Parkin interacts with Klokin1 for mitochondrial import and maintenance of membrane potential

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Parkin is a multifunctional protein, including maintaining mitochondrial homeostasis. Recent evidence suggests that Parkin is recruited from the cytoplasm to damaged mitochondria with low membrane potential. We found that intracellular localization of Parkin changed with cellular growth phase. Parkin was preferentially localized in the mitochondria of cultured cells. The mitochondria with large amounts of Parkin showed preserved membrane potentials even during treatment with carbonyl cyanide m-chlorophenylhydrazone. Here we report a novel protein named Klokin 1 that transports Parkin to the mitochondria. Klokin 1 was localized to the mitochondria and enhanced mitochondrial expression of Parkin. Klokin 1 enhanced cell viability in Parkin-silenced cells. Klokin 1 expression was enhanced in the brains of Parkin-deficient mice but not in an autopsied PARK2 brain. Our findings indicate that mitochondrial Parkin prevents mitochondrial depolarization and that Klokin 1 may compensate for Parkin deficiency.

INTRODUCTION

Parkinson’s disease (PD) is a prevalent age-associated progressive neurodegenerative movement disorder primarily characterized by the death of nigrostriatal dopaminergic neurons and the presence of intracytoplasmic proteinaceous inclusions, termed Lewy bodies. Although PD is a sporadic disorder of unclear etiology, recent studies have demonstrated the importance of genetic contributions to PD that may provide insight into the mechanistic details of disease pathogenesis. Mutation of the gene-encoding Parkin (PARK2) plays a major etiopathogenic role in autosomal recessive juvenile parkinsonism (1). PARK2 contains RING finger motifs and functions as a ubiquitin–protein ligase for protein degradation (2,3).

Recently, important insight has been obtained into the mechanism by which Parkin regulates mitochondrial homeostasis. Parkin translocates from the cytoplasm to accumulate on depolarized mitochondria and promotes their degradation by autophagy (4). Several studies have suggested that PTEN-induced putative kinase 1 (PINK1) is required for Parkin-mediated mitochondrial autophagy, wherein it recruits Parkin to dysfunctional mitochondria and promotes their degradation (5–7). However, we and others have reported that Parkin can associate directly with mitochondria under basal conditions (8–10). Recent studies have detected Parkin in the mitochondria of untreated cultured cells, although it is mainly present in the cytoplasm (5,10–12). We previously reported that Parkin is localized in the mitochondrial matrix during proliferation and is rapidly released to the cytosol in...
differentiated or quiescent states. We also found that Parkin enhances mitochondrial transcription and replication in vitro and in vivo (9), which was confirmed by a recent study (10).

Here we report that Parkin is present in both the cytoplasm and mitochondria at basal conditions and that its intracellular localization changes with growth phase. Parkin was diffusely located in the cytoplasm in the early culture phase, but a portion of Parkin appeared to be located in the mitochondria of mature cells. Unlike previous reports, its mitochondrial localization was not associated with reduced membrane potential during the log growth phase. We found a novel protein—Klokin 1—that transports Parkin to the mitochondria. Klokin 1, a splice variant of human chondroitin polymerizing factor (ChPF), may attenuate cellular apoptosis with or without Parkin.

RESULTS
Intracellular localization of Parkin changes with cellular growth phase

We mainly utilized a cell line derived from kidney cells, COS-1, and a cell line derived from cervical cancer cells, HeLa, in this study because they are highly transfectable, making them convenient for statistical analyses. The endogenous Parkin signal was weak in COS-1 and HeLa cells and insufficient for intracellular localization analysis (Supplementary Material, Fig. S1). Low expression of endogenous Parkin in HeLa cells has also been reported in a previous study (13). The authors reported that carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment recruits Parkin from the cytoplasm to the mitochondria in HeLa cells (13). In the present study, we identified novel intracellular dynamics of Parkin in COS-1 and HeLa cells using exogenously expressed green fluorescent protein (GFP)-Parkin.

