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Stress-induced alterations in parkin solubility promote parkin aggregation and compromise parkin's protective function

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Mutations in parkin are currently recognized as the most common cause of familial Parkinsonism. Emerging evidence also suggests that parkin expression variability may confer a risk for the development of the more common, sporadic form of Parkinson's disease (PD). Supporting this, we have recently demonstrated that parkin solubility in the human brain becomes altered with age. As parkin apparently functions as a broad-spectrum neuroprotectant, the resulting decrease in the availability of soluble parkin with age may underlie the progressive susceptibility of the brain to stress. Interestingly, we also observed that many familial-PD mutations of parkin alter its solubility in a manner that is highly reminiscent of our observations with the aged brain. The converging effects on parkin brought about by aging and PD-causing mutations are probably not trivial and suggest that environmental modulators affecting parkin solubility would increase an individual's risk of developing PD. Using both cell culture and *in vivo* models, we demonstrate here that several PD-linked stressors, including neurotoxins (MPP+, rotenone, 6-hydroxydopamine), paraquat, NO, dopamine and iron, induce alterations in parkin solubility and result in its intracellular aggregation. Furthermore, the depletion of soluble, functional forms of parkin is associated with reduced proteasomal activities and increased cell death. Our results suggest that exogenously introduced stress as well as endogenous dopamine could affect the native structure of parkin, promote its misfolding, and concomitantly compromise its protective functions. Mechanistically, our results provide a link between the influence of environmental and intrinsic factors and genetic susceptibilities in PD pathogenesis.

INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder characterized pathologically by the rather selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the presence of intraneuronal protein inclusions known as Lewy bodies (LB). Although the molecular mechanism of dopaminergic neuronal loss in the more common, sporadic form of PD is not well understood, the recent focus on the study of genes linked to rare inherited forms of PD has yielded significant insights into the pathogenesis of sporadic PD (1–3). In particular, the linkage of genetic mutations in the parkin gene to familial Parkinsonism and the seminal discovery that parkin functions

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as a ubiquitin ligase associated with protein homeostasis have helped position the ubiquitin proteasome system as a key focal point in PD pathogenesis (1–4).

Mutations in parkin are currently recognized as one of the most common cause of familial Parkinsonism. To date, the descriptions of parkin-related PD include patients with homozygous mutations, compound heterozygous mutations as well as those with a single mutation (5). Although PD due to parkin mutations is classically transmitted in an autosomal recessive inheritance, the existence of patients with single mutations raises the possibility of an expanded risk associated with parkin haploinsufficiency (5–7). Supporting this possibility is the recent association of parkin gene promoter with late-onset PD (8). Parkin variability could thus be considered as a risk factor for the development of PD.

The importance of parkin expression to neuronal survival is probably related to the multitude of neuroprotective roles it appears to serve (9). Parkin apparently confers protection to neurons against a diversity of cellular insults including manganese-induced cell death (10), α-synuclein toxicity (11), proteasomal dysfunction (12), Pael-R (13) and p38/JTv-1 (14) accumulation and kainate-induced excitotoxicity (15). Additionally, parkin also confers neuronal resistance towards stimuli that promote mitochondria-dependent apoptosis and dopamine-mediated toxicity (16,17). Given the multipurpose neuroprotective roles of parkin, it is conceivable that any depletion in the level or activity of parkin would significantly compromise neuronal survivability. Indeed, familial PD-linked mutations of parkin frequently lead to a reduction or ablation of parkin catalytic competency (18,19). Similarly, the inhibition of parkin activity by BAG5 enhances dopamine neuron death in an *in vivo* model of PD (20). Conversely, animals with overexpressed parkin have reduced α-synuclein-induced neuronal pathology compared with normal control animals (21,22).

Recently, we demonstrated that parkin solubility in the brain becomes altered with age (23). As parkin appears to function as a broad-spectrum neuroprotectant, the resulting increase in parkin insolubility with age may underlie the progressive susceptibility of the neurons to stress. Interestingly, we also observed that many familial PD-linked mutations of parkin produce alterations in its solubility in a manner that is highly reminiscent of our observations with the aged brain (24,25). The converging effects on parkin brought about by aging and PD-causing mutations are probably not trivial and suggest that age-dependent modulators affecting the parkin protein in the same way caused by clinically relevant parkin mutations would increase an individual's risk of developing PD.

Here we used both cellular and animal models to investigate the effects of various environmental and intrinsic factors that are linked to PD on parkin solubility. We found that several of these stressors induce alteration in parkin solubility and result in its intracellular aggregation. Furthermore, the depletion of soluble, functional forms of parkin is associated with reduced proteasomal activities and increased cell death. Interestingly, we also observed variations in parkin distribution in post-mortem human PD brains relative to normal controls, which provide further support to our findings. Taken together, our results suggest that conditions promoting parkin insolubility compromise parkin's protective function and predispose the neurons to degeneration when they are exposed to continual stress.

RESULTS

Various PD-linked stressors induce changes in parkin solubility

To facilitate our investigation on the effects of exogenous modulators on parkin solubility, we have generated SH-SY5Y neuroblastoma cells stably expressing FLAG-tagged parkin (SH-Parkin) or containing vector alone as a control (SH-Vector). Three individual parkin-positive clones (numbers 1, 2 and 6) were selected for our experiments to minimize clonal variation. All of these parkin-positive clones express parkin at a much higher level when compared with vector control or parental cells but are otherwise similar morphologically (Fig. 1A and B). Using the sequential fractionation method described in Materials and Methods, we observed that the majority of the expressed FLAG-parkin resides in the detergent-soluble (S) fractions (Fig. 1C).

As the treatment of cells with an acute dose (20 mM) of hydrogen peroxide (H_2O_2) has previously been shown by Winklhofer *et al.* (26) to induce parkin to adopt a detergent-insoluble conformation, we initially performed the earlier mentioned experiment with our parkin stable cell lines to see if we could reproduce the observed effect on parkin. Consistent with Winklhofer's finding, we observed a significant shift in parkin extractibility towards the detergent-insoluble (P) fractions following H_2O_2-induced oxidative stress (Fig. 1C). We also treated the cells with lower concentrations of H_2O_2 (2–10 mM) and recorded essentially the same observations (data not shown). Interestingly, treatment of cells with the proteasome inhibitor, MG-132, led to a similar shift in parkin extractibility (Fig. 1C). Thus, both oxidative and proteolytic stress appear to be capable of modifying parkin solubility.

We have recently described the S-nitrosylation of parkin in sporadic PD (27). To examine whether nitrosative stress induces a similar effect on parkin solubility observed earlier, we treated SH-Parkin cells with NOC-18, an NO-donor we have previously described to be effective in bringing about the S-nitrosylation of parkin (27). Although the treatment of SH-Parkin cells with 100 μM of NOC-18 for 24 h did not significantly alter its solubility (data not shown), the amount of detergent-soluble parkin showed a dramatic relocalization to the P-fractions when a higher amount (500 μM) of NOC-18 was used to treat the cells (Fig. 1D). NO assays performed with 2,3-diaminonapthalene revealed that parkin is S-nitrosylated more prominently in cells treated with 500 μM NOC-18 than those treated with 100 μM NOC-18 (Fig. 1E). These results suggest that the S-nitrosylation of parkin could alter the protein solubility in a manner that mirrors that induced by H_2O_2 and MG-132.

