Molecular interaction between parkin and PINK1 in mammalian neuronal cells

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Abstract

Parkinson’s disease (PD) is characterized by the deterioration of dopaminergic neurons in the pars compacta of substantia nigra and the formation of intraneuronal protein inclusions. The etiology of PD is not known, but the recent identification of several mutation genes in familial PD has provided a rich understanding of the molecular mechanisms of PD pathology. Mutations in PTEN-induced putative kinase 1 (PINK1) and parkin are linked to early-onset autosomal recessive forms of familial PD. Here we show molecular and functional interactions between parkin and PINK1. Parkin selectively binds to PINK1 and upregulates PINK1 levels. In addition, PINK1 reduces the solubility of parkin, which induces the formation of microtubule-dependent cytoplasmic aggresomes. Our findings reveal that parkin and PINK1 affect each other’s stability, solubility and tendency to form aggresomes, and have important implications regarding the formation of Lewy bodies.

Introduction

Parkinson disease (PD) is a devastating neurodegenerative disease characterized by a distinct set of motor symptoms, including muscle rigidity, tremor, postural instability and bradykinesia (Olanow and Tatton, 1999). Pathological hallmarks of PD include the progressive degeneration and death of dopaminergic neurons in the substantia nigra pars compacta and the presence of cytoplasmic inclusions known as Lewy bodies (LBs) (Moore et al., 2005a,b). The etiology of PD remains poorly understood. The familial form of PD is influenced by genetic mutations. Recently, several genetic loci have been linked to early-onset autosomal recessive forms of familial PD. Here we show molecular and functional interactions between parkin and PINK1. Parkin selectively binds to PINK1 and upregulates PINK1 levels. In addition, PINK1 reduces the solubility of parkin, which induces the formation of microtubule-dependent cytoplasmic aggresomes. Our findings reveal that parkin and PINK1 affect each other’s stability, solubility and tendency to form aggresomes, and have important implications regarding the formation of Lewy bodies.

Parkinson’s disease (PD) is characterized by the deterioration of dopaminergic neurons in the pars compacta of substantia nigra and the formation of intraneuronal protein inclusions. The etiology of PD is not known, but the recent identification of several mutation genes in familial PD has provided a rich understanding of the molecular mechanisms of PD pathology. Mutations in PTEN-induced putative kinase 1 (PINK1) and parkin are linked to early-onset autosomal recessive forms of familial PD. Here we show molecular and functional interactions between parkin and PINK1. Parkin selectively binds to PINK1 and upregulates PINK1 levels. In addition, PINK1 reduces the solubility of parkin, which induces the formation of microtubule-dependent cytoplasmic aggresomes. Our findings reveal that parkin and PINK1 affect each other’s stability, solubility and tendency to form aggresomes, and have important implications regarding the formation of Lewy bodies.

Neurodegeneration in PD is accompanied by abnormal protein accumulation, aggregation and the sequestration of a wide range of proteins into LBs. LBs contain various proteins, including the most abundant α-synuclein (Spillantini et al., 1997), neurofilaments (Schmidt et al., 1991), ubiquitin and ubiquitinated proteins (McNaught et al., 2002; Lennox et al., 1989). LBs also contain the components of the UPS, including proteasomal subunits, ubiquitination/de-ubiquitination enzymes and proteasome activators (Li et al., 1997; Lowe et al., 1990). It has been found that some PD-associated gene products are functionally linked to one another. Such interactions can lead to the activation of common pathogenic pathways of neuronal degeneration and the emergence of PD. For example, parkin directly ubiquitinates or cleave α-synuclein (Shimura et al., 2001; Kim et al., 2003). Parkin also interacts with pathogenic DJ-1 mutant (Moore et al., 2005a,b) and LRRK2 (Smith et al., 2005). In addition, PINK1 physically interacts with DJ-1 and the resulting PINK1/DJ-1 complex protects cells against stress (Tang et al., 2006).

The intracellular levels of parkin and PINK1 are modulated via UPS (Tang et al., 2006; Choi et al., 2000; Muquit et al., 2006; Lin and Kang, 2008). In PD patients, proteasomal activity has been reported to be significantly reduced (McNaught et al., 2003), which results in the...
abnormal accumulation of parkin and PINK1 proteins. This hypothesis was further supported by the finding that PINK1 and parkin are localized in LBs (Gandhi et al., 2006; Schlossmacher et al., 2002). In addition, a number of recent studies by using *Drosophila melanogaster* and mammalian cells indicated that parkin and PINK1 share a common pathway to maintain mitochondrial function (Clark et al., 2006; Exner et al., 2007; Park et al., 2006; Yang et al., 2006) as well as to regulate its morphology towards mitochondrial fission (Deng et al., 2008; Poole et al., 2008). In the present study, we investigated the biological and functional relationship between PINK1 and parkin. We show that PINK1 physically interacts with parkin in cultured cells and brain tissues, which results in the alteration of parkin solubility and PINK1 levels.

