Mutations of parkin are linked to early onset Parkinson disease. Here we show that stable transfection of parkin in the human dopaminergic neuroblastoma cell line SH-SY5Y markedly reduced the activities of both monoamine oxidase (MAO) A and B. The amount of 3,4-dihydroxyphenylacetic acid, which is produced during dopamine oxidation by MAO, was greatly reduced by parkin overexpression. Radioligand binding assays showed that MAO binding sites were decreased accordingly. Consistent with these, MAO-B protein level was much lower, whereas the amount of MAO-A protein was not determined due to the lack of a suitable antibody. Co-transfection of either MAO with parkin in HEK293 cells did not significantly alter ubiquitination and degradation of each MAO. When we measured MAO expression by real-time quantitative reverse transcription-PCR, marked reductions were seen in SH-SY5Y cells stably expressing parkin compared with the parental cells or a control line stably transfected with luciferase. In addition, parkin mutants defective in E3 ligase activity exhibited different effects on MAO expression. We found that parkin also significantly decreased mRNA levels of both MAOs in the mouse fibroblast cell line NIH3T3. Furthermore, MAO expression was significantly increased in human B lymphocyte cell lines derived from Parkinson disease patients with homozygous but not heterozygous deletion of exon 4 of parkin. Together these results suggest that parkin suppresses MAO expression. This function may limit the production of reactive oxygen species generated by MAO in dopamine oxidation and would, thus, be beneficial to the survival of dopaminergic neurons.

Parkinson disease is characterized by the selective degeneration of dopaminergic (DA)[2] neurons in substantia nigra. Mutations of several genes, including α-synuclein, parkin, DJ-1, PINK-1, and LRRK2, have been definitively linked to familial forms of Parkinson disease (1). Evidence accumulated so far shows that parkin mutations represent the most frequent cause of recessively inherited PD (2, 3). In addition, parkin mutations, presumably at the heterozygous state, play a significant role in idiopathic PD, especially in cases with an early age at onset (4–6).

Parkin has a protein-ubiquitin E3 ligase activity (7) that targets a variety of substrates for ubiquitin-dependent proteolysis by the 26 S proteasome (8). Although it is generally assumed that accumulation of parkin substrates in the absence of functional parkin may underlie the selective degeneration of DA neurons in Parkinson disease, recent studies suggest that parkin may have functions independent of its E3 ligase activity. Our previous studies have shown that parkin strongly binds to tubulin and microtubules (9) and stabilizes microtubules against depolymerizing agents through three redundant interaction domains in an E3-independent manner (10). Parkin has a RING-IBR-RING motif in the C terminus that is predicted to be involved in the regulation of gene expression (11). A number of proteins containing this motif play important roles in transcription regulation. For example, RBCK1 binds to DNA and activates transcription in *in vitro* assays (12, 13). parc (p53-associated, parkin-like cytoplasmic protein) also has the RING-IBR-RING motif in the C terminus. Although parc has an E3 ligase activity and can ubiquitinate itself, it does not ubiquitinate p53, to which it binds strongly. Instead, parc suppresses p53-dependent gene transcription and apoptosis by tethering p53 in the cytosol (14).

A key culprit for the selective death of DA neurons in PD may be dopamine itself (15). Oxidation of dopamine by monoamine oxidase (MAO) produces large amounts of reactive oxygen species (ROS) that are toxic to the cell (15). Monoamine oxidase has two isoforms, MAO-A and MAO-B, which are encoded by two distinct genes. Although dopamine can be oxidized by either MAO *in vivo* (16), MAO-A is predominantly expressed in catecholaminergic neurons, whereas MAO-B is abundant in glial cells (17). The catabolism of dopamine has two pathways, depending on whether dopamine is first deaminated by monoamine oxidase or first methylated by catechol *O*-methyltransferase. In the latter case, the product 3-methoxytyramine (3-MT) is then deaminated by MAO. In both pathways the aldehydes produced in the deamination reaction are quickly oxidized by aldehyde dehydrogenase to 3,4-dihydroxyphenylacetic acid (DOPAC) or homovanillic acid (HVA), respectively. H_2O_2 is produced during the oxidative deamination of dopamine (or 3-MT). It can be converted into many different reactive oxygen species in DA neurons. In addition, dopamine aldehyde generated in the deamination reaction is 1000-fold more toxic *in vivo* than DA (18).

Studies done *in vivo* (19) and in cell lines (20) have demonstrated that dopamine induces cell death through the generation of reactive oxygen species. Injection of dopamine into rat striatum causes selective death of DA neurons, and the effect is significantly attenuated by antioxidants (19). Dopamine treatment of HEK293 cells or rat striatal neuronal cultures induces apoptosis through a mechanism dependent on ROS (20). Thus, endogenous dopamine in DA neurons represents a significant toxicity to the cell. This notion is consistent with the observations that basal protein oxidation is 2-fold higher in substantia nigra pars compacta than other regions in the normal human brain (21) and is significantly increased in brain tissues from PD compared with incidental Lewy body disease (22).

Our previous studies have shown that overexpression of parkin in the human dopaminergic neuroblastoma cell line SH-SY5Y reduces dopamine-induced apoptosis by decreasing ROS production (23). In the present study we provide evidence that this is achieved through the

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[2] The abbreviations used are: DA, dopamine; PD, Parkinson disease; MAO, monoamine oxidase; MAO-A, monoamine oxidase A; MAO-B, monoamine oxidase B; 3-MT, 3-methoxytyramine; DOPAC, 3, 4-dihydroxyphenylacetic acid; HVA, homovanillic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ct, threshold cycle; E3, protein-ubiquitin ligase; ROS, reactive oxygen species; HPLC, high performance liquid chromatography; HA, hemagglutinin; RT, reverse transcription; SH cells, SH-SY5Y cells; PKN, parkin.

ability of parkin to suppress the expression of monoamine oxidases at the RNA level. This novel function of parkin may limit the expression of MAO and, thus, restrict ROS produced during dopamine oxidation by MAO. Such a function would protect DA neurons from their own toxin, dopamine.

EXPERIMENTAL PROCEDURES

Generation of Stable Cell Lines—Human dopaminergic neuroblastoma SH-SY5Y cells and mouse fibroblast NIH3T3 cells were purchased from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). SH-SY5Y cell lines stably expressing parkin, its mutants, or luciferase control were generated previously (23). NIH3T3 cells were transfected with 5 μg of linearized parkin expression construct (9) using Lipofectamine 2000 (Invitrogen). Transfected cells were selected in 800 μg/ml Geneticin (Invitrogen) and tested for the expression of transfected genes by Western blotting.

Human B Lymphocyte Cell Lines with Parkin Mutation—Epstein-Barr virus-transformed human B lymphocyte cell lines were purchased from Coriell Cell Repositories (Camden, NJ). ND01037 was derived from a PD patient (male Caucasian, 33 years of age at sampling) with homozygous deletion of exon 4 of parkin (24). ND01038 (male Caucasian, 41 years) and ND01040 (female Caucasian, 51 years) were derived from unaffected siblings of ND01037 and carried heterozygous deletion of exon 4 (24). Two unrelated and unaffected controls were used, ND00092 (male Caucasian, 37 years) and ND05706 (male Caucasian, 39 years). These cells were maintained in RPMI 1640 medium containing 2 mM L-glutamine and 15% heat-inactivated fetal bovine serum (Invitrogen). Cells were seeded at a density of 4×10^5 cells/ml and subcultured every 3 days. The protocol covering the use of these cell lines has been determined to meet exempt criteria 45 CFR 46.101(b) (4) by the Health Sciences Institutional Review Board of the State University of New York at Buffalo.