We next examined the effects of treating the parkin stable cell lines with the parkinsonian neurotoxins, MPP+, rotenone and 6-hydroxydopamine (6-OHDA), because these agents are well known to trigger oxidative stress and selective

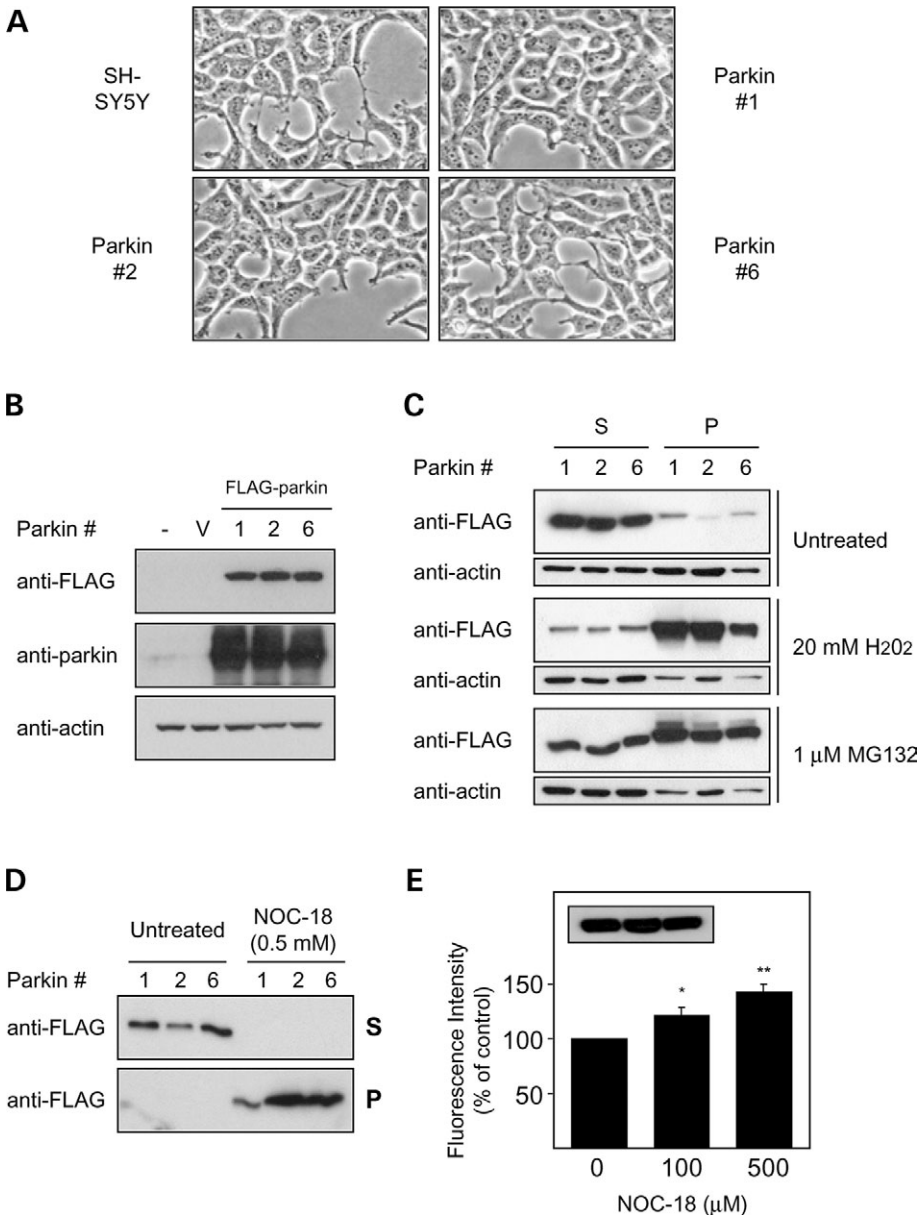

Figure 1. Effects of oxidative, proteolytic and nitrosative stress on parkin stable cell lines. (**A**) Phase-contrast images of normal SH-SY5Y neuroblastoma cells and three individual clones stably expressing FLAG-tagged parkin (nos 1, 2 and 6) showing no apparent difference in their morphology. (**B**) Anti-FLAG and anti-parkin immunoblots of total cell lysates prepared from normal SH-SY5Y (−), SH-Vector (V) and the parkin stable clones (nos 1, 2 and 6). (**C** and **D**) Anti-FLAG immunoblots of cell extracts sequentially prepared with Triton X-100 (S) and SDS (P) from untreated parkin stable cell lines or those treated either with 20 mM H_2O_2 (30 min), 1 μM MG-132 (16 h) or 0.5 mM NOC-18 (24 h) as indicated. Equal loading of the different cell lysates was verified by anti-β-actin immunoblotting. (**E**) Bar graph showing S-nitrosylated parkin as detected by a fluorometric method described by Chung *et al*. (27). Anti-FLAG immunoblot (inset) shows equivalent amount of FLAG-parkin immunoprecipitated. These results represent mean ± SEM of five separate experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ versus control group (one-way ANOVA).

dopaminergic neuronal death in injected animals. Not surprisingly, treatment of the SH-Parkin cells with all the three neurotoxins resulted in a significant population of parkin being shifted to the P-fractions (Fig. 2A). These neurotoxin-mediated alterations in parkin solubility appear to be dose- and time-dependent. In all three cases, a progressive shift in parkin extractibility from the S- to the P-fractions was apparent with increasing dose of the neurotoxic treatment (Fig. 2B) (data not shown for 6-OHDA). Although the solubility of parkin was relatively unaffected at lower doses of these neurotoxins, prolonging the treatment at these doses for a few days likewise led to a shift in parkin extractibility towards detergent insolubility (Supplementary Material, Fig. S1) (Fig. 4C).

Expectedly, we observed a similar alteration in parkin solubility, as previously described, when the stable cell lines were treated with the PD-linked herbicide, paraquat, as well as with the metal, iron (Fe^{2+}) (Fig. 2C). Further, we also examined the