**Results**

**Parkin interacts with PINK1**

To investigate the relationship between parkin and PINK1, we determined whether parkin binds to PINK1 in mammalian cells using a co-immunoprecipitation assay. After HEK293 cells were transfected with Flag-tagged PINK1 and Myc-tagged parkin, immunoprecipitation was performed with an anti-Myc antibody, followed by immunoblotting with an anti-Flag antibody. Consistent with previous reports (Silvestri et al., 2005; Abou-Sleiman et al., 2006; Bellina et al., 2005), PINK1 appeared in two bands, as shown by Western blot analysis (Fig. 1A). The upper band represents full-length PINK1, whereas the lower band represents the processed form of PINK1 from which the N-terminal mitochondrial-targeting motif has been removed.

In addition, we found that parkin binds to PINK1 (Fig. 1A). Based on a previous report showing that PINK1 is degraded by the ubiquitin–proteasome pathway (Tang et al., 2006; Muqit et al., 2006; Lin and Kang, 2008), cells were treated with proteasomal inhibitor, MG132, and co-immunoprecipitation was performed. As expected, the levels of PINK1 protein were significantly accumulated; enhanced binding of parkin to PINK1 was observed (Fig. 1B). Moreover, PINK1 was precisely colocalized with parkin, primarily in the cytoplasm (Fig. 1C).

To determine whether endogenous parkin binds to endogenous PINK1 in mammalian cells, a rabbit polyclonal PINK1 antibody against 157–170th residues was prepared and its specificity was checked. As shown in Supplementary material, Fig. S1, the antibody recognized endogenous PINK1 band with the expected size in human dopaminergic neuroblastoma SH-SY5Y cells. The band mobility was similar to that observed with transfected Myc-tagged wild-type (WT) PINK1 (Supplementary material, Fig. S1A). In order to show the antibody specificity, when cell lysates were immunoblotted with preimmune serum, there was no band with the similar size (Supplementary material, Fig. S1B). In addition, when cells were transfected with either PINK1-siRNA or control-siRNA, the level of endogenous PINK1 was remarkably reduced by PINK1-siRNA, but not by nonspecific control-siRNAs (Supplementary material, Fig. S1C). These data confirmed that antiserum raised by PINK1 antigen can specifically detect the PINK1 protein. By using this anti-PINK1 antibody co-immunoprecipitation assay of SH-SY5Y cell lysates was performed, followed by the immunoblotting with anti-parkin antibody. As shown...
in Fig. 2A, endogenous parkin interacts with endogenous PINK1 (Fig. 2A). The specific interaction between PINK1 and parkin was also observed in PC12 cells (Supplementary material, Fig. S2A). Confocal microscopic analysis revealed that parkin was colocalized with PINK1 in SH-SY5Y cells (Fig. 2B). Furthermore, the parkin/PINK1 complex was predominantly localized in the cytoplasm, while only very small amounts were found in the nucleus (Fig. 2B).

We then examined whether the association between parkin and PINK1 also occurs in the mammalian CNS. As shown in Fig. 2C, parkin selectively binds to PINK1 in the substantia nigral and striatal regions of rat brain, whereas no obvious interaction band was found for immunocomplex samples prepared with preimmune IgG as a control. The binding of parkin to PINK1 was also observed in rat CNS cortical tissues (Supplementary material, Fig. S2B). Taken together, these results suggest that the physical association of PINK1 with parkin is not merely an artifact observed in cell culture and occurs in the mammalian CNS.

To clarify whether PINK1 binds to parkin directly or indirectly, we conducted an in vitro GST pull down assay with recombinant GST fused PINK1 and in vitro translated parkin. We found that parkin can interact with GST-PINK1 directly, but not with GST as a negative control (Supplementary material, Fig. S3).