Measurement of Dopamine and Its Metabolites Using Reverse-phase HPLC—Cells in 10-cm dishes were washed 3 times with ice-cold phosphate-buffered saline, collected, and homogenized in 0.1 M perchloric acid with 1 mM EDTA, 0.1 mM sodium metabisulfite, and 0.1 μg/ml 3,4-dihydroxybenzylamine hydrobromide as an internal standard. The homogenates were centrifuged at 13,000 rpm for 45 min in 4 °C. The supernatant were filtered using filters with a 0.45-μm pore size and stored at −80 °C until analysis. The pellet was dissolved in 1 ml 0.5 N NaOH. Protein content was determined by DC protein assay kit (Bio-Rad). Dopamine, DOPAC, HVA, and 3-MT in the supernatant were quantified by reverse-phase HPLC coupled with electrochemical detection. Samples were injected onto a Supelco Discovery C18 (150 × 3.0 mm, 3 μM) column perfused with MD-TM mobile phase (ESA, Inc., Chelmsford, MA) at 0.5 ml/min. The eluates were analyzed using an ESA Coulochem II electrochemical detector (model 5200A) with a coulometric analytic cell (model 5014). Data were analyzed with built-in software from ESA.

MAO Activity Assay—MAO activities were determined as described previously (25, 26). Briefly, $\sim 5 \times 10^6$ cells were homogenized in 250 μl of 50 mM sodium phosphate buffer, pH 7.4. Assays were performed in 100-μl reaction mixtures containing 50 mM sodium phosphate buffer, pH 7.4, 1 mM tyramine doped with 0.1 μCi of [^{14}C]tyramine (55 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO), and 40 μl of cell homogenates. After 20 min of incubation at 37 °C, the reaction was terminated by the addition of 10 μl of 6 N HCl. Blank samples were prepared by adding HCl before the substrate (tyramine). The reaction products were extracted with 600 μl of water-saturated toluene:ethyl acetate (1:1, v/v). After vortexing for 1 min and centrifugation at 13,000 rpm for 5 min, the top organic layer was withdrawn and mixed with 5 ml of scintillation fluid. Radioactivity of the reaction products was determined in a liquid scintillation counter. To measure MAO-A or MAO-B activity, 1 μl of 100 μM pargyline or clorgyline was first incubated with the 40 μl of cell homogenate for 30 min at room temperature to selectively block MAO-B or MAO-A activity. The remaining activity, determined in the same manner as above, was for MAO-A or MAO-B, respectively.

[^{3}H]Clorgyline and [^{3}H]Deprenyl Binding Assay—Binding of ^{3}H-labeled inhibitors of MAO-A or MAO-B was performed as previously described (27). Briefly, SH or SH/PKN cells were plated at 2×10^5 cells/ml in 12-well plate. After 24 h of incubation, cells were washed 3 times with pre-warmed Dulbecco's modified Eagle's medium without serum and treated with various concentrations of [^{3}H]clorgyline (1.8 Ci/mmol, Moravek Biochemicals, Brea, CA) or [^{3}H]deprenyl (85 Ci/mmol, American Radiolabeled Chemicals) for 1 h at 37 °C. After the cells were washed 3 times with cold Dulbecco's modified Eagle's medium without serum, they were lysed in 0.5 ml of 1% SDS for 15 min at room temperature. Whole cell lysates were mixed with 5 ml of scintillation fluid for the determination of bound radioactivity in a liquid scintillation counter. To remove the influence of nonspecific binding, a duplicate culture was preincubated with 1 μM unlabeled clorgyline or deprenyl for 1 h at 37 °C and then treated with ^{3}H-labeled inhibitors as described above. The difference between the duplicate cultures in bound radioactivity, which was converted to quantity of radiolabeled inhibitors, represented specific binding of the radiolabeled inhibitors to MAO-A or MAO-B. Because all specific binding sites were saturated by the nonradioactive inhibitors, any radioactivity in the duplicates was due to nonspecific binding. For human B lymphocyte cell lines, 2×10^6 cells were incubated with 50 nM [^{3}H]clorgyline for 1 h at 37 °C to measure MAO-A binding sites as described above. For MAO-B, 4×10^6 cells were incubated with 100 nM [^{3}H]deprenyl for 1 h at 37 °C.

In Vivo Ubiquitination of MAO—Parkin, HA-tagged ubiquitin, and Myc-tagged MAO-A or MAO-B were co-transfected into HEK293 cells using FuGENE 6 (Roche Applied Science) according to the manufacturer's protocol. Forty-eight 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) with protease inhibitor mixture tablets (Roche Applied Science) for 20 min. Lysates were centrifuged at 4 °C at 16,000 × *g*, and supernatant fractions were incubated with antibody against Myc overnight at 4 °C followed by incubation with protein A/G plus-agarose (Santa Cruz Biotechnology) under the same conditions. Immunoprecipitates were washed 3 times with the lysis buffer, then boiled in 2× SDS loading buffer for 5 min and separated on a 7.5% SDS-polyacrylamide gel. Western blotting was performed with anti-HA to detect the ubiquitination of MAO. Cell lysates containing equal amounts of total protein (50 μg) were boiled in a sample buffer, separated on 7.5% SDS-polyacrylamide gel, and analyzed by Western blot with anti-Myc or anti-parkin. Western blotting was carried out using the ECL method according to the manufacturer's protocol (Amersham Biosciences).

Real-time Quantitative RT-PCR—Total RNA was extracted using TRIZOL Reagent (Invitrogen). First-strand complementary DNA (cDNA) was synthesized with oligo(dT) or random hexamers as primers using the SuperScript first-strand synthesis system according to the manufacturer's protocol (Invitrogen). An equal volume mixture of the products was used as the template for PCR amplification. The primers

for MAO-A were CTGATCGACTTGCTAAGCTAC and ATGCACTGGATGTAAAGCTTC (fragment length, 102 bp). The primers for MAO-B were GCTCTCTGGTTCCTGTGGTATGTG and TCCGCTCACTCACTTGACCAGATC (fragment length, 118 bp). The primers for GAPDH were GACAACAGCCTCAAGATCATCAG and ATGGCATGGACTGTGGTCATGAG (fragment length, 122 bp). Reactions were performed in a 25-μl volume with 200 nM each of forward and reverse primers, and iQ^{TM} SYBR Green Supermix (Bio-Rad) using iCyler and iQ software (Bio-Rad). Each sample was run in triplicate. PCR conditions included an initial denaturation step of 4 min at 95 °C followed by 40 cycles of PCR consisting of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Average threshold cycle (Ct) values from the triplicate PCR reactions for MAO-A or MAO-B were normalized against the average Ct values for GAPDH from the same cDNA sample. -Fold change of MAO transcript levels between SH and SH/PKN cells equals $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{MAO} - Ct_{GAPDH}$, and $\Delta\Delta Ct = \Delta Ct_{SH/PKN} - \Delta Ct_{SH}$.