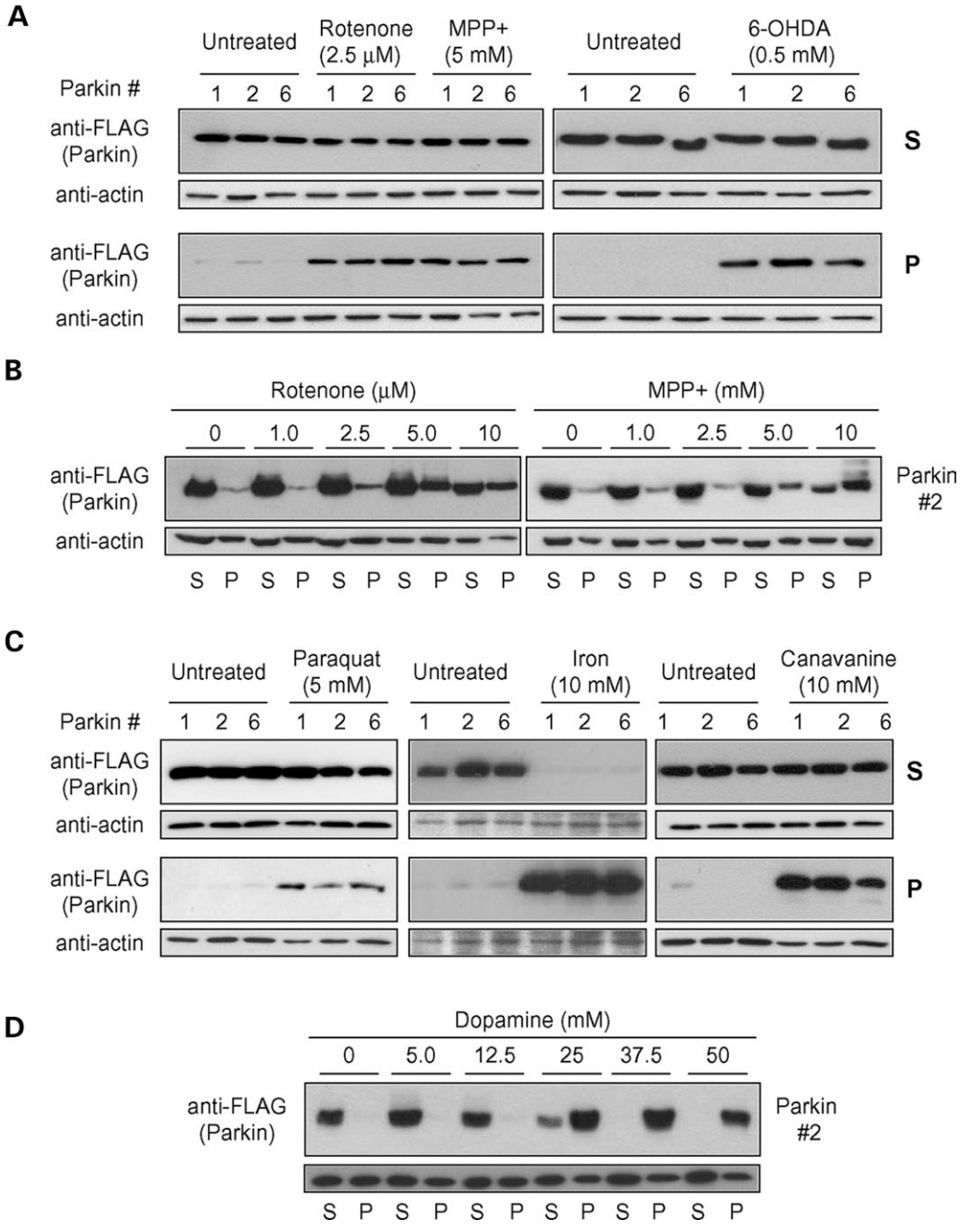

Figure 2. Stress-induced alterations in parkin solubility. Anti-FLAG immunoblots of cell extracts sequentially prepared with Triton X-100 (S) and SDS (P) from untreated parkin stable cell lines or those treated with the indicated doses of (**A**) rotenone (24 h), MPP+ (24 h), 6-OHDA (3 h). (**C**) Paraquat (24 h), iron and canavanine (16 h). (**B** and **D**) Anti-FLAG immunoblots of cell extracts similarly prepared showing a dose-dependent shift in parkin solubility towards the P-fractions following rotenone, MPP+ and dopamine treatment (20 h), as indicated. Equal loading of the different cell lysates was verified by anti-β-actin immunoblotting. Each of these experiments was repeated at least three times.

effects of dopamine treatment on parkin solubility properties because dysregulated dopamine homeostasis is known to cause significant endogenous stress (28). Consistent with our earlier observations, the translocation of soluble parkin into the detergent-insoluble fractions following dopamine treatment of SH-Parkin cells occurs in a dose-dependent manner (Fig. 2D). Taken together, our results suggest that various forms of cellular stress, originating both exogenously and endogenously, could modify parkin structure and lead to its misfolding, thereby altering the protein solubility. Supporting this, we could reproduce our earlier observations by treating the SH-Parkin cells with canavanine (29), an amino acid analog that promotes protein misfolding by substituting for arginine during translation (Fig. 2C). Presumably, misfolded parkin aggregates and becomes resistant to normal detergent extraction.

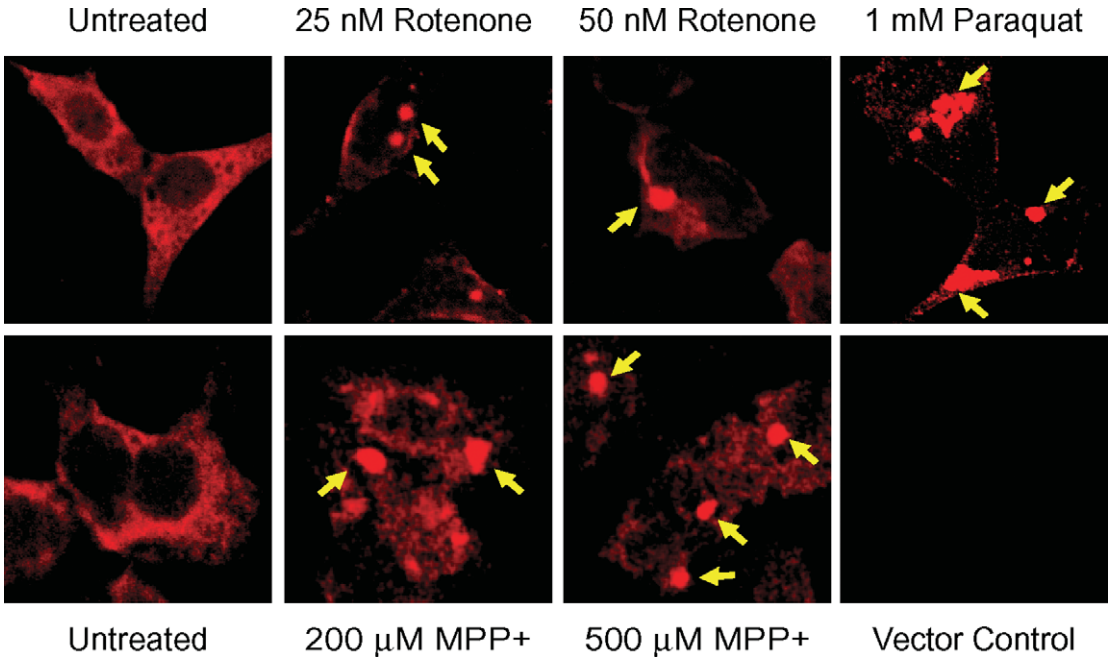

Figure 3. Stress-induced alterations in parkin solubility promote the formation of intracellular parkin-positive inclusions. Representative confocal images of anti-FLAG immunostained (red) parkin stable cells showing uniform cytoplasmic staining in untreated cells and FLAG-positive inclusions (arrows) in cells treated with the various stressors, as indicated. Anti-FLAG immunostained SH-Vector controls cells showed no background staining.

Stress-induced alterations in parkin solubility promote parkin aggregation

To investigate if stress-induced alteration in parkin solubility is associated with its propensity to form intracellular aggregates, we examined the localization of FLAG-parkin in untreated SH-Parkin cells as well as in cells treated with rotenone, MPP+ and paraquat. As the percentage of cell death is markedly increased at acute doses of these toxins, we performed our localization studies here with lower doses of these stressors in order to reduce the amount of cell death. Consistent with our previous report (24,25), we found that parkin in untreated cells is uniformly expressed throughout the cytoplasm (Fig. 3). In SH-Parkin cells treated with the parkinsonian neurotoxins rotenone and MPP+ at their respective dose that would produce alteration in parkin solubility (Supplementary Material, Fig. S1) (Fig. 4C), we observed prominent parkin aggregates within the cell, which accumulate in the perinuclear region (Fig. 3). Similarly, treatment of SH-Parkin cells with paraquat produced FLAG-parkin positive inclusions. These results are reminiscent of our previous observation that parkin mutations that alter the protein solubility tend to trigger its intracellular aggregation (24,25). Our results here show that stress-induced alteration in parkin solubility similarly results in parkin's tendency to aggregate within the cell.