After resuscitation of frozen cell lines, cells initially pass through a quiescent or lag phase and then enter the log phase, which is followed by a plateau phase. We observed the expression pattern of GFP-Parkin in cultured cells during these growth phases. GFP-Parkin was diffusely localized in the cytoplasm in the early culture phase. A portion of Parkin appeared to be located in the mitochondria in COS-1 cells (3.73 ± 0.11%, mean ± SE) and HeLa cells (1.33 ± 0.11%). Therefore, GFP-Parkin was localized in the cytoplasm in the majority of cells (termed C-type) and in the mitochondria of small portion of cells (termed M-type). The mitochondrial localization gradually increased with cellular growth. M-type cells showed strong GFP signals compared with C-type cells (Fig. 1A). Mitochondrial membrane potentials were assessed by measuring tetramethylrhodamine methyl ester (TMRM) signals. TMRM signals were markedly stronger in M-type cells than in C-type cells (Fig. 1B). We quantified the GFP signal per cell for M- and C-type cells. Because GFP signal/cell was >16 000 in the majority of M-type cells and <16 000 in the majority of C-type cells (Fig. 1C), a signal over 16 000 was defined as ‘High Signal’ and a signal under 16 000 was defined as ‘Low Signal’. From the late log phase to the plateau phase, GFP-Parkin was detected in the mitochondria of 24.94 (+1.21%) of COS-1 cells and 23.27 (+1.15%) of HeLa cells (Fig. 1D). GFP signals of M-type cells with ‘High Signal’ accounted for 95.2% of COS-1 cells and 96.8% of HeLa cells from a total of 500 cells. This is concordant with previous immunoblotting results that revealed Parkin in the mitochondrial fraction (9). We determined whether endogenous Parkin was localized in the mitochondria of cell lines other than COS-1 and HeLa cells. Unlike in a previous study (4), endogenous Parkin expression was found to be relatively weak in a human embryonic kidney cell line, HEK293 (Supplementary Material, Fig. S1). However, we clearly detected endogenous signals for Parkin in a neuroblastoma cell line, SH-SY5Y, and a rhabdomyosarcoma cell line, RD cells that preferentially corresponded to mitochondria at the late log growth phase (Supplementary Material, Fig. S1).

Mitochondrial Parkin prevents CCCP-induced mitochondrial depolarization

Mitochondrial localization of Parkin gradually increased after the early log growth phase. We determined whether CCCP treatment changed the initial mitochondrial localization. After CCCP treatment, M-type cells increased in number and the GFP signal levels ranged from strong to weak (Fig. 1A and C). In addition, cells with ‘High’ GFP signal levels showed high TMRM signal levels (Fig. 1B). GFP signal levels decreased after CCCP treatment, but 25.9% of cells showed ‘High Signal’ (Fig. 1C). CCCP treatment increased the number of M-type cells with ‘Low Signal’ and decreased the number of C-type cells (Fig. 1D). M-type cells with ‘High Signal’ showed TMRM signals 19 and 16 times larger than C-type cells (COS-1, \(P = 0.00063\); HeLa, \(P = 0.00082\)) (Fig. 1E). However, M-type cells with ‘Low Signal’ showed similar TMRM signal levels to C-type cells (COS-1, \(P = 0.134\); HeLa, \(P = 0.0414\)). CCCP-induced increase in number of M-type cells with ‘Low Signal’ suggests that Parkin has been translocated to mitochondria in the cells. These results are consistent with previous findings that CCCP treatment reduces mitochondrial membrane potential and recruits Parkin from the cytoplasm to the mitochondria (4). Our results suggest that Parkin translocates from the cytoplasm to depolarized mitochondria and that the GFP signal in depolarized mitochondria was weak and could be easily distinguished from the GFP signal in mitochondria where Parkin was initially present. Parkin initially located in the mitochondria seemed to prevent CCCP-induced mitochondrial depolarization.