RESULTS

Parkin Decreases Dopamine Oxidation in the Human Dopaminergic Neuroblastoma Cell Line SH-SY5Y—Our previous studies have shown that overexpression of parkin in the human dopaminergic neuroblastoma cell line SH-SY5Y significantly attenuates dopamine-induced apoptosis by suppressing the production of reactive oxygen species (23). To study the mechanism further, we measured the amounts of dopamine and its metabolites in SH-SY5Y cells (SH) and SH-SY5Y cells stably transfected with wild-type human parkin (SH/PKN). As illustrated in Fig. 1*A*, enzymatic oxidation of DA by MAO produces dopamine aldehydes (3,4-dihydroxyphenylacetaldehyde and 3-methoxy-4-hydroxyphenylacetaldehyde) and hydrogen peroxide. These aldehydes are highly unstable and are quickly converted to DOPAC and HVA by aldehyde dehydrogenase. Only a portion of DOPAC is methylated by catechol *O*-methyltransferase to become HVA. Thus, the amount of DOPAC in the cell is indicative of ROS produced during dopamine oxidation. We performed HPLC analysis of DA and its metabolites in SH and SH/PKN cells treated without or with dopamine (75 μM for 4 h). In the absence of DA treatment, the amount of DOPAC was markedly reduced in SH/PKN cells compared with SH cells. No significant difference was found for DA, HVA, and 3-MT between SH and SH/PKN cells (Fig. 1*B*). The reduction of DOPAC in SH/PKN cells suggests that MAO activities are attenuated when parkin is overexpressed. The lack of significant change in the amount of 3-MT suggests that catechol *O*-methyltransferase is not significantly affected by parkin overexpression.

After dopamine treatment, a significant reduction of DOPAC and HVA and a marked increase of 3-MT were observed in SH/PKN cells compared with SH cells (Fig. 1*C*). These data are consistent with decreased MAO activities in SH/PKN cells, which would result in less dopamine oxidation through the upper pathway and accumulation of 3-MT in the lower pathway of Fig. 1*A*. Taken together, these data suggest that MAO activities are lowered by parkin expression. Dopamine treatment led to a huge increase in DA content in SH/PKN cells compared with SH cells. This could be due to reduced catabolism of dopamine by MAO and/or parkin-mediated increase in dopamine uptake through the dopamine transporter (28).

Overexpression of Parkin in SH-SY5Y Cells Attenuates MAO Activities—To investigate whether decreased dopamine oxidation in SH/PKN cells was due to reduced activities of monoamine oxidases, we measured total MAO activities of cell lysates from SH-SY5Y and SH/PKN cells using [^{14}C]tyramine, a good substrate for both MAO-A and MAO-B (17). Clorgyline, a selective inhibitor of MAO-A, was added in the reaction mix to derive MAO-B activity. In parallel experiments we used pargyline, which specifically blocks MAO-B, to assay the activity of MAO-A. As shown in Fig. 2, total MAO activities as well as those of MAO-A and MAO-B were markedly reduced in SH/PKN cells compared with SH cells ($p < 0.01$, $n = 6$).

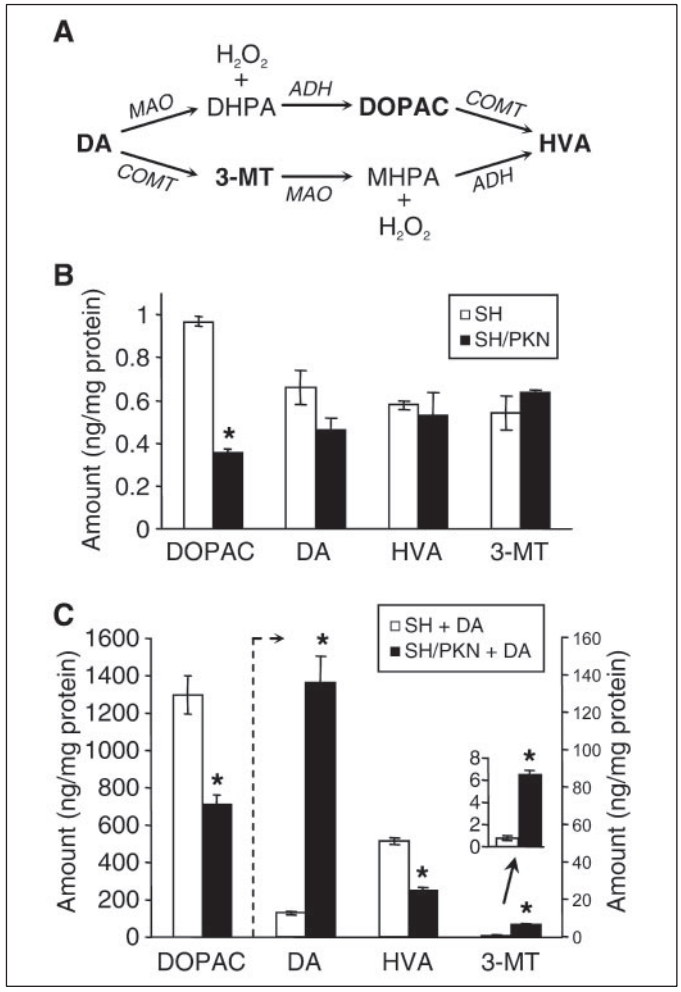

FIGURE 1. **Parkin decreases dopamine oxidation in the human dopaminergic neuroblastoma cell line SH-SY5Y.** *A*, dopamine catabolism is catalyzed by monoamine oxidase in two alternative pathways. *ADH*, aldehyde dehydrogenase; *COMT*, catechol-*O*-methyltransferase. 3,4-Dihydroxyphenylacetaldehyde (*DHPA*) and 3-methoxy-4-hydroxyphenylacetaldehyde (*MHPA*) are unstable and difficult to detect. *B*, basal levels of dopamine and its metabolites in SH-SY5Y cells (*SH*) or SH-SY5Y cells stably expressing parkin (*SH/PKN*). *, $p < 0.05$, SH/PKN *versus* SH, $n = 5$. *C*, the amount of dopamine and its metabolites in SH and SH/PKN cells after dopamine treatment (75 μM for 4 h). The value for DOPAC was according to the *left axis*, whereas those for DA, HVA, and 3-MT were according to the *right axis*. *Inset*, enlarged graph for 3-MT. *, $p < 0.05$, SH *versus* SH/PKN, $n = 5$.

Reduced Expression of MAO in SH-SY5Y Cells Stably Expressing Parkin—The significant reductions in MAO activities could be due to attenuated catalytic activity or decreased protein levels. To distinguish the two possibilities, we carried out binding assays using ^{3}H-labeled clorgyline or deprenyl (specific inhibitors for MAO-A or MAO-B, respectively). These irreversible MAO inhibitors are membrane-permeable; they bind to and covalently inactivate MAOs in the cell. Thus, the maximum amount of bound radioactivity correlates with levels of MAO proteins inside the cell. Both radioligands bound to their target proteins in a dose-dependent and saturable manner (Fig. 3, *A–B*). Much less binding for [^{3}H]clorgyline and [^{3}H]deprenyl was observed in SH/PKN cells than in SH cells at all concentrations tested. Scatchard analyses of data from six independent experiments showed that the maximal amount of MAO-A binding sites for [^{3}H]clorgyline in SH/PKN

cells was 4.65 ± 0.26 pmol/mg of protein, which was significantly less than that in SH cells (12.06 ± 1.32 pmol/mg of protein, $p < 0.05$, Fig. 3*A*). Similarly, maximal binding sites of MAO-B for [^{3}H]deprenyl were significantly reduced from 12.61 ± 1.86 pmol/mg of protein in SH cells to 3.15 ± 0.38 pmol/mg of protein in SH/PKN cells ($p < 0.05$, $n = 6$, Fig. 3*B*).