Stress-induced alterations in parkin solubility compromise parkin's protective function

It is conceivable that the changes in parkin solubility triggering its aggregation could deplete the cell of soluble functional parkin and concomitantly compromise parkin's protective function. To enable us to ascertain this, we treated the SH-Parkin and control cells with the lipid-soluble neurotoxin, rotenone, to first establish parkin's potential protective effects towards this neurotoxic insult. For this experiment, we treated the cells with low doses of rotenone to better recapitulate the slow progression of dopaminergic neuronal death in PD. After 6 days of chronic rotenone treatment, especially at the 25 and 50 nM doses, we noticed a dramatic morphological difference between the SH-Parkin and control cells (Fig. 4A). Although a significant population of the SH-Vector cells at these doses of rotenone treatment became rounded and reflective, i.e. indicative of dying cells, it is apparent that the SH-Parkin cells were relatively spared of these features (Fig. 4A). Quantitative measurement of cell death at this time point by means of flow cytometry (Fig. 4B) and Trypan blue exclusion method (data not shown) correlates well with our morphological observations. Although we observed some cell death (20%) in the untreated control cells after 6 days of culture, a significantly higher average percentage of cell death was evident when these cells were treated with 25 (50%) and 50 nM rotenone (60%) (Fig. 4B). Cells expressing exogenous parkin recorded a dramatically better survival rate at these doses of rotenone treatment. Indeed, although the average percentage survivability of SH-Parkin cells at 50 nM rotenone treatment is apparently reduced when compared with untreated SH-Parkin cells, it is still significantly better compared with SH-Vector cells treated with either 50 or even 25 nM rotenone (Fig. 4B). Our results thus indicate that overexpressed parkin could afford cellular protection against rotenone-induced cell death.

To examine whether an association exists between the parkin solubility and the degree of its cellular protection,

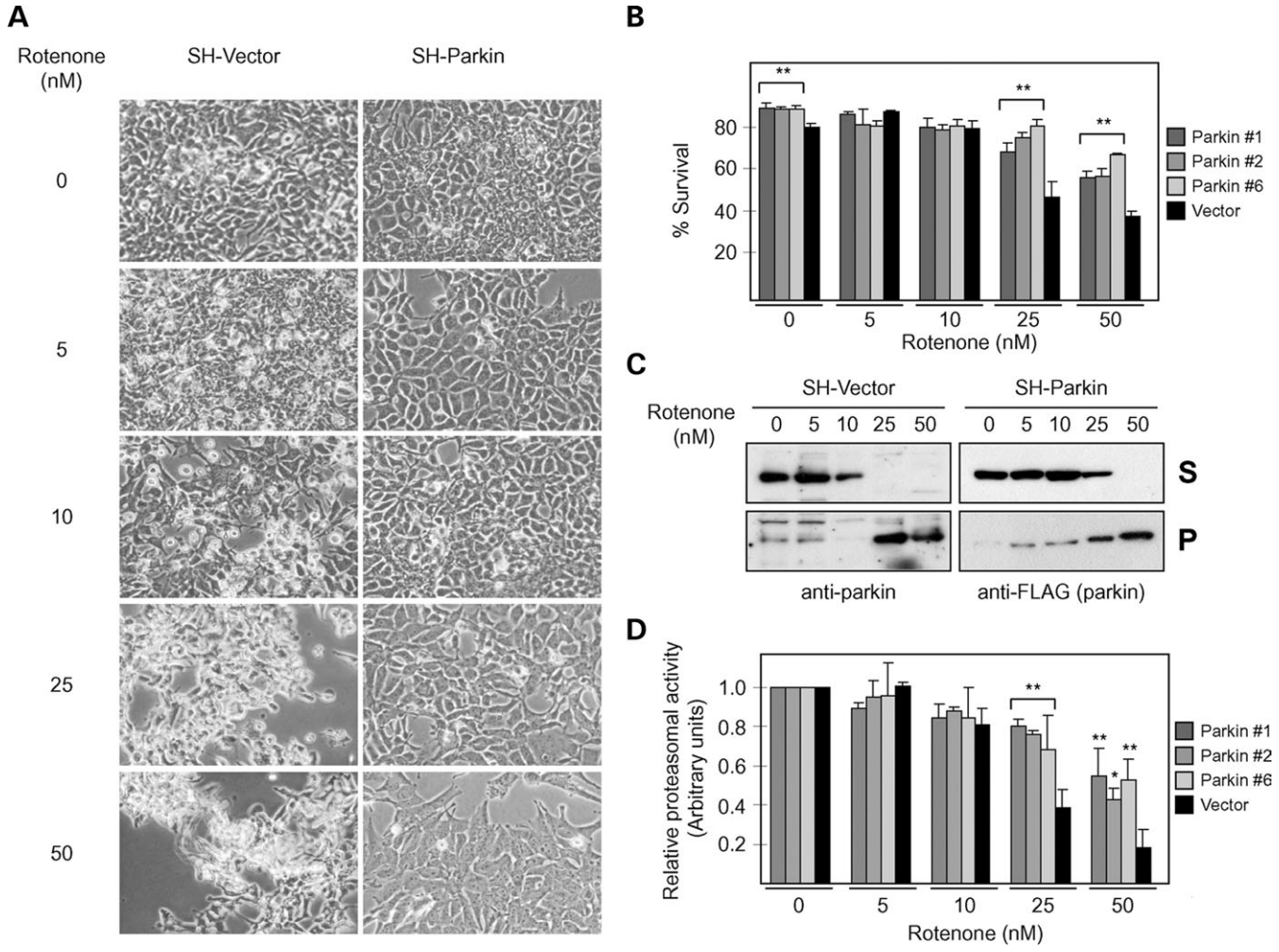

Figure 4. Stress-induced alterations in parkin solubility compromise parkin protective function. (**A**) Representative phase-contrast images of SH-SY5Y control cells (SH-Vector) and cells stably expressing parkin (SH-Parkin) showing a dose-dependent increase in the population of rounded, refractile cells 6 days after treatment with the indicated doses of rotenone. (**B**) The cell viability was measured and plotted as a bar graph (SH-Parkin nos 1, 2 and 6 depicted by dark, medium and light shade bars, respectively, and SH-Vector depicted by black bars). Data represent mean ± SEM of three duplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.001$ versus control group (one-way ANOVA). (**C**) Anti-parkin and anti-FLAG immunoblots of cell extracts sequentially prepared with Triton X-100 (S) and SDS (P) from vector and parkin stable cell lines 6 days after treatment with the indicated doses of rotenone. (**D**) Bar graph showing the dose-dependent decrease in the chymotrypsin-like proteasome activities of SH-Parkin (nos 1, dark; 2, medium; 6, light shade) and control cells (black) following 6 days of rotenone treatment relative to their respective untreated counterparts. Data represent mean ± SEM of three duplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.001$ versus control group (one-way ANOVA).

we performed anti-parkin or anti-FLAG immunoblotting of lysates prepared from rotenone-treated or -untreated SH-Parkin and controls cells. Anti-parkin immunoblotting of lysates prepared from SH-Vector cells treated with rotenone at doses above 25 nM reveals a complete shift of the endogenous parkin protein to the detergent-insoluble fractions (Fig. 4C). It is noteworthy that at these doses of rotenone treatment, the control cells showed significantly more cell death when compared with untreated cells (Fig. 4B). Although a dose-dependent shift in the population of exogenous parkin to the detergent-insoluble fractions is also observed in lysates prepared from rotenone-treated SH-Parkin cells, as revealed by anti-FLAG immunoblotting, the complete shift of FLAG-tagged parkin from S- to P-fractions occurred only at the highest dose of rotenone used (Fig. 4C). At 25 nM rotenone treatment, a significant population of exogenous parkin remained in the detergent-soluble fractions, which correlates with the higher survival rate seen in these cells compared with their corresponding control cells (Fig. 4B). We obtained essentially similar results when we repeated the earlier mentioned experiments with MPP+ in place of rotenone (Supplementary Material, Fig. S1). Our results thus suggest that stress-induced relocalization of soluble parkin into detergent-insoluble fractions is inversely related to the extent of its cellular protection.