We then investigated whether the interaction between PINK1 and parkin depends on PINK1 kinase activity. Towards this end, we used a triple kinase-dead (KD) mutant (K219A/D362A/D384A) of PINK1 that has been confirmed to have no kinase activity (Beilina et al., 2005). In contrast to wild type PINK1, this KD kinase-deficient PINK1 mutant fails to bind to parkin (Fig. 3A), indicating that PINK1 kinase activity is required to the association with parkin. To identify the PINK1-binding domain of parkin, several deletion mutants of parkin were cotransfected with GFP-tagged PINK1 into HEK293 cells. As shown in Fig. 3C, PINK1 interacts with deletion parkin mutants spanning 1–415 or 77–465 amino acids. However, 217–465 and 295–465 mutants of parkin containing RING domains or its 1–100 mutant
containing ubiquitin-like domain did not bind to PINK1 (Fig. 3C). These data implied that the 101–216th region of parkin appears to be crucial for the interaction with PINK1, despite the multiple domains of parkin could be required for PINK1-association.

Parkin and PINK1 levels are mutually modulated

Interestingly, it was consistently found that cotransfection of parkin plus PINK1 causes a concomitant reduction of parkin with a significant increase of PINK1. To further examine this phenomenon, HEK293 cells were transfected with Myc-parkin, GFP-tagged wild type PINK1, or its KD mutant. Subsequently, 1% NP-40-soluble (S) and-insoluble fractions (P) were prepared from cell lysates. Overexpression of parkin plus PINK1 resulted in marked increase of 1% NP-40-insoluble parkin level with a concomitant decrease of its soluble level to a similar extent, compared to cells overexpressing parkin or PINK1 alone, (Fig. 4A). Interestingly, parkin solubility was reduced by wild type PINK1, but not by KD mutant (Fig. 4A). Moreover, PD-linked PINK1 mutant (G309D) had no effect on parkin solubility (Fig. 4B). Furthermore, parkin upregulated the levels of wild type PINK1 in soluble and particulate fraction, but not with KD- or PD-linked mutants (Figs. 4A and B). After HEK293 cells were cotransfected with GFP, PINK1, and parkin, Western blotting with anti-GFP antibody showed that GFP expression was not affected by parkin and PINK1 (Supplementary material, Fig. S4). In order to determine whether this finding may be an artifact caused by protein overexpression, the change of endogenous PINK1 levels was analyzed in mouse neuroblastoma N2A cells to overexpress parkin. This cell line was simply chosen and used to elevate the transfection efficiency of parkin. As shown in Fig. 4C, the level of premature form of endogenous PINK1 was higher in both soluble and particulate fractions by the ectopic overexpression of parkin. In addition, when PINK1 was overexpressed, endogenous parkin level in soluble fraction was reduced, whereas its insoluble level increased (Fig. 4D). Taken together, these data indicate that PINK1 reduces parkin solubility, which depends on the kinase activity, whereas parkin is capable of upregulating the PINK1 levels.

In addition to protein solubility, we further explored whether the subcellular localizations of two proteins are altered by co-expression. After cell lysates were fractionated into the cytosolic, mitochondrial, and nuclear fractions, a small amount of PINK1 within the cytosol appeared to be relocated into mitochondrial by parkin, but not any remarkable change of their localization (Supplementary material, Fig. S5). These results indicate that the solubility of parkin and PINK1, but not their subcellular locations, are mutually affected by ectopic coexpression of these two proteins.

Based on the previous reports that oxidative stresses and S-nitrosylation induce the reduction of parkin solubility (LaVoie et al., 2007; Yao et al., 2004), we examined whether PINK1-induced...
reduction of parkin solubility might be caused by such stresses. As shown in Supplementary material, Fig. S6, the PINK1-overexpression still reduced the parkin-solubility in the presence of antioxidants, such as NAC or PTIO, suggesting that PINK1-induced reduction of parkin level is not mediated via the oxidative protein modification.

PINK1-induced attenuation of parkin solubility promotes parkin aggregation

Next we examined whether the alteration of parkin solubility is associated with the propensity of PINK1 to form intracellular aggregates. The localization of parkin was determined using fluorescent microscopy. When PINK1 was coexpressed with GFP as a control, GFP proteins were expressed evenly throughout the cytosol and nucleus (Fig. 5A). When PINK1-Myc was coexpressed with GFP-tagged wild type parkin, parkin aggregates formed (Fig. 5B). Additional experiments revealed that the formation of parkin aggregates was caused by wild type PINK1, but not by KD- or two PD-linked (i.e. G309D, L347P) mutants of PINK1 (Fig. 5C). This is consistent with previous results (Figs. 4A and B). When the number of cells expressing parkin diffusely, in a punctated manner, or localized to the nucleus were counted, wild type PINK1 significantly increased the number of cells showing punctated localization of parkin aggregates (Fig. 5D). This result indicates that PINK1 regulates parkin solubility and consequently induces parkin-containing aggregates.