To confirm these findings, we performed Western blotting of total cell lysates from SH and SH/PKN cells with an antibody that specifically recognized MAO-B (29). As shown in Fig. 3*C*, the amount of MAO-B protein was much less in SH/PKN cells than that in SH cells. As a control we blotted the same cell lysates with an antibody against tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. The levels of tyrosine hydroxylase protein in these cells were very similar (Fig. 3*C*). Because of the lack of a good antibody for human MAO-A protein in SH-SY5Y cells, we were not able to determine the expression level of MAO-A in these cells by Western blotting.

To confirm that parkin-induced reduction in DOPAC was indeed caused by the decrease in MAOs, we transiently transfected SH/PKN cells with an expression construct for MAO-A or MAO-B. After the cells were treated with 75 μM dopamine for 4 h, the amounts of DOPAC and other dopamine metabolites were measured by HPLC. As shown in Fig. 3*D*, expression of exogenous MAO-A or MAO-B significantly attenuated the decrease of DOPAC induced by parkin ($p < 0.05$, $n = 5$). Consistent with these, the amount of HVA was significantly increased by either MAO, whereas the amount of 3-MT was not significantly affected (data not shown). Because SH-SY5Y cells are very difficult to transfect, the effects of transfected MAOs were modest but significant ($p < 0.05$).

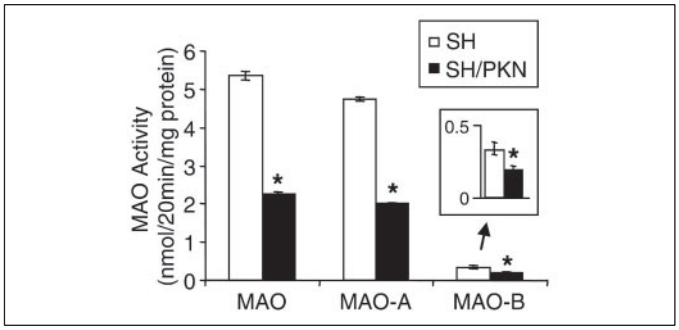

FIGURE 2. **Parkin reduces the activities of monoamine oxidases in SH-SY5Y cells.** Total MAO activities as well as MAO-A and MAO-B activities in lysates from SH or SH/PKN cells were measured as described in the methods. Significant reductions of MAO activities were seen in SH/PKN cells compared with SH cells. *Inset*, enlarged graph for MAO-B. *, $p < 0.01$, $n = 6$.

Parkin Does Not Directly Alter the Expression, Activity, Ubiquitination, and Degradation of Exogenously Expressed MAOs—Because parkin has a protein-ubiquitin E3 ligase activity, one possibility for the decreased levels of MAO proteins may be that parkin ubiquitinates them and facilitates their degradation. To examine this issue, we cloned the cDNAs for MAO-A and MAO-B by RT-PCR using total RNA isolated from SH-SY5Y cells and subcloned them into pCMV-Tag3B, which produced Myc-tagged MAO-A or MAO-B when transfected into HEK293 cells (Fig. 4*A*, *middle panel*). After HEK293 cells were transfected with various combinations of Myc-tagged MAO-A or MAO-B, HA-tagged ubiquitin, and parkin, cleared cell lysates were immunoprecipitated with anti-Myc to pull down MAO-A or MAO-B. Western blotting of precipitated proteins with anti-HA showed that coexpression of HA-tagged ubiquitin with either MAO-A or MAO-B caused significant polyubiquitination of MAO. However, co-transfection of parkin did not further increase MAO ubiquitination. In fact, it reduced the level of ubiquitination a little (Fig. 4*A*, *top panel*). These data suggest that MAO-A and MAO-B are ubiquitinated by another endogenous E3 ligase(s) in HEK293 cells but not by transfected parkin. The slight reduction in MAO ubiquitination in the presence of parkin is perhaps due to the competition between parkin and the unknown E3 ligase(s) for HA-tagged ubiquitin.

The level of MAO-A or MAO-B expression was similar across the lanes in the absence or presence of transfected parkin (Fig. 4*A*, *middle panel*). It suggests that parkin does not significantly affect MAO protein levels when these proteins are produced heterologously under the cytomegalovirus promoter. Thus, decreased expressions of endogenous MAOs in SH/PKN cells is very unlikely to be caused by the ubiquitination of MAO, which was not catalyzed by parkin.

To determine whether parkin directly inhibits the activities of monoamine oxidases, we transfected HEK293 cells without or with MAO-A or MAO-B in combination with parkin or a luciferase expression con-

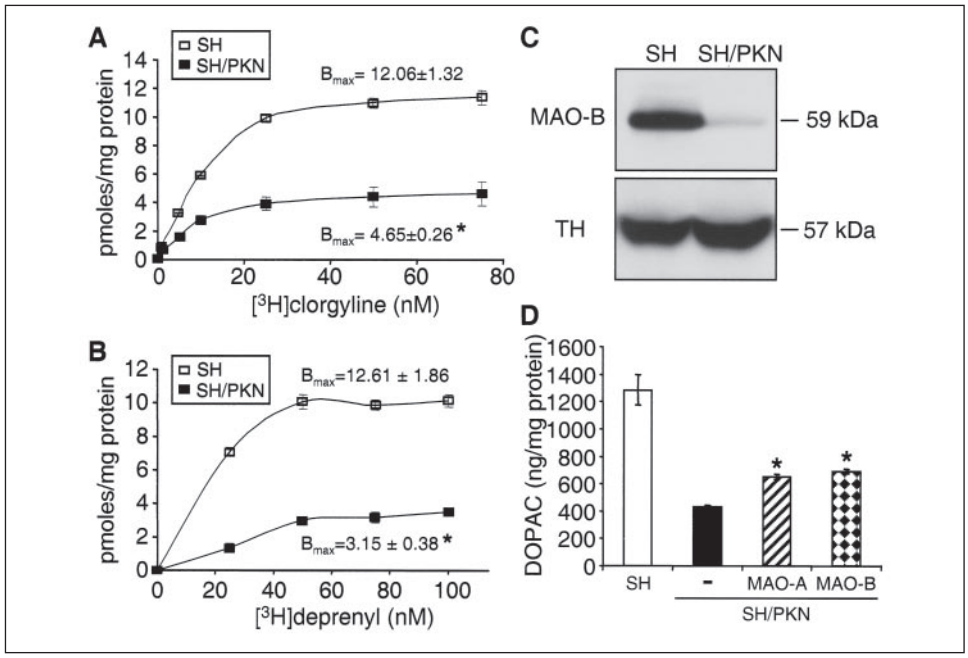

FIGURE 3. **Parkin decreases radioligand binding sites and protein expression level of monoamine oxidases.** *A*, [^{3}H]clorgyline, which binds to MAO-A, had significantly fewer binding sites in SH/PKN cells compared with SH cells. *, $p < 0.05$, $n = 6$. *B*, [^{3}H]deprenyl, which binds to MAO-B, had significantly fewer binding sites in SH/PKN cells compared with SH cells. *, $p < 0.05$, $n = 6$. *C*, Western blots of total lysates from SH or SH/PKN cells with an antibody against MAO-B or tyrosine hydroxylase (*TH*). *D*, SH/PKN cells were transiently transfected with MAO-A or MAO-B. Parkin-induced decrease in DOPAC was significantly reversed by MAO-A or MAO-B. *, $p < 0.05$, $n = 5$.