Protective effect of parkin related to its ability to preserve proteasomal function

We were curious about the mechanism of parkin-mediated cellular protection against rotenone-induced stress and wondered, in accordance with our recent speculation (30) and also reports

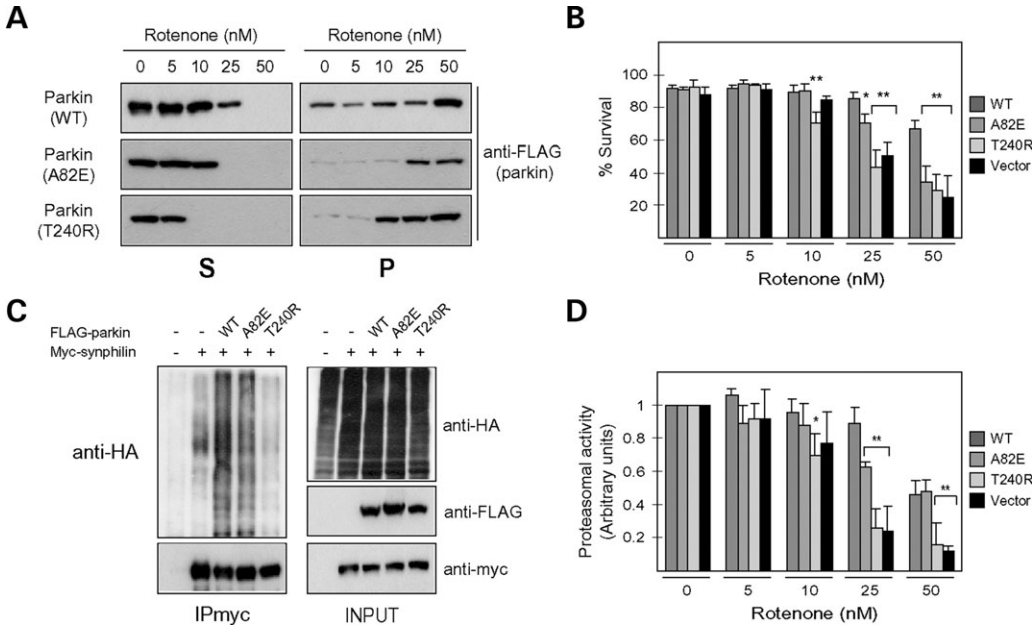

Figure 5. Familial PD-linked parkin mutants predispose parkin to stress-induced solubility alterations. (**A**) Anti-FLAG immunoblots of cell extracts sequentially prepared with Triton X-100 (S) and SDS (P) from wild-type and mutant parkin stable cell lines 6 days after treatment with the indicated doses of rotenone. (**B**) Bar graph showing the percentage cell viability in SH-Parkin WT (dark shade), SH-Parkin A82E (medium shade), SH-Parkin T240R (light shade) and SH-Vector (black) measured at 6 days after rotenone treatment. Data represent mean ± SEM of three duplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.001$ versus control group (one-way ANOVA). (**C**) Anti-HA and anti-myc immunoblots of synphilin immunoprecipitates (*IPmyc*) prepared from SH-SY5Y cells transfected with myc-tagged synphilin-1 in the presence of HA-tagged ubiquitin without or with the various FLAG-tagged parkin forms. Lysates prepared from these variously transfected cells were also subjected to anti-myc or anti-FLAG immunoblotting to show their expression (INPUT). These experiments were duplicated. (**D**) Bar graph showing the dose-dependent decrease in the chymotrypsin-like proteasome activities of SH-Parkin WT (dark shade), SH-Parkin A82E (medium shade), SH-Parkin T240R (light shade) and SH-Vector (black) following 6 days of rotenone treatment relative to their respective untreated counterparts. Data represent mean ± SEM of three duplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.001$ versus control group (one-way ANOVA).

by others (11,12), whether parkin could exert its protective functions by preserving proteasome function in times of cellular stress. To investigate this, we conducted *in vitro* proteasomal assays with lysates prepared from rotenone-treated SH-Parkin and SH-Vector cells as previously described. We compared the respective extent of proteasomal impairment in each of these cell types following stress application and plotted the bar graph accordingly by assigning a single arbitrary proteasomal activity unit to each of the untreated cell types (Fig. 4D). It becomes immediately apparent that there is a dose-dependent decrease in the proteasome activities in these cells following their treatment with rotenone (Fig. 4D). However, at higher doses of rotenone treatment, the SH-Vector cells appear to be much more susceptible to rotenone-induced proteasomal impairment among the cell lines examined (Fig. 4D). Collectively, SH-Parkin clones showed a statistically higher proteasomal activity when compared with SH-Vector cells at 25 nM or higher rotenone treatment (Fig. 4D). The drastic decrease in the proteasomal activities of control cells at these rotenone doses compared with untreated cells is consistent with the dramatic increase in their cell death as described earlier (Fig. 4B). Conversely, there is an association between the better maintenance of functional proteasomes in SH-Parkin cells and the greater survivability of these cells in times of stress (compare Fig. 4B and D). Our results therefore strengthen the suggestion that one mechanism by which parkin could afford cellular protection against stress-induced cell death is through the preservation of proteasomal activity (11,12,30).

Familial PD-linked parkin mutations predispose parkin solubility alterations by stress and compromise its protective function

We have previously classified familial PD-linked parkin missense mutants into two broad groups on the basis of their detergent solubility properties, i.e. detergent-soluble versus-insoluble mutants (24). Here, we examined the effect of stress on the properties of the detergent-soluble parkin mutants. To facilitate our investigation, we generated stable SH-SY5Y cells expressing the detergent-soluble parkin A82E (SH-A82E) and T240R mutants (SH-T240R). We then repeated the experiments described in Figure 4 with these parkin mutant stable cell lines. After 6 days of chronic rotenone treatment, detergent-soluble and -insoluble lysates were prepared from the neurotoxin-treated or -untreated SH-SY5Y stable cell lines. Following anti-FLAG immunoblotting of the fractionated lysates, we noticed a dose-dependent shift of FLAG-parkin into the P-fractions similar to what we have observed earlier (compare Figs 5A and 4C). Interestingly, both the parkin mutants examined appear to be more susceptible to rotenone-induced solubility alteration (Fig. 5A). Although the wild-type FLAG-tagged parkin apparently shifted completely to the P-fractions at 50 nM rotenone

treatment, the phenomenon occurs at earlier rotenone doses, i.e. 25 and 10 nM, respectively, for the A82E and T240R parkin mutants (Fig. 5A). Quantitative measurement of cell death in these rotenone-treated cell lines reveals that stable SH-SY5Y cell lines expressing the parkin T240R mutant are as susceptible to neurotoxin-induced cell death as the SH-Vector control (Fig. 5B). Associated with this, the proteasome activities in the SH-T240R cells, relative to SH-Vector control, showed no enhanced resistance to impairment caused by the treatment (Fig. 5D). In contrast, the A82E parkin mutant afforded significant cellular protection against cell death and proteasomal impairment brought about by the neurotoxin. However, at 25 nM rotenone treatment, where the A82E mutant relocalizes completely to the P-fraction, its protective effects decrease significantly compared with wild-type FLAG-parkin (Fig. 5B and D).

Although the degree of cellular protection afforded by these parkin mutants correlates well with their propensity to become directed to the detergent-insoluble fractions in the presence of rotenone, it is apparent that their respective catalytic competency plays a deterministic role in improving the survivability of cells that express them. To examine their enzymatic activity relative to wild-type parkin, we conducted *in vivo* ubiquitination assays using synphilin-1, a known parkin target, as a substrate. Consistent with our previous report (19), the parkin T240 mutant exhibited a significantly reduced catalytic activity compared with wild-type parkin (Fig. 5C). In contrast, the A82E mutant ubiquitinates synphilin-1 almost as efficiently as wild-type parkin (Fig. 5C). Taken together, our results support the importance of parkin catalytic activity for its protective functions and at the same time suggest that the higher propensity of soluble parkin mutants to stress-induced alteration is an alternative mechanism underlying their dysfunction, particularly for those mutants that retain substantial catalytic activity.