The morphology of PINK1-induced parkin aggregates appeared similar to that of aggresomes (Kopito, 2000). It has been reported that microtubule-dependent retrograde transport plays a role in aggresome formation. To test whether the parkin aggregates were indeed aggresomes, we examined the effect of microtubule-depolymerizing drug, nocodazole, on the formation of PINK1-induced parkin aggregates. As shown in Fig. 6A, when parkin and PINK1 were coexpressed, there were no parkin aggregates in nocodazole-treated cells. In addition, PINK1-induced reduction in parkin solubility was not observed in the cells treated with nocodazole (Fig. 6B). This finding suggests that PINK1-induced formation of parkin aggregates is dependent on the intact microtubule structure. Furthermore, when cells were immunostained with anti-parkin or anti-ubiquitin antibodies as an aggresome marker, parkin was colocalized with ubiquitin in the form of aggresome in the presence of PINK1, whereas ubiquitin was evenly localized in the cytoplasm and nucleus in control cells transfected with GFP-parkin alone (Fig. 6C). These data strongly indicated that parkin aggregates are indeed aggresomes.

Accumulating evidences indicated that aggresomes are formed to avoid the accumulation of potentially toxic proteins in the cytoplasm and consequently allow cells to minimize misfolded protein-induced cytotoxicity (Olanow et al., 2004; Kopito, 2000). Based on those reports, we examined whether the formation of PINK1-induced parkin-aggresomes produces a cytoprotective effect. To this end, we measured the effect of parkin and/or PINK1 on the occurrence of proteasomal inhibitor-induced cell death. While the addition of MG132 caused cell death, reducing the cell viability by approximately 30%, parkin or PINK1 alone showed a relative increase of cell viability by ∼10% (Supplementary material, Fig. S7). These results were consistent with the previous reports that parkin and PINK1 act as cellular protectors against proteasome inhibition-induced cell toxicity (Yang et al., 2007; Valente et al., 2004). Moreover, when parkin and PINK1 were expressed together, the cell viability additively increased by ∼10% more (Supplementary material, Fig. S7). These data indicates that parkin and PINK1 synergistically protects cells against MG132-induced cell death, in which the formation of PINK1-induced parkin-positive aggresomes may play a role.

PINK1 is upregulated by overexpression of parkin

Next we determined which domain(s) of parkin are critical for the upregulation of PINK1. As shown in Supplementary material, Fig.
S8A, wild type parkin and its 77–465 or R42P mutants markedly increased PINK1 levels. However, other parkin deletion mutants (i.e. 1–415, 217–465 or 295–465) showed no significant effect on PINK1 level (Supplementary material, Fig. S8A). These results suggest that RING2 domain of parkin is critical for the upregulation of PINK1 as well as PINK1 binding. The RING2 domain, including a RING-IBR-RING structure, was reported to be necessary for the intrinsic ubiquitin E3 ligase activity of parkin (Capili et al., 2004), implicating that the enzymatic activity of parkin is crucial to the upregulation of PINK1.

Based on the previous finding that the levels of PINK1 protein, especially its cleaved form, are much higher in the presence of MG132, the current finding was further examined after the pretreatment of cells with MG132, followed by co-transfection with Myc-parkin and PINK1-Flag. As shown in Supplementary material, Fig. S8A, the increase of cleaved PINK1 levels was greatly enhanced by MG132, suggesting that parkin may regulate PINK1 levels by acting on the premature form of PINK1. We then tested whether the upregulation of PINK1 is specific to parkin, or could similarly be mediated by other ubiquitin E3 ligases. Interestingly, PINK1 levels were not significantly changed in response to NEDD4-1 or NEDD4-2, which have HECT domains and act as ubiquitin E3 ligase, irrespective of the presence of MG132 (Supplementary material, Fig. S8B). Moreover, cIAP1, which acts as RING domain-dependent ubiquitin E3 ligase, had no effect on PINK1, (Supplementary material, Fig. S8C). These results demonstrate that the upregulation of PINK1 is a parkin-specific event.

**Parkin decreases PINK1 ubiquitination**

To investigate the mechanism by which parkin upregulates PINK1, in vivo ubiquitination assay of parkin and PINK1 was conducted in mammalian cells. First, to clarify the dominant protease system that regulates the stability of PINK1, the pattern of PINK1 proteolysis was compared in the absence or presence of several protease inhibitors, such as inhibitor(s) of proteasome, calpain, or lysosomal proteases. Lactacystin and epoxomicin are highly specific proteasome inhibitors; MG132 acts as a potent but less specific proteasome inhibitor. To test whether the proteasome inhibitors also affect PINK1 stability, we used calpeptin, which acts as a calpain inhibitor and E-64 is used as a lysosomal protease inhibitor. Consistent with previous reports (Tang et al., 2006; Muqit et al., 2006; Lin and Kang, 2008), only proteasome inhibitors substantially restored the
decrease in PINK1 levels in HEK293 cells, but neither calpain nor lysosomal protease inhibitors (Fig. 7A).