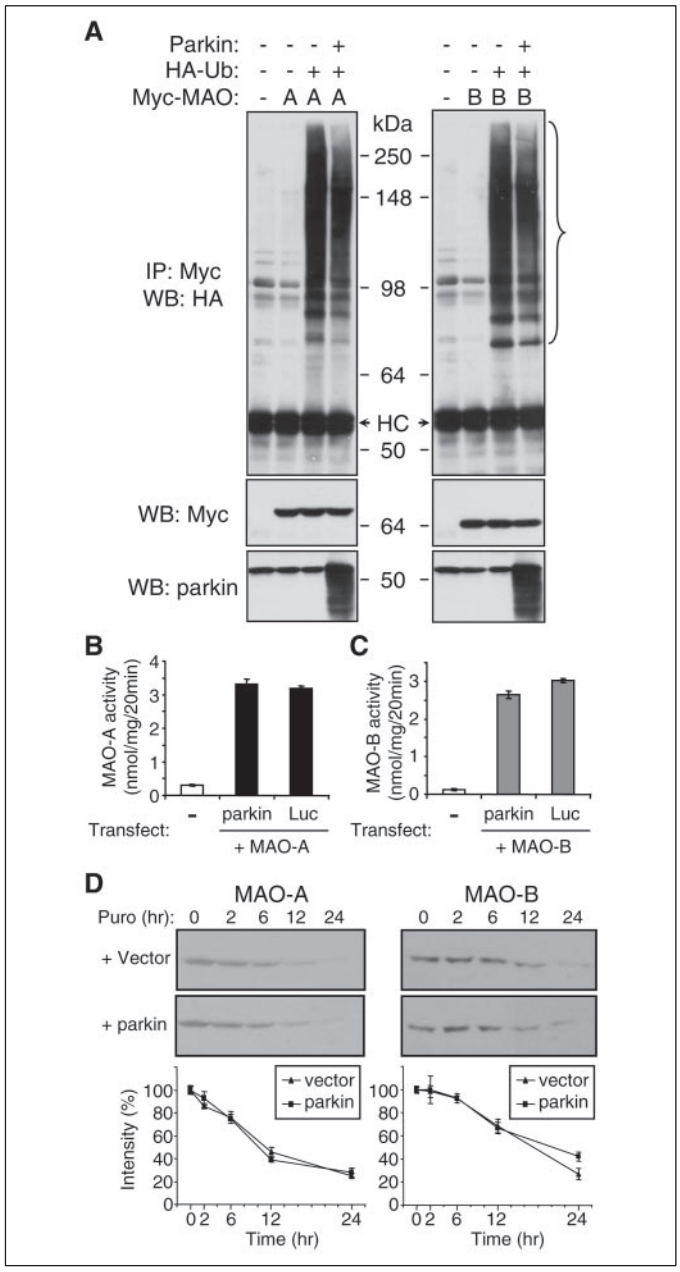

FIGURE 4. **Parkin does not directly alter the expression, activity, ubiquitination, and degradation of exogenously expressed MAOs.** *A*, parkin, HA-tagged ubiquitin (*Ub*), and Myc-tagged MAO-A or MAO-B were co-transfected into HEK293 cells in combinations as indicated. Cell lysates were immunoprecipitated (*IP*) with anti-Myc. Precipitated proteins were blotted (*WB*) with anti-HA to detect MAO ubiquitination (marked by the *bracket*). *HC*, IgG heavy chain. Cell lysates were also blotted with anti-Myc or anti-parkin to show the expression level of MAO-A, MAO-B, or parkin, respectively. *B* and *C*, the activity of transfected MAO-A (*B*) or MAO-B (*C*) in HEK293 cells was not significantly affected by co-transfection of parkin or the luciferase control (*Luc*), $p > 0.05$, parkin *versus* luciferase, $n = 6$. *D*, HEK293 cells co-transfected with Myc-tagged MAO-A or MAO-B as well as parkin or empty vector were treated 48 h later with puromycin (100 μM) for various durations to inhibit new protein synthesis. Western blotting with anti-Myc showed that degradation of MAO-A or MAO-B in the presence of parkin or the empty vector was not significantly different. Results from three independent experiments were quantified in the graph ($p > 0.05$ for all time points).

struct as control. MAO-A activity was very similar in HEK293 cells co-transfected with MAO-A and parkin or MAO-A and luciferase (Fig. 4*B*, $p > 0.05$, $n = 6$). It suggests that coexpression of parkin with MAO-A does not directly change the catalytic activity of MAO-A. A similar result was obtained for MAO-B (Fig. 4*C*, $p > 0.05$, $n = 6$).

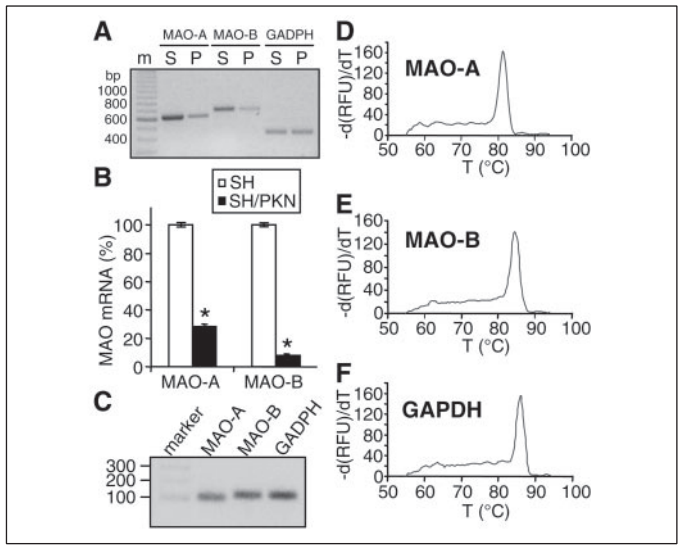

FIGURE 5. **Parkin decreases the mRNA levels of monoamine oxidases.** *A*, RT-PCR amplification of MAO-A (612 bp), MAO-B (695 bp), or GAPDH (400 bp) fragments using total RNA isolated from SH or SH/PKN cells. Image of the gel was in inverted pixels to show the bands more clearly. *m*, marker; *S*, SH-SY5Y cells; *P*, SH/PKN cells. *B*, the relative amount of MAO-A or MAO-B mRNA as measured by real-time quantitative RT-PCR normalized against GAPDH. *, $p < 0.001$, SH *versus* SH/PKN, $n = 5$. *C–F*, agarose gel electrophoresis (*C*) and melting curves of real-time quantitative RT-PCR products for MAO-A (*D*), MAO-B (*E*), and GAPDH (*F*) showed that only a single species was amplified for each gene. *RFU*, relative fluorescence units.

Furthermore, we examined whether the degradation of MAO-A or MAO-B was affected by parkin. HEK293 cells were transfected with Myc-tagged MAO-A or MAO-B together with parkin or the empty vector. Cells were treated with the protein synthesis inhibitor puromycin (100 μM) for various durations. Degradation of transfected MAO-A or MAO-B was followed by Western blotting with anti-Myc. As shown in Fig. 4*D*, degradation of either MAO in the presence of parkin or the empty vector was not significantly different ($p > 0.05$ for all time points examined, $n = 3$). Together, these data indicate that parkin does not directly alter the expression, activity, ubiquitination, and degradation of exogenously expressed monoamine oxidases.