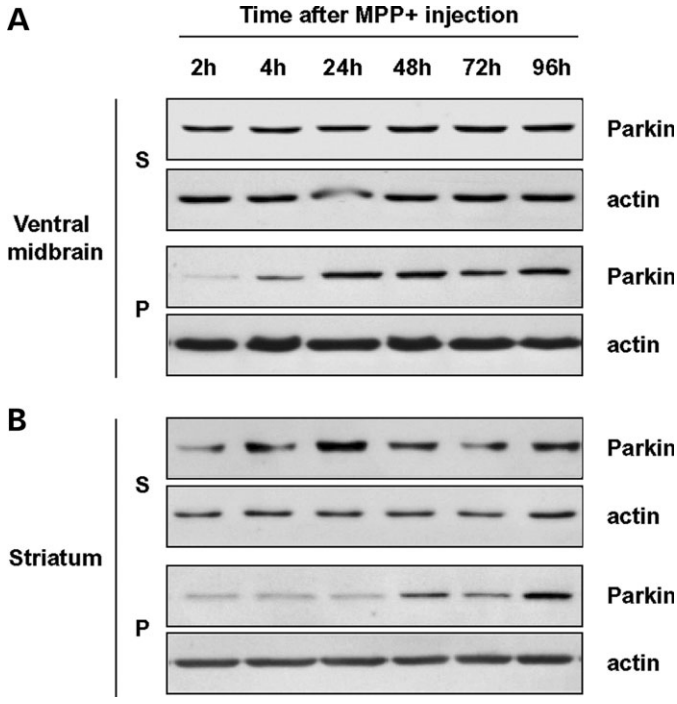

Figure 6. Significant increase in detergent-insoluble parkin in the mouse brain following MPP+ treatment. Detergent-soluble and -insoluble fractions of brain lysates prepared from (**A**) ventral midbrain and (**B**) striatum of mice after treatment with MPP+ for 2, 4, 24, 48, 72 and 96 h were subjected to SDS–PAGE and immunoblotted with anti-parkin and anti-actin antibodies.

Parkin solubility alterations in the brains of MPP+-treated mice

To extend our earlier mentioned cell culture studies further, as well as to investigate the physiological relevance of parkin solubility alterations following neurotoxic stress, we examined the effects of MPP+ on the biochemical distribution of mouse brain parkin *in vivo*. We performed anti-parkin immunoblotting of detergent-soluble and -insoluble lysates prepared from the ventral midbrain and striatal regions of mice treated with an acute paradigm of MPP+. Consistent with our cellular studies described earlier, we observed a time-dependent increase in the amounts of insoluble parkin in both the ventral midbrain (Fig. 6A) and striatal regions (Fig. 6B) of mice after the last MPP+ treatment following an acute intoxication paradigm. Significant amounts of insoluble parkin immunoreactivity started appearing at 24 and 48 h post-MPP+ injection in the ventral midbrain and striatal preparations, respectively, and persisted through the time course examined (Fig. 6A and B). Interestingly, the level of soluble striatal parkin increased transiently at 24 h post-MPP+ injections and its subsequent decrease corresponded to the appearance of the insoluble forms (Fig. 6B). The results we gathered from our cell culture and animal studies here are thus in general agreement with each other and collectively point towards the pathophysiological relevance of altered parkin function associated with its modification by PD-linked stressors.

Variations in parkin distribution between normal and PD human brains

Previously, we have demonstrated an age-dependent decrease in parkin solubility in human brains (23). Given the neuroprotective roles of parkin, we wondered whether the aging brain undergoing stress linked to PD would respond by elevating its levels of parkin to counteract the protein depleted into the insoluble fraction. The replacement of soluble parkin would be conceivably important in helping the brain mitigate the ill effects posed by the insults and concomitantly prolong its neuronal survival. Accordingly, we performed anti-parkin immunoblotting with detergent-soluble and -insoluble lysates prepared from post-mortem normal and PD human brains. The mean age of these samples is 83.6 and 81.5 years, respectively (Table 1). The sequential detergent extraction procedure used to extract the brain tissues, as described in Materials and Methods, is similar to the one we have described previously (23), except that 1% (instead of 2%) SDS was used in our final extraction. Consistent with our previous report (23), we observed a dramatic relocalization of brain parkin from the detergent-soluble to the detergent-insoluble fractions in the caudate and cingulate cortex regions from control brains (Fig. 7). Although the amount of detergent-insoluble parkin

Table 1. Brain region, diagnosis, age and post-mortem delay

Subjects	Brain region	Diagnosis	Age	Post-mortem delay
Control 1	Caudate	—	83	5
Control 2	Caudate	—	79	10
Control 3	Caudate	—	81	20
Control 4	Cingulate	—	83	5
Control 5	Cingulate	—	92	19
Patient 1	Caudate	PD/LB CHG LIMBIC, Porencephalic Cyst	81	8.75
Patient 2	Caudate	PD/LB CHG Neocortical	80	19
Patient 3	Caudate	PD/LB CHG	84	17.5
Patient 4	Caudate	PD/LB CHG Limbic	83	14
Patient 5	Caudate	PD/DLB	77	5
Patient 6	Caudate	PD/LB CHG Neocortical	84	13
Patient 7	Caudate	PD/LB CHG	88	19.5
Patient 8	Caudate	PD	73	6
Patient 9	Caudate	PD W/Dementia, LBD Neocortical	84	5

associated with the samples from PD brains are expectedly significantly higher than those derived from control brains (Fig. 7), the phenomenon could be related to a remarkable corresponding increase in soluble parkin levels in the majority of the PD brains. Although the average amount of detergent-insoluble parkin in PD brains increased by 16% over their control counterparts, the mean elevation of soluble parkin in PD brains is a dramatic 2.5-fold in the PD brains relative to their controls (Fig. 7). It would thus appear that an increase in endogenous parkin level in the brain accompanies the disease, presumably to counter the loss of the protein into insoluble pools in a bid to preserve neuronal survivability.

DISCUSSION

Although the debate surrounding the respective roles of environmental and genetic factors in the etiology of sporadic PD has persisted since the disease was first described in the early 19th century, current evidence favors a synergistic interaction between exogenous toxic agents and endogenous elements in bringing about the neurodegenerative process (1,31). Indeed, several reports have demonstrated a pathogenic relationship between the environmental stress factors and the PD-linked gene product, α-synuclein (32–34). In our current report, we show that several environmental stress factors linked to PD, as well as endogenous dopamine, produce alterations in parkin solubility and concomitantly compromise its protective function. Our results therefore further strengthen the relationship between environmental factors and genetic susceptibilities in the development of PD.

In recent years, it has become increasingly clear that parkin plays a pivotal role in maintaining dopaminergic neuronal survivability. We have previously demonstrated that familial PD-linked mutations in parkin led to its dysfunction either through direct catalytic impairment or through the alteration of the protein's structural and biochemical properties, leading to a decrease in its detergent solubility (18,19,24,25). Hence, it is conceivable that exogenous and/or endogenous factors that affect parkin in a way that mirrors the impact of parkin mutations on the protein would increase the vulnerability of dopaminergic neurons to degenerate. Interestingly, brain parkin becomes progressively more detergent-insoluble with normal aging (23). As age represents an unequivocal risk factor for PD, the depletion of soluble parkin with age is unlikely to be a trivial association. We believe that age-mediated parkin solubility changes are likely a cumulative event promoted by the continuous spectrum of insults experienced by the brain throughout its lifetime. Shedding some light on these, our results indicate that oxidative, nitrosative and proteolytic stress could modify parkin in a manner analogous to that mediated by several of its clinically relevant mutations, as well as by normal aging. The resulting relocalization of detergent-soluble parkin into insoluble fractions suggests that one mechanism of environmental neurotoxicity triggering the demise of dopaminergic neurons in PD is through the progressive depletion of soluble functional parkin.