Interestingly, PINK1 ubiquitination was dramatically reduced by parkin overexpression, which led to the intracellular accumulation of PINK1 (Fig. 7B). To confirm that the reduction of PINK1 ubiquitination is specifically caused by parkin, we tested the effect of other E3 ubiquitin ligase containing RING domain, such as Siah-1. As shown in Fig. 7C, there was no significant change in ubiquitinated PINK1 levels in the absence or presence of Siah1, indicating that the reduction of PINK1-ubiquitination selectively depends on parkin. Furthermore, the self-ubiquitination of parkin was significantly reduced by PINK1, which was caused by the reduction in 1% NP-40 soluble parkin levels (Fig. 7B).

Increased steady state levels of PINK1 in cells stably overexpressing wild type parkin, but not its dominant-negative mutant

We next determined whether the overexpression of parkin affects the steady-state level of endogenous PINK1. Stable SH-SYSY cell lines overexpressing either Myc-tagged wild type parkin (SH-SYSY/WT-parkin) or deletion mutant (amino acids: 1–415) (SH-SYSY/1–415-parkin) was generated. Since the RING2 domain was previously reported to be necessary for the intrinsic E3 ligase activity (Capilli et al., 2004), stable SH-SYSY cell line overexpressing a RING2-deleted mutant was established as a ‘loss-of-parkin-function’ control. When immunocytochemistry was performed with anti-PINK1 and anti-parkin antibodies, PINK1 level increased only in SH-SYSY/WT-parkin cells, but not in SH-SYSY/1–415-parkin cells (Fig. 8A). In addition, the measurement of fluorescent intensity from each protein indicated that the steady-state PINK1 level is significantly increased in SH-SYSY/WT-parkin cells, compared with parental control or SH-SYSY/1–415-parkin cells (Fig. 8B).

Western blot analysis using anti-PINK1 or anti-parkin antibodies showed that both premature and cleaved form levels of PINK1 are accumulated in SH-SYSY/WT-parkin cells, compared with parental control or SH-SYSY/1–415-parkin cells (Fig. 8C). However, when real time-PCR was performed, there was no change of endogenous PINK1 mRNA level in SH-SYSY/WT-parkin cells compared with parental control cells (Fig. 8D), indicating that parkin does not affect on the transcription of PINK1.

Next we checked whether and how the PINK1 level is being altered in parkin KO mice (PaKO). The PaKO was generated by the
targeted deletion of exon 3, which results in a frameshift after 57th amino acid of parkin (Stichel et al., 2007). Western blotting of 1% NP-40-soluble fraction prepared from brain extracts showed that parkin level is completely abolished in three individuals of PaKO. In addition, parkin was not detected in NP-40-insoluble fraction. Furthermore, the levels of PINK1 in the NP-40-soluble and-insoluble fraction were down-regulated in the PaKO (Fig. 9A). When the intensity of PINK1 bands was quantified statistically, the soluble and insoluble PINK1 levels in the PaKO were reduced by 60% and 45%, respectively, compared with the control mice (Fig. 9B). Taken together, these results indicate that parkin overexpression causes the upregulation of endogenous PINK1 levels in dopaminergic neuroblastoma cells, and the deficiency of parkin induces the downregulation of PINK1 expression in vivo.

Discussion

There has been increasing evidences that mutation gene products in familial PD, such as PINK1, parkin and/or DJ-1, could interact and play an important role in mitochondrial function and resistance to oxidative stress in PD. For example, PINK1 physically interacts with DJ-1 and collaborates to protect cells against stress via complex formation (Tang et al., 2006). Co-expression of PINK1 and DJ-1 with PD-linked double mutation in cultured cells enhanced susceptibility to MPP+-induced cell death (Tang et al., 2006). In addition, parkin was reported to interact with a pathogenic DJ-1 mutant and to modulate the stability of the DJ-1 mutant (Moore et al., 2005a,b).