Parkin Decreases the mRNA levels of MAO-A and MAO-B—After ruling out a direct effect of parkin on MAOs at the protein level, we compared the amount of mRNA for MAO-A and MAO-B in SH and SH/PKN cells. A simple RT-PCR analysis of total RNA isolated from the two cell lines showed that both MAO-A and MAO-B were expressed at much lower levels in SH/PKN cells compared with SH cells. In contrast, expression of the housekeeping gene GAPDH was unchanged (Fig. 5*A*). To confirm this we measured the amounts of mRNA for MAO-A and MAO-B by real-time quantitative RT-PCR. Levels of MAO transcripts were normalized against those of GAPDH in the same cell line, which were virtually identical between SH and SH/PKN cells. In contrast, the expression level of MAO-A in SH/PKN cells was 28.8 ± 1.2% that in SH cells, whereas MAO-B transcripts in SH/PKN cells were reduced to 8.3 ± 0.3% that of those in SH cells (Fig. 5*B*). These significant reductions ($p < 0.001$, $n = 5$) in MAO expression are consistent with decreased MAO activities (Fig. 2), binding sites, and protein levels (Fig. 3) in SH/PKN cells compared with SH cells.

When these real-time PCR products were separated on an agarose gel, only one band at the correct size was observed for each gene (Fig. 5*C*). This was corroborated by melting curves of the real-time PCR products, which showed that only one species of amplicon was produced for MAO-A, MAO-B, or GAPDH (Fig. 5, *D–F*).

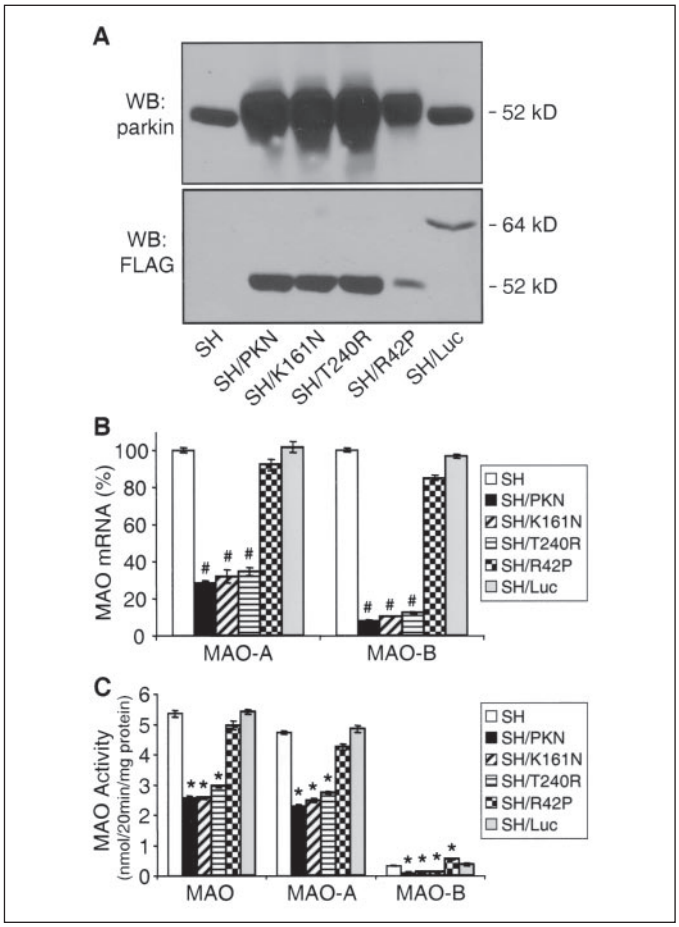

FIGURE 6. **PD-linked mutations of parkin differentially affect its ability to suppress MAO expression.** *A*, total cell lysates from SH-SY5Y cells (*SH*) and SH-SY5Y cells stably expressing FLAG-tagged wild-type parkin (*PKN*), K161N, T240R, or R42P mutant parkin or luciferase (*Luc*) were blotted (*WB*) with anti-parkin or anti-FLAG to show expression levels of total parkin and FLAG-tagged parkin constructs respectively. *B*, the relative amount of MAO-A or MAO-B mRNA as measured by real-time RT-PCR in these stable cell lines. #, $p < 0.001$, *versus* SH, $n = 5$. *C*, total MAO activities as well as MAO-A and MAO-B activities in lysates from these cell lines. *, $p < 0.01$, *versus* SH, $n = 6$.

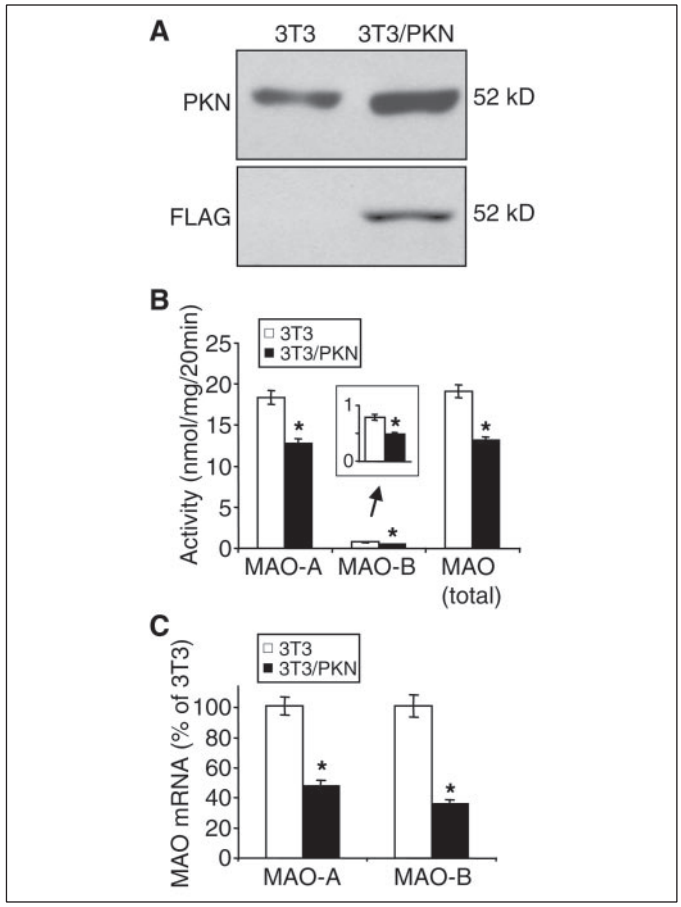

FIGURE 7. **Parkin decreases MAO expression and activities in the mouse fibroblast cell line NIH3T3.** *A*, NIH3T3 cell line stably expressing human wild-type parkin was generated (3T3/PKN). Total lysates from 3T3 and 3T3/PKN were blotted with anti-parkin or anti-FLAG to compare the level of endogenous and transfected parkin. *B*, total MAO activities as well as MAO-A and MAO-B activities in lysates from 3T3cells were significantly reduced by overexpression of parkin. *Inset*, enlarged graph for MAO-B. *, $p < 0.05$, *versus* 3T3, $n = 8$. *C*, the relative amount of MAO-A or MAO-B mRNA as measured by real-time quantitative RT-PCR. *, $p < 0.05$, *versus* 3T3, $n = 8$.

PD-linked Mutations of Parkin Differentially Impact Its Ability to Suppress MAO Expression—To examine the effects of PD-linked mutations of parkin on its ability to attenuate the expression of MAO, we used our previously generated SH-SY5Y cell lines that were stably transfected with FLAG-tagged K161N, T240R, or R42P mutant parkin or luciferase (23). Expression levels of total parkin as well as transfected parkin constructs were monitored by Western blotting with anti-parkin or anti-FLAG, respectively (Fig. 6*A*).