As parkin functions as a broad-spectrum neuroprotectant, the susceptibility of the stress-prone dopaminergic neurons to degeneration in the absence of functional parkin is easy to comprehend. This is indeed the case with our cell culture studies described earlier. Although overexpressed parkin helps the SH-SY5Y neuroblastoma cells mitigate externally applied stress (rotenone and MPP+) rather effectively, it could do so only until a point, beyond which soluble parkin becomes fully depleted and could afford no extra protection to the cells expressing it. Similarly, the depletion of soluble endogenous parkin coincides with the onset of significant rotenone-induced cell death in control cells, suggesting again that parkin is protective only until a certain point. This phenomenon is at the same time curiously paradoxical, as it appears that an important factor chosen by the cell to provide a critical defense against environmentally triggered stress would later become the victim itself. An important corollary to this is that during times of heightened stress, increasing the level of endogenous parkin expression to counter the loss of the protein into insoluble pools would help ensure neuronal survivability. Supporting this, we found that parkin mRNA expression in SH-SY5Y cells increased several fold in response to various stressors including rotenone, iron and paraquat (Supplementary Material, Fig. S2). Furthermore, the unfolded protein stress also promotes the elevation of parkin expression, which acts to relieve the stress (35). Conceivably, cells that fail to produce functional parkin in sufficient amount would be less

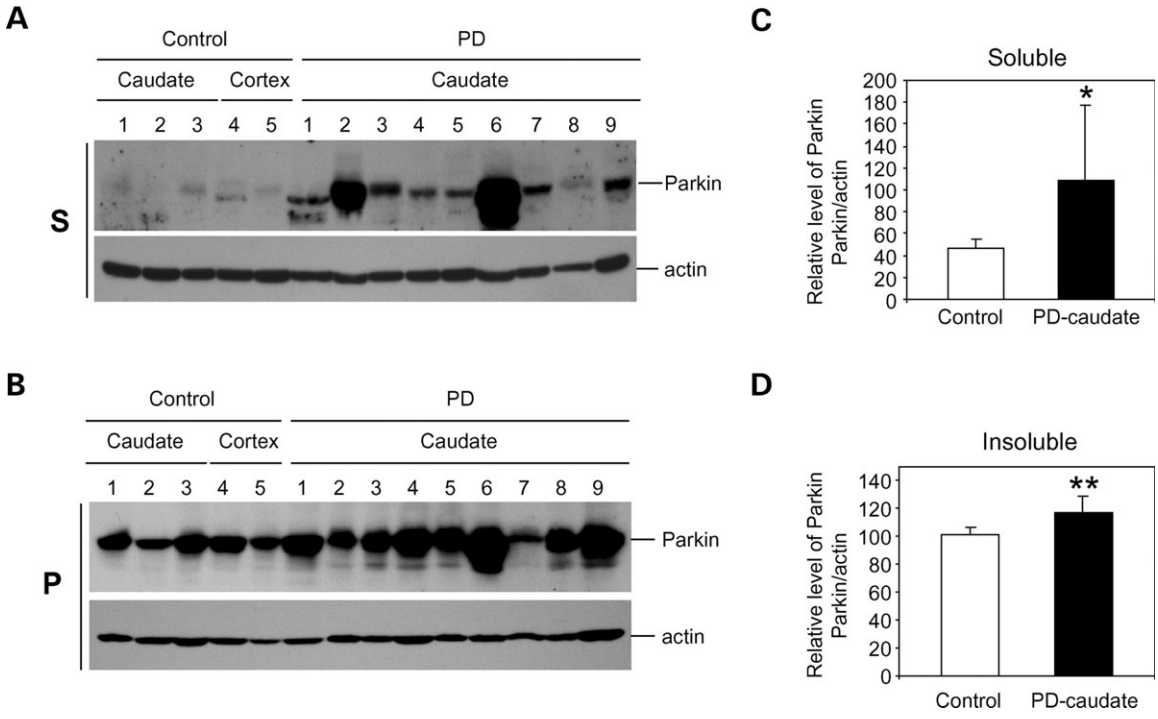

Figure 7. Variations in brain parkin distribution between normal and PD individuals. (**A**) Soluble fractions and (**B**) insoluble fractions of brain lysates from caudate ($n = 3$) and cingulate cortex ($n = 2$) of control and sporadic PD caudate brains ($n = 9$) were subjected to SDS–PAGE and immunoblotted with anti-parkin and anti-actin antibodies. The levels of parkin protein in the (**C**) soluble and (**D**) insoluble fractions were normalized to their respective actin levels and quantified and the results obtained plotted on a histogram ($^*P < 0.05$, $^{**}P < 0.01$, Student's *t*-test).

able to cope with stress. In this regard, it is important to note that the caudate region of the PD brain, where a significant upregulation of parkin levels occurs (Fig. 7), is relatively spared from degeneration during the disease progression. Although it is tempting to think that the selective vulnerability of dopaminergic neurons seen in sporadic PD may be related to their failure in replacing cytosolic parkin lost to the insoluble pool, a more detailed analysis of parkin distribution in different regions of the PD brains would be required to evaluate this speculation. Nonetheless, such a scenario remains a formal possibility. Indeed, a selective loss of the proteasome α-subunit in the SNc but not in the frontal cortex and striatum in PD has been recently reported (36). Further, an important component of the proteasome complex (PA700) failed to upregulate in the SNc, although it occurred in the frontal cortex and striatum in PD compared with control (36). Our results may thus help to explain the expanded risk for PD associated with parkin haploinsufficiency, as well as with normal aging. Indirectly, our results also support the utility of parkin gene therapy as a means to mitigate the progression of the disease.

Recently, Jiang *et al.* (17) demonstrated that overexpression of parkin in SH-SY5Y cells significantly reduced the amount of intracellular oxyradicals, suggesting that the protective effect of parkin is afforded by its ability to suppress oxidative stress. Consistent with this, we found here that parkin attenuates rotenone-induced cell death. It is evident from our results that a plausible mechanism responsible for the protective function of parkin against stress-induced toxicity is through the preservation of proteasome activity. This is consistent with previous reports by others (11,12). The maintenance of a viable proteasome by parkin is clearly dependent on its E3 ligase activity. However, whether parkin-mediated K48-linked ubiquitination, or a newly described K63-linked ubiquitination activity of parkin (37,38), is required in this protection is unknown. Interestingly, mutations in parkin that do not overtly alter the protein activity or solubility appear to predispose the protein to solubility alteration by environmental stress. Hence, the A82E parkin mutant afforded significantly less protection against rotenone-induced toxicity when compared with the wild-type protein, despite having comparable catalytic activity. In contrast, being catalytic impaired and even more susceptible to stress-induced alteration than the A82E parkin mutant, the T240R mutant offers virtually no protection against rotenone-induced toxicity. Taken together, our previous (18,19,24,25) and current work on the parkin mutants demonstrate that mutations in parkin could impair the protein function in more than one way, including frank catalytic impairment, direct alteration of the protein solubility and localization or simply a predisposition to such alterations.