Moreover, recent studies from Drosophila demonstrated that PINK1 and parkin act in a linear genetic pathway required for the maintenance of mitochondrial function (Clark et al., 2006; Park et al., 2006), and regulates mitochondrial morphology by promoting mitochondrial fission (Deng et al., 2008; Poole et al., 2008). However, the possibility that PINK1 directly binds to parkin, and whether parkin could be a potential substrate for PINK1 kinase, or vice versa, has not been determined. The current study provides the first evidence of direct interaction between these two proteins. This study also confirms the previous finding that PINK1 and parkin are functionally linked and have a cytoprotective activity against proteasomal dysfunction-induced cell toxicity, possibly through a common mechanism.

We showed that parkin is colocalized with PINK1 in the cytoplasm (Fig. 1C), and that a considerable amount of PINK1 is localized in cytosol as well as in mitochondria (Supplementary material, Fig. S5). These results are well supported by the previous reports that PINK1 localizes either in both cytosol and mitochondria (Silvestri et al., 2005; Beilina et al., 2005; Pridgeon et al., 2007) or only in cytosol (Takatori et al., 2008; Weihofen et al., 2008). We also demonstrated that parkin causes an increase of PINK1 level in NP-40-soluble and -insoluble fraction (Fig. 4). This could occur because the ubiquitinated PINK1 level becomes dramatically reduced by parkin (Fig. 7B). It has been shown that PINK1 is degraded via a proteasomal system (Tang et al., 2006; Muqit et al., 2006; Lin and Kang, 2008). PINK1 may be presumed to be ubiquitinated by an unknown endogenous E3 ubiquitin ligase.
Fig. 8. PINK1 is upregulated in SH-SY5Y cells stably expressing wild type parkin. (A) SH-SY5Y cells stably expressing pcDNA3.1 vector as a control (Vector), Myc-tagged wild type parkin (WT) or its deleted mutant (amino acids: 1–415, MT) were fixed, permeabilized and labeled with anti-parkin (Cell signaling) or anti-PINK1 antibodies, followed by staining with FITC-conjugated goat anti-rabbit IgGs or TRITC-conjugated goat anti-mouse IgGs. Immunostained preparations were visualized by confocal microscopy. (B) The graph indicates the relative level of each protein, calculated by dividing the fluorescent intensity of each protein by the intensity of DAPI in randomly chosen fixed areas (n = 10) of three independent experiments (**p < 0.01, *p < 0.05). The fluorescent intensity was measured with a confocal microscopic analysis program. (C) Cell lysates were analyzed by immunoblotting with anti-PINK1 or anti-parkin (Cell signaling) antibodies. To determine equal loading, cell lysates were analyzed by immunoblotting with anti-Hsp90 antibody. (D) Total mRNAs were prepared from SH-SY5Y cells stably expressing pcDNA3.1 vector as a control (Con) or Myc-tagged wild type parkin (Parkin). The levels of PINK1 mRNA in each sample were measured by real time PCR. Values are normalized to β-actin level in each sample, and experiments were performed in triplicates ± S.D. “n.s” means statistically not significant.

Fig. 9. Down-regulation of PINK1 in parkin KO mice. (A) Detergent-soluble and -insoluble fraction of brain lysates from parkin KO mice (PaKO; n = 3) or age-matched control mice (Con; n = 3) were resolved by SDS-PAGE, followed by immunoblotting with anti-PINK1 or anti-parkin (Cell signaling) antibodies. As a control for equal sample loading, cell lysates were immunoanalyzed with anti-α-tubulin antibody. (B) The PINK1 level in the soluble and insoluble fraction was quantified using ImageJ program (NIH), and normalized by α-tubulin content. The graph represents the mean of three independent experiments (*p < 0.05).
which could be negatively modulated by parkin in a direct or indirect way. This speculation was further supported by the current finding that 1–415 mutant of parkin interacts with PINK1 but fails to upregulate the level of PINK1 (Fig. 3C and S8), indicating that RING2 domain of parkin is critical for the up-regulation of PINK1 as well as PINK1-binding. As the RING2 domain, including a RING–IBR–RING structure, was reported to be necessary for the intrinsic ubiquitin E3 ligase activity of parkin (Caplli et al., 2004), these results strongly suggest that the enzymatic activity of parkin is crucial to the up-regulation of PINK1.

Moreover, PINK1 induced the formation of microtubule-dependant parkin-positive aggresomes in cytoplasm (Fig. 6). It has been reported that the solubility of parkin is reduced by various stresses such as proteasomal dysfunction, PD-linked drugs or oxidative stress (Wang et al., 2005; LaVoie et al., 2005; Wong et al., 2007; LaVoie et al., 2007). However, the reduction of parkin solubility by PINK1 still occurred under treatment of antioxidants (Fig. S6), which indicated that the molecular mechanism for PINK1-induced reduction of parkin solubility is not mediated by oxidative stress or S-nitrosylation.