Real-time quantitative RT-PCR amplification of total RNA from these cell lines showed that the levels of MAO-A and MAO-B transcripts were greatly reduced in SH/K161N and SH/T240R cells compared with the parental SH-SY5Y cells (Fig. 6*B*). The degrees of reduction were similar to that in SH/PKN cells. In contrast, there was no significant decrease in the mRNA level of MAO-A or MAO-B in SH/R42P cells. The control cell line SH/Luc behaved as the parental SH-SY5Y cells, indicating that the process of generating a stable cell line did not cause the change.

We treated SH or SH/PKN cells with lactacystin (1 μM for 12 h) and examined MAO expression by quantitative real-time RT-PCR. Lactacystin reduced MAO-A expression to 64.8 ± 9.3% that of the original amount in SH cells and to 73.9 ± 7.5% that of the basal level in SH/PKN cells. On the other hand, lactacystin decreased MAO-B expression to 37.5 ± 6.4 or 41.5 ± 6.2% of the untreated level in SH or SH/PKN cells, respectively. Thus, proteasome inhibition exerted similar influences on MAO expression in SH and SH/PKN cells ($p > 0.05$, $n = 8$), which suggests that the effect of parkin on MAO expression is not affected by proteasome inhibition.

We also measured total MAO activities as well as that of MAO-A or MAO-B in these cell lines. As shown in Fig. 6*C*, the K161N or T240R mutant behaved like wild-type parkin in decreasing MAO activities. R42P had no significant effect on total MAO activities or MAO-A activity but had a slight and significant increase in MAO-B activity compared with SH or SH/Luc cells. Thus, the data on MAO activities are largely in agreement with its expression levels.

The Effect of Parkin on MAO Is Replicated in the Mouse Fibroblast Cell Line NIH3T3—To confirm our findings on the ability of parkin to suppress MAO expression, we stably transfected the mouse fibroblast cell line NIH3T3 with human wild-type parkin. This cell line (3T3/PKN) expressed a much higher level of FLAG-parkin compared with endogenous parkin in the parental NIH3T3 cells (Fig. 7*A*). We compared total MAO activities as well as that of MAO-A or MAO-B in 3T3 and 3T3/PKN cells. As shown in Fig. 7*B*, parkin overexpression in 3T3 cells significantly reduced total MAO activities and that of MAO-A or MAO-B ($p < 0.05$, $n = 8$). To examine whether MAO expression was attenuated, we performed real-time quantitative RT-PCR amplification

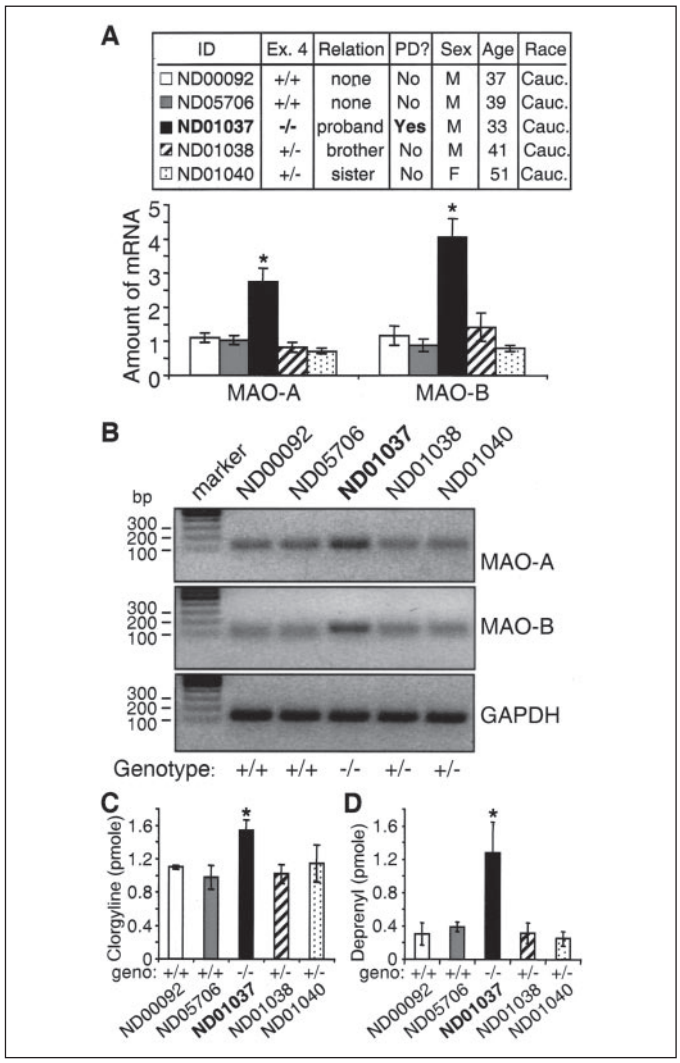

FIGURE 8. **MAO expression is significantly increased in human PD patients with homozygous deletion of parkin exon 4.** *A*, B lymphocyte cell lines derived from two normal controls and a family with exon 4 deletion in parkin were used to measure the amounts of MAO-A and MAO mRNA by quantitative real-time RT-PCR. GAPDH was used as an internal control, which showed no significant variation. Data for each MAO were normalized against the unaffected control ND00092, respectively. *, $p < 0.01$, *versus* ND00092, $n = 8$. *Cauc.*, Caucasian. *B*, products of the above real-time RT-PCR reactions were separated on gels and showed that the amounts of MAO-A and MAO-B amplicons were markedly increased in ND01037 compared with the other four samples, which had similar levels of expression. The image of the gel was in inverted pixels to show the bands more clearly. *C* and *D*, radioligand binding assays using [^{3}H]clorgyline (*C*) and [^{3}H]deprenyl (*D*) showed that the amount of MAO-A (*C*) or MAO-B (*D*) binding sites was significantly increased only in ND01037. *, $p < 0.05$, *versus* ND00092, $n = 6$ (*C*) or $n = 5$ (*D*).

of MAO-A, MAO-B, and GAPDH transcripts using total RNA isolated from both cell lines. Utilizing GAPDH as an internal control, which did not show any significant change, we found that mRNA levels of MAO-A and MAO-B were significantly decreased in 3T3/PKN cells compared with those in 3T3 cells ($p < 0.05$, $n = 8$). Together, these results suggest that overexpression of parkin suppresses MAO expression also in NIH3T3 cells.

MAO Expression Is Significantly Increased in PD Patients with Homozygous Deletion of Parkin Exon 4—To further substantiate the observation that parkin regulated MAO expression, we used B lymphocyte cell lines derived from a family with deletion of exon 4 of parkin (24). As shown in Fig. 8*A*, the PD patient ND01037 is a homozygous carrier of the mutation, whereas his unaffected brother (ND01038) and unaffected sister (ND01040) are heterozygotes (24). We also used B lymphocyte cell lines derived from two unrelated and unaffected controls (ND00092 and ND05706). Quantitative real-time RT-PCR amplification of MAO-A and MAO-B from these cells showed that the amounts of MAO-A and MAO-B mRNA were significantly increased in the homozygous carrier of parkin exon 4 deletion compared with normal controls ($p < 0.01$, $n = 8$). In contrast, neither heterozygous carrier exhibited any significant change in MAO expression (Fig. 8*A*). The housekeeping gene GAPDH was used as an internal control, which had very similar expression levels in these cells. When products of these real-time RT-PCR reactions were separated on agarose gels, it was obvious that MAO-A and MAO-B expression was markedly increased only in ND01037 compared with the rest, which were at similar levels (Fig. 8*B*).