Parkin associates with Klokin 1, a splice variant of ChPF

Because Parkin lacks a mitochondrial targeting sequence (MTS), it seems likely that an unidentified protein transports Parkin to the mitochondria. We searched for human Parkin-associated proteins using a yeast two-hybrid system. From ~10^7 transformants containing cDNA, 118 positive colonies were identified and 12 clones with MTS were obtained. We named them Klokin 1–12 and subcloned them into the pCMV-HA vector. Klokin 1 was associated with Parkin in human cells, and this endogenous protein was detected in human tissues. We identified Klokin 1 (HSI10665; GenBank accession number AK026331) as being a novel Parkin-interacting protein. A basic local alignment search tool analysis of the GenBank database revealed that Klokin 1 is homologous in its 3’-end with ChPF (GenBank accession number AB095813)
Figure 1. Intracellular localization of exogenous Parkin. (A) Mitochondria were visualized with CMXRos. GFP-Parkin was localized in the cytoplasm of some cells (termed C-type) and in the mitochondria of other cells (termed M-type). Cells of M-type showed strong GFP signals compared with those of C-type. After CCCP treatment, M-type cells were predominant and GFP signal levels ranged from strong to weak. Scale bars, 10 μm. (B) Mitochondrial membrane potential was visualized with TMRM. TMRM signals were markedly stronger in M-type cells compared with C-type cells. CCCP-treated cells with high GFP signal levels showed high TMRM signal levels. Scale bars, 10 μm. (C) Histogram of GFP signals per cell for M- and C-type cells. GFP signal/cell was >16 000 in the majority of M-type cells and <16 000 in the majority of C-type cells. A signal over 16 000 was defined as ‘High Signal’ and a signal under 16 000 was defined as ‘Low Signal’. GFP signal levels decreased after CCCP treatment, but 25.9% of M-type cells still showed ‘High Signal’. (D) Ratio of M- and C-type cells. From the late log phase to the plateau phase, GFP-Parkin was detected in the mitochondria of 24.94 ± 1.21% (mean ± SE) of COS-1 cells and 23.27 ± 1.15% of HeLa cells. CCCP treatment increased the number of M-type cells with ‘Low Signal’ and decreased the number of C-type cells. (E) Relative TMRM signal in M- and C-type cells. M-type cells with ‘High Signal’ showed TMRM signals 19 and 16 times larger than C-type cells (COS-1, \( P = 0.00063 \); HeLa, \( P = 0.00082 \)). However, M-type cells with ‘Low Signal’ showed TMRM signals similar to C-type cells (COS-1, \( P = 0.13 \); HeLa, \( P = 0.0414 \)).
Klokin 1 lacks the 5′-terminal transmembrane domain of ChPF. However, the N-terminal sequences of Klokin 1 are rich in positive charges, indicating MTSs (Fig. 2B). We also identified another splice variant of ChPF, named ChPF<sub>D996</sub> (GenBank accession number AL136814). ChPF<sub>D996</sub> lacks the 5′-terminal transmembrane domain of ChPF but contains the latter part of the ChPF domain. Klokin 1 and ChPF<sub>D996</sub> cDNAs were found in the human leukocyte Marathon Ready cDNA library (data not shown). To confirm that Klokin 1 and ChPF<sub>D996</sub> are splice variants of ChPF, we performed an immunoblot analysis using an anti-Klokin 1 antibody. When ChPF, ChPF<sub>D996</sub> and Klokin 1 were overexpressed in COS-1 cells, three, two and one protein bands were detected, respectively (Supplementary Material, Fig. S2A). These bands (95, 70 and 37 kDa) were detected in peripheral blood mononuclear cells, RD and SH-SY5Y cells. We examined protein expression of the ChPF family in human tissues (Supplementary Material, Fig. S2B). ChPF and ChPF<sub>D996</sub> were detected in the majority of tissues, and additional indistinct bands at 80–90 kDa were also present. Klokin 1 expression was limited to tissues of muscle, brain, spleen, ovary and testis. The immunoblotting results indicate that the splicing patterns of the ChPF family and their individual expression vary in tissues, and that there are unidentified members of the family.