Although the molecular mechanism underlying the regulation of parkin solubility by PINK1 remains to be elucidated, wild type PINK1 directly binds to and possibly phosphorylates parkin, which then induces a conformational change of parkin and lead to the formation of parkin aggregates. This speculation was supported by the finding that the KD mutant of PINK1 could not bind to parkin (Fig. 3A) nor reduces parkin solubility (Fig. 4A). Furthermore, Kim et al. recently reported that PINK1 directly phosphorylates parkin at T175 residue of Linker domain (Kim et al., 2008). Understanding the role of protein inclusion in the pathogenesis of neurodegenerative diseases remains one of the key questions of neurodegeneration research (McNaught and Olanow, 2006). There have been reports that both PINK1 and parkin are localized to aggresomes under the conditions of proteasomal inhibition (Muquit et al., 2006; Junn et al., 2002). PINK1 is detected within a subset (5–10%) of LBs (Gandhi et al., 2006). In addition, parkin is co-localized with α-synuclein in LBs (Schlossmacher et al., 2002). There is increasing evidence to support the hypothesis that LBs may be formed by an aggresomal process in vivo (Olanow et al., 2004). Although the formation of protein inclusions are cytotoxic or cytoprotective depending on the cellular condition, several reports indicated that aggresomes are cytoprotective proteinaceous inclusions formed at the centrosome that segregate and facilitate the degradation of excess amounts of unwanted and possibly cytotoxic proteins (Olanow et al., 2004). Sequestration of misfolded proteins into aggresomes can allow cells to minimize misfolded protein-induced cytotoxicity (Kopito, 2000). In this context, we propose that parkin could be recruited into aggresomes and then ubiquitinate misfolded proteins in these inclusions. Concerning the neuroprotective roles of parkin and PINK1, the upregulation of PINK1 levels and the subsequent formation of parkin-mediated aggresomes could also be a cellular counteraction for survival against toxic stress. This speculation was further confirmed by the finding that parkin and PINK1 together exhibit an additive cytoprotection against MG132–induced cell death (Supplementary material, Fig. S7). Moreover, the current finding that parkin solubility is not changed by PD-linked PINK1 mutants (G309D, L347P) (Figs. 4B and 5C) suggests that cytoprotective parkin-aggresomes may be not formed when PD-linked PINK1 mutant proteins are present. Further characterization of the molecular mechanisms leading to mutual regulation of parkin and PINK1 levels will help to clarify the biological functions of PINK1.

The presented study demonstrates the functional relevance and cooperativity of PINK1 and parkin. The two proteins affect each other’s stability, solubility and tendency to form cytoprotective aggresomes. Our report suggests that both of PINK1 and parkin appear to play an important role to regulate the formation of LBs, and advances the current understanding of PD pathogenesis.

Experimental methods

Materials

 Peroxidase-, FITC-, TRITC-conjugated anti-rabbit and anti-mouse antibodies were purchased from Zymed Laboratories Inc. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), horse serum (HS) and LipofectAMINE PLUS reagents were obtained from Invitrogen. Protein A-Sepharose and Glutathione-Sepharose 4B were purchased from Amersham Pharmacia Biotech, and enhanced chemiluminescence (ECL) reagents were obtained from Perkin Elmer Life Sciences. Protein marker was from Fermentas. Clasto-Lactacystin β-lactone and MG132 were purchased from A. G. Scientific, while calpeptin and nocardazole were purchased from Calbiochem. Anti-HA, anti-GFP, anti-GST, anti-Hsp90, anti-Sp1, anti-α-tubulin, anti-ubiquitin and anti-Myc antibodies were purchased from Santa Cruz Biotechnology. Anti-Flag antibodies, N-acetylcysteine (NAC), PTIO, and E-64 were purchased from Sigma-Aldrich. A monoclonal anti-parkin antibody was purchased from Cell Signaling; polyclonal anti-parkin antibody from Chemicon; and anti-CoxVI antibody was purchased from Molecular Probes, Inc.