Notably, most, if not all, of the stressors we have used in this study are associated with oxidative stress. The occurrence of oxidative stress in PD is consistently supported by both post-mortem studies and by studies demonstrating the capacity of oxidative stress and oxidizing toxins to induce nigral cell degeneration (39). Although a myriad of cellular macromolecules could be damaged by oxidative free radicals, enzymes whose structure and function are dependent on catalytic cysteine are expected to be more susceptible to the consequence of oxidative modification. The tyrosine phosphatase family of enzymes, all of which contain catalytic

cysteines in their active sites, is one example (40). RING-finger E3 ligases, such as parkin, are also characterized by their cysteine-rich catalytic moiety that represent targets for oxidative modification. Furthermore, we and others have demonstrated the importance of these RING-finger cysteines in maintaining the native structure of parkin (24,41–43). Thus, it is not surprising that several of the stress factors we have examined could produce alterations in the structural and biochemical properties of parkin, as they are associated with the production of oxidative free radicals. Similarly, nitrosative modification of parkin has been shown to occur on the protein's RING finger cysteines (27,44), which would explain the associated solubility changes observed. Further, our results showing that S-nitrosylated parkin's tendency to adopt a detergent-insoluble conformation would support a negative role for this type of modification on parkin.

Taken together, our results provide a mechanistic link between putative etiologic environmental (and endogenous) factors and dysfunction of a key PD-linked gene product and support a role for parkin in the pathogenic cascade leading to idiopathic PD.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were used: polyclonal anti-parkin (Cell Signalling Technologies), monoclonal anti-Flag peroxidase (Sigma, St Louis, MO, USA), monoclonal anti-myc (Roche, Indianapolis, IN, USA), monoclonal anti-c-myc-peroxidase conjugates (Roche), monoclonal anti-HA-peroxidase conjugate (Roche), monoclonal anti-β-actin (Sigma) and rhodamine-conjugated anti-mouse IgG (Molecular Probes). The FLAG-tagged wild-type parkin expression construct was a kind gift from R. Takahashi and the FLAG-tagged parkin missense mutants A82E and T240R have been described previously (24). All other chemicals, unless otherwise stated, were purchased from Sigma. Stock solutions of the following chemicals were prepared and stored at −20°C: MG-132 (1 M in DMSO); rotenone (10 mM in DMSO); MPP+ (10 mM in dH_2O); paraquat (1 M in PBS); canavanine (0.5 M in H_2O); $FeCl_2$ (1 M in 0.1 M HCl); dopamine (0.5 M in H_2O supplemented with 0.1 M vitamin C).

Generation of stable cell lines, preparation of cell lysate and western blot analysis

SH-SY5Y neuroblastoma cells were transfected with pCDNA3 plasmid vector alone or bearing the FLAG-tagged parkin constructs using LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instructions. Two days later, stably transfected cells were selected for in media containing 800 μg/ml geneticin (Invitrogen). Subsequently, at least 20 clonal colonies were picked for each cDNA construct and screened by means of western blot analysis. All positive cell lines used for the experiments described here were maintained in serum containing DMEM supplemented with 200 μg/ml geneticin (Invitrogen) to prevent extrusion of the integrated constructs. Sequential fractionation of cell lysates into Triton X-soluble (S) and SDS-soluble (P) fractions was performed as previously described (24). Equivalent amount of proteins among different S- and P-fractions was resolved by means of SDS–PAGE and the levels of various proteins were analyzed by means of western blotting procedures using ECL detection reagents (Amersham).

Immunocytochemistry and confocal microscopy

Cellular localization studies of parkin by means of immunocytochemistry and confocal microscopy were performed as previously described (24). For these studies, parkin stable cells were either untreated or treated with rotenone (25 or 50 nM for 6 days), MPP+ (200 or 500 μM for 4 days) and paraquat (1 mM for 4 days). The cells in culture were replaced with fresh media daily, each containing appropriate dose of the various stressors.

Cell viability and proteasome activity analysis

Quantitation of cell survival was performed using two methods: flow cytometry and Trypan blue exclusion assay. For the flow cytometric analysis, cells were seeded at a concentration of 1×10^6/ml. After treatment, cells were trypsinized and propidium iodide (1 μg/ml) was added 5 min before flow cytometric analysis by the Coulter imaging system (BD). A count of 10 000 cells were taken and percentage of cell death was calculated using winMDIv2.8 (BD). Proteasomal activities after chronic rotenone exposure were determined by incubating lysates (10–15 μg of protein) with substrates Suc-LLVY-AMC for 1.5 h at 37°C. The relative amount of AMC released was measured using a fluorometer equipped with a 380/460 nm filter set (TECAN). Statistical significance for all the quantitative data obtained was analyzed using one-way ANOVA (SPSS 13.0) ($^*P < 0.05$, $^{**}P < 0.001$) unless otherwise stated.

Animals and MPP+ injections

All procedures involving animals were approved by and conformed to the guidelines of the Institutional Animal Care Committee of Johns Hopkins University. Eight-week-old male C57/bl mice (Charles River Laboratories, Wilmington, MA, USA) ($n = 5$ per group) received an acute paradigm of four intraperitoneal injections of MPP+ and HCl (20 mg/kg free base; Sigma) in saline at 2 h intervals on 1 day and control mice received equivalent volumes of saline at the same frequency. Animals were sacrificed by decapitation at 2, 4, 24, 48, 72 and 96 h after the last MPP+ injection and their ventral midbrain and striata were quickly dissected out on ice and immediately frozen in −80°C and processed for western blot analysis.

Human tissues

Human brain tissue was obtained through the brain donation program of the Morris K. Udall Parkinson's Disease Research Center at Johns Hopkins Medical Institutions (JHMI) according to HIPAA regulations. This research proposal involves anonymous autopsy material that lacks identifiers of gender, race or ethnicity. The JHMI Joint Committee on Clinical

Investigations decided that the studies in this proposal are exempt from Human Subjects Approval because of Federal Register 46.101 exemption number 4.

Preparation of human and mouse brain tissues

Caudate tissues from three control brain and frontal cortical tissues from two control and nine caudate tissues from PD/DLB brains were age matched and used to analyze parkin solubility. Detergent-soluble and -insoluble fractions were prepared from human brain tissues and mouse brain tissue, such as ventral midbrain and striatum, by homogenization of samples in lysis buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 10 mM Na-β-glycerophosphate, phosphate inhibitor mixture I and II (Sigma), complete protease inhibitor mixture (Roche)] by using a Diax 900 homogenizer (Heidolph, Cinnaminson, NJ, USA). After homogenization, samples were rotated at 4°C for 30 min for complete lysis, then the homogenate was centrifuged (10 000*g*, 4°C, 20 min) and the resulting pellet and supernatant fractions were collected. The pellet fractions were washed once in lysis buffer containing detergent, and the resulting pellet was solubilized in lysis buffer containing 1% SDS. Protein concentrations of both soluble and insoluble fractions were determined using the BCA kit (Pierce, Rockford, IL, USA) with BSA standards and analyzed by western blot. Western blotting was carried out with anti-parkin (PRK8 mouse monoclonal) and anti-β-actin (Sigma) antibodies. Detection was performed with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

Quantitative real-time PCR

Total RNA was isolated from SH-SY5Y cells either untreated or treated with 50 nM rotenone, 5 mM paraquat or 10 mM iron for 24 h using the RNAeasy Mini Kit (Qiagen). Subsequently, the isolated RNA was reverse transcribed using the Superscript First-Strand Synthesis System (Invitrogen). Real-time PCR was carried out in a Light Cycler (Roche) using the FastStart DNA Master Plus Sybergreen I system (Roche) according to the manufacturer's protocol. A pair of parkin-specific primers (forward: 5′-GGAAGTCCAGCAGGTAGA TCA; Reverse: 5′-ACCCTGGGTCAAGGTGAG) were generated for this purpose. Concurrently, real-time PCR with a primer pair specific for GAPDH (forward: 5′-GAAGGT GAAGGTCGGAGTCAACG; Reverse: 5′-TGCCATGGGT GGAATCATATTGG) was also included in the same run as an internal control. A single PCR end product was obtained in each case and the validity of these products was verified by direct sequencing. Consistent with the role of GAPDH as a general housekeeping gene, we found no significant change in its mRNA level during each stress treatment.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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