Intracellular localization of Klokin 1

We examined the association of Klokin 1 with Parkin in the mitochondria of COS-1 cells cotransfected with His-Parkin and HA-Klokin 1. Immunoprecipitation results revealed that His-Parkin associates with HA-Klokin 1 in the mitochondrial fraction (Supplementary Material, Fig. S2C). Similar results were obtained in the total cell lysate. The endogenous association of Klokin 1 with Parkin was also observed in mouse brain tissue (Supplementary Material, Fig. S2D and E) and human skeletal muscle tissue (data not shown). Next, we examined the intracellular localization of Klokin 1 in SH-SY5Y cells transfected with Klokin 1. Klokin 1 was only detected in the mitochondrial fraction, but ChPF and ChPF<sub>D996</sub> were mainly detected in the crude cytosolic fraction. To determine its precise localization, the mitochondrial fraction was partially digested with proteinase K (0.05 mg/ml). The immunoreactivity pattern of Klokin 1 was similar to that of the inner membrane protein ANT1. However, the signals for ChPF and ChPF<sub>D996</sub> disappeared rapidly.

Intracellular localization of Klokin 1

Figure 2. Characterization of the ChPF family. (A) Genetic organization of ChPF, ChPF<sub>D996</sub> and Klokin 1. Exon regions are denoted by boxes. Closed boxes represent the coding sequence. ChPF has a transmembrane domain (TMD) at its 5′ end, but ChPF<sub>D996</sub> and Klokin 1 lack the TMD. The two boxes with slanted lines show the regions homologous to β-1,3-Gal transferase (β-1,3-Gal T) and β-1,4-Gal transferase (β-1,4-Gal T). (B) Amino acid sequence of Klokin 1 in the N-terminal region. There are five arginines in the 30 amino acids of the N-terminal end, indicating a MTS. (C) Intracellular localization of Klokin 1. Immunoblotting of Klokin 1 in SH-SY5Y cells transfected with His-Klokin 1. Klokin 1 was detected in the mitochondrial fraction but not in the cytosolic (nonmitochondrial) fraction. A weak signal was detected in the nuclear fraction. The respiratory chain complex 3 was only detected in the mitochondrial fraction, but β-COP was detected in the crude cytosolic fraction. Histone H2B was only detected in the nuclear fraction. (D) Immunoblotting of endogenous ChPF/ChPF<sub>D996</sub>/Klokin 1 in SH-SY5Y cells. Klokin 1 was only detected in the mitochondrial fraction, but ChPF and ChPF<sub>D996</sub> were mainly detected in the crude cytosolic fraction. (E) Limited digestion of mitochondria with proteinase K (0.05 mg/ml). The immunoreactivity pattern of Klokin 1 was similar to that of the inner membrane protein ANT1.
proteinase K. The immunoreactivity pattern of Klokin 1 was similar to that of the inner membrane protein adenine nucleotide translocator 1 (ANT1) (Fig. 2E), but the immunoreactivity patterns of ChPFΔ996 and ChPF were similar to those of cytochrome c (Cy-c) and voltage-dependent anion channel 1 (VDAC1), respectively. These results indicate that Klokin 1 is located only within the mitochondria. Similar results were obtained by partial trypsin treatment (data not shown). We previously reported that Parkin is detectable inside the inner mitochondrial membrane under proliferative conditions (9). Our findings suggest that Klokin 1 is associated with Parkin inside the inner mitochondrial membrane. We microscopically examined the endogenous expression of Klokin 1 in various cell lines. Klokin 1 in the mitochondria was mainly detected in SH-SY5Y and RD cells but rarely in COS-1, HeLa and HEK293 cells (data not shown). This endogenous expression pattern is similar to that of Parkin. We also found that endogenous signals for Parkin and Klokin 1 colocalized in mitochondria of SH-SY5Y and RD cells (Supplementary Material, Fig. S3A). In addition, signals for GFP-Parkin and GFP-Klokin 1 exactly corresponded to those of mitochondria in COS-1 cells (Supplementary Material, Fig. S3B). Some signals for GFP-ChPF and GFP-ChPFΔ996 were detected in the mitochondria, while some signals were detected outside the mitochondria (Supplementary Material, Fig. S3B). These results are consistent with the immunoblot (Fig. 2C and D) and the partial digestion results (Fig. 2E).