Rabbit polyclonal PINK1 antibodies were raised by immunizing two rabbits with synthetic peptides corresponding to unique amino acid residues at positions 157–170, which are well conserved between species such as rat, monkey, and human. Anti-serum was affinity-purified on columns against the synthetic peptides (ATGen, Korea). Mammalian expression vectors encoding for Myc-tagged human parkin, pcDNA3.1-Myc-parkin, and its deleted mutants spanning amino acids 77–465, 1–415, 217–465, 295–465 were kindly provided by K. Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). A mammalian expression vector encoding GFP-tagged wild type parkin was a gift from H. Rhim (The Catholic University, Seoul, Korea). Plasmids encoding for Myc-tagged PINK1, PINK1–GFP (wild type, kinase-inactive mutant, G309D, and L347P) and recombinant GST–PINK1, were gifts from M.R. Cookson (National Institute on Aging, MD, USA). Plasmids encoding PINK1–Flag and Flag-parkin were provided by M. Mouradian (University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, NJ, USA). Plasmid encoding Flag-Siah1 was kindly provided by W.S. Park (The Catholic University, Seoul, Korea). Plasmids encoding HA-NEDD4–1 and NEDD4-2 were provided by P.M. Snyder (University of Iowa College of Medicine, Iowa City, USA), and Flag-clAP1 by T.H. Lee (Yonsei University, Seoul, Korea).

Cell culture and preparation of cell lysates

Human embryonic kidney 293 (HEK293) cells, African green monkey kidney COS7 cells, murine neuroblastoma N2A cells and dopaminergic neuroblastoma SH-SY5Y cells were maintained in DMEM containing 10% FBS and 100 U/ml of penicillin–streptomycin. Pheochromocytoma 12 (PC12) cells were maintained in DMEM containing 5% FBS and 10% HS. To prepare cell lysates, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and solubilized in lysis buffer containing Tris, pH 7.4, 1.0% Nonidet P-40 (NP-40; USB Corporation), 150 mM NaCl, 10% glycerol, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride. To further purify the cell lysates into 1% NP-40–soluble (S) and insoluble (P) fraction, cells were lysed in lysis buffer and sedimented at 16,000 x g for 30 min at 4 °C. Supernatants from the initial fractionation were saved as S fraction. The pellets were washed once with PBS before re-extraction with SDS buffer containing 2% SDS. After sedimentation
Generation of parkin stable cell lines

SH-SY5Y cells were transfected with pcDNA3.1 plasmid vector expressing either Myc-tagged wild type parkin or its deleted mutant (amino acids: 1–415) using LipofectAMINE PLUS reagents (Invitrogen). Two days later, the cells stably transfected with parkin were selected in DMEM containing 2 mg/ml of G-418 (Sigma-Aldrich). Consequently, at least 30 clonal cells were picked; the amount of parkin expression for each cell line was measured by Western blot analysis with anti-Myc or anti-parkin antibodies. All positive cell lines used for the experiments described here were maintained in DMEM containing 500 μg/ml of G-418 to prevent extrusion of the integrated constructs.

Immunoprecipitation and Western blot analysis

One microgram of suitable antibodies was incubated with cell extracts (1 mg) prepared in lysis buffer overnight at 4 °C. A 1:1 suspension of protein A-Sepharose beads (30 μl) was added and incubated for 2 h at 4 °C with gentle rotation. The beads were pelleted and washed five times with cell lysis buffer. The immunocomplexes were dissociated by boiling in SDS-PAGE sample buffer. Whole protein samples were separated on an SDS-PAGE gel and transferred to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked in TBST buffer containing detergent and solubilized in lysis buffer at 16,000 × g for 30 min for complete lysis. The homogenate was then centrifuged at 16,000 × g for 20 min at 4 °C; the resulting pellet and supernatant fractions were collected. The pellet fraction was washed once in lysis buffer containing detergent and solubilized in lysis buffer containing 2% SDS.

Quantitative real time RT-PCR analysis of PINK1 mRNA levels

After total cellular mRNAs were isolated from SH-SY5Y cells, first strand cDNA was synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR for cDNA quantification was performed with SYBR Green PCR master mix (Applied Biosystems) and ABI 7300 Real-Time PCR System, according to the manufacturer’s instruction. The data were analyzed by 7300 System Software v 1.4.0. The primers used were as follows: human PINK1 forward primer, 5′-GAGTATCGTAGGGCGACTCCATT-3′; human PINK1 reverse primer, 5′-CTCTCTTGGATTTTCTGAAGT-3′; β-actin forward primer, 5′-TGTTCAACCTGCGAGAC-3′; β-actin reverse primer, 5′-TCTACGCTGTGAGTGAAG-3′.

Statistical analysis

Statistical significance of cell counting data or fluorescent intensity measurements was determined by unpaired Student’s t-test using Sigma Plot 9.0. Values were expressed as mean S.E.M. Acknowledgments

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References