To provide another independent measurement of MAO levels, we performed radioligand binding assays on these cells using [^{3}H]clorgyline and [^{3}H]deprenyl. As shown in Fig. 8*C*, the amount of MAO-A binding sites for clorgyline was significantly increased in ND01037 compared with the two normal controls ($p < 0.05$, $n = 6$). Both heterozygotes (ND01038 and ND01040) showed no significant change. Similarly, the homozygote (ND01037) had significantly more MAO-B binding sites for deprenyl ($p < 0.05$, $n = 5$), whereas the heterozygotes exhibited no significant change (Fig. 8*D*). Thus, these two different readouts confirmed each other and suggest that MAO expression is significantly increased in the PD patient with homozygous deletion of parkin exon 4.

DISCUSSION

Mutations of parkin are linked to Parkinson disease and its pathological hallmark, the selective degeneration of dopaminergic neurons in substantia nigra (30). Despite intensive research efforts in the past few years, it remains unclear how mutations of parkin lead to the demise of nigra DA neurons and ensuing PD symptoms. On the other hand, many lines of evidence strongly suggest that oxidative stress stemming from dopamine oxidation may play a critical role in the selectivity of the neurodegeneration process in PD (15). Our previous study has shown that parkin attenuates dopamine toxicity on human dopaminergic neuroblastoma cell line SH-SY5Y by suppressing the production of reactive oxygen species (23). In the present study we showed that parkin reduced the expression of monoamine oxidases, enzymes responsible for the oxidative deamination of dopamine.

Although parkin has a protein-ubiquitin E3 ligase activity toward many substrates (31), the ability of parkin to suppress MAO expression does not appear to be dependent on its E3 ligase activity. Parkin did not increase the ubiquitination of MAO-A or MAO-B, which was very efficiently ubiquitinated by endogenous E3 ligase(s) in HEK293 cells in the absence of transfected parkin (Fig. 4*A*). Consistent with this, parkin did not have any significant effect on the steady-state level of exogenous MAO-A or MAO-B co-transfected in HEK293 cells (Fig. 4*A*). It suggests that parkin does not exert its effect on MAOs at the protein level. This is corroborated by the results that parkin did not change the activity of MAO-A or MAO-B exogenously expressed in HEK293 cells (Fig. 4, *B* and *C*). Furthermore, the degradation rates of MAO-A and MAO-B proteins were not significantly affected by co-expression of parkin in HEK293 cells (Fig. 4*D*).

Rather intriguingly, parkin strongly decreased the amounts of mRNA for MAO-A and MAO-B (Figs. 5 and 7). This effect of parkin was not significantly affected by its K161N and T240R mutations but was abrogated by its R42P mutation (Fig. 6). The former two mutations are known to inactivate the E3 ligase activity of parkin on many substrates (9, 32, 33), whereas the latter mutation reduces the stability of parkin

(23, 34) and its association with the Rpn10 subunit of the 26 S proteasome (35). The lack of a significant effect for the R42P parkin mutant may be linked to its poor expression (23) and/or increased tendency to form aggregates in the cell (36). Together, these results suggest that parkin regulates the levels of MAO transcripts through a mechanism independent of its E3 ligase activity.

The effect was observed in two totally different cell lines, the human dopaminergic neuroblastoma cell SH-SY5Y (Fig. 5) and the mouse fibroblast cell NIH3T3 (Fig. 7). It was also confirmed from the opposite direction in B lymphocyte cell lines derived from PD patients with exon 4 deletion of parkin (Fig. 8). Homozygous, but not heterozygous deletion of parkin led to a significant increase in MAO expression and MAO binding sites (Fig. 8). Previous studies have shown that lymphocytes produce and degrade endogenous catecholamines including dopamine (37). Our results are in agreement with recent studies that show an increased level of DOPAC (38) and elevated MAO-B activity (39) in parkin-deficient mice compared with wild-type controls. The differential effects of PD-linked mutations of parkin on its ability to suppress MAO expression suggest that the effect alone does not explain the causative link between parkin mutations and PD. Other factors, including those dependent on the E3 ligase activity of parkin, must also contribute to the protective function of parkin.

It is unclear how parkin decreases MAO expression at the RNA level. One possibility is that parkin may suppress the transcription of MAO in a manner analogous to the regulation of p53 by parc (14). parc is p53-associated, parkin-like cytoplasmic E3 ligase that also has the RING-IBR-RING motif at the C terminus. By binding to rather than ubiquitinating p53, parc tethers this transcription factor in the cytosol to keep it away from the transcriptional machinery in the nucleus (14). Previous studies have also shown that p53 is anchored in the cytoplasm through interaction with microtubules (40). It seems likely that parc and microtubules may act in a concerted manner to sequester a portion of p53 in the cytoplasmic pool, away from its transcriptional activities in the nucleus (41). Our previous studies have found that parkin tightly binds to microtubules (9) through strong, redundant interactions mediated by its linker, RING1 and RING2 domains (10). One can envision a scenario where a transcription activator of MAO may be anchored in the cytosol by parkin through the strong association between parkin and microtubules. Overexpression of parkin would, thus, lead to increased sequestration of this transcription factor and reduced expression of MAO. Although we do not have solid evidence at present to support this model, our ongoing studies using microarrays showed that overexpression of parkin induced changes in gene expression (including MAOs) in a coordinated manner. It suggests that parkin may have a role in transcription regulation.

On the other hand, several studies suggest that parkin is involved in mitochondrial functions. In parkin-deficient *Drosophila*, abnormal mitochondrial morphology is seen in indirect flight muscle and spermatids (42, 43). Increased expression of certain mitochondrial proteins (44) and elevated oxidative stress (43) are observed in these flies. Consistent with these, parkin-deficient mice exhibit alterations in the levels of mitochondrial proteins and increased peroxidation of proteins and lipids (45). Furthermore, parkin has been shown to be associated with mitochondria and may interact with an unknown target that is critical for mitochondrial functions (46). Both MAO-A and MAO-B are located on the cytoplasmic side of the mitochondrial outer membrane through a C-terminal tail anchored in the membrane (47, 48). MAO-catalyzed oxidative deamination of monoamines produces H_2O_2 that has been shown to damage mitochondrial DNA (49). By controlling the mRNA level of MAOs, parkin may limit the amount of MAOs anchored on the mitochondrial outer membrane and restrict the impact of reactive oxygen species on mitochondria. Thus, the action of parkin on MAO expression is consistent with previous studies that implicate the involvement of parkin in mitochondrial functions. Through this action, parkin could protect DA neurons, since inhibition of MAO activities by drugs such as deprenyl (selegiline) is effective in delaying the progression of PD symptoms (50).

Converging lines of evidence have indicated that mitochondrial dysfunction is a key factor in the selective degeneration of DA neurons in Parkinson disease (51). On the other hand, oxidative stress induced by dopamine oxidation plays a critical role in the specific death of dopaminergic neurons (52). The ability of parkin to suppress the expression of monoamine oxidases, mitochondrial enzymes responsible for the oxidative catabolism of dopamine, may provide a possible link between mitochondrial dysfunction and dopamine-induced oxidative stress. Together with its E3-dependent functions, the effect of parkin on MAO expression, which appears to be independent of its E3 ligase activity, may contribute to the selective degeneration of dopaminergic neurons and Parkinson disease.

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