**Klokin 1 enhances the mitochondrial localization of Parkin**

We determined whether endogenous expression of Klokin 1 changes in M-type COS-1 cells with strong GFP-Parkin expression. Tetramethylrhodamine isothiocyanate (TRITC) signals for Klokin 1 were markedly stronger in M-type cells than C-type cells or GFP-Parkin-negative cells (Fig. 3A). While endogenous Klokin 1 expression was relatively weak under basal conditions, the expression seemed to be enhanced in response to Parkin overexpression. In addition, signals for Tom 20 were preferentially strong in M-type cells. This is concordant with our observation that Parkin enhances mitochondrial biogenesis (9).

We next determined whether Klokin 1 affects the intracellular localization of Parkin by cotransfected discosoma red (DsRed)-Parkin and GFP-Klokin 1. Figure 3B shows the localization of DsRed-Parkin and GFP-Klokin 1. Strong expression of GFP-Klokin 1 was detected in the cells with M-type expression of DsRed-Parkin. The cells with C-type expression of DsRed-Parkin did not express GFP-Klokin 1. The right graph of Figure 3B shows the expression pattern of GFP-Klokin 1 in DsRed-Parkin-positive cells. The cells that showed M-type patterns of both DsRed-Parkin and GFP-Klokin 1 were the most common (35%). The cells that showed a C-type expression pattern of DsRed-Parkin tended not to express GFP-Klokin 1. When only GFP-Parkin was transfected into COS-1 cells, 25% of cells displayed an M-type expression pattern (Fig. 1D). In contrast, when DsRed-Parkin and GFP-Klokin 1 were cotransfected, 64% of cells showed an M-type expression pattern of GFP-Parkin.

We examined the effect of Klokin 1 on the intracellular localization of Parkin by interfering with Klokin 1 expression. We prepared three small-interfering RNA (siRNA) molecules (RNAs 1–3) to attenuate Klokin 1 expression. Exogenous Klokin 1 was attenuated by RNA3 treatment down to 3–10% (data not shown). We confirmed the effect of Klokin 1 knockdown at mRNA level. Results of real-time polymerase chain reaction demonstrated that RNA3 attenuated Klokin 1 mRNA down to 24.8% but not glyceraldehyde 3-phosphate dehydrogenase mRNA (data not shown). The strong signals of GFP-Parkin were attenuated in cells treated with RNA3 (Fig. 3C). The proportion of cells with M-type Parkin expression significantly decreased from 25.48% (± 5.895%) to 10.17% (± 1.37%) after RNAi treatment (\( P = 0.00029 \)).

**Klokin 1 may transport Parkin to the mitochondria**

We examined the in vitro import of Parkin into mitochondria. For this study, His-Parkin and His-Klokin 1 were purified from *Escherichia coli* (Fig. 4A). As shown in Figure 4B, a portion of His-Parkin was imported into mitochondria with proteinase K treatment in the absence of His-Klokin 1 (lane 1). Similar results were reported in our previous study (9). On the other hand, Klokin 1 itself is easily imported into mitochondria (lane 8) (0.869 ± 0.045), and the mitochondrial import ratio of His-Klokin 1 (lane 5) was greater than that of His-mitochondrial transcription factor A (TFAM) (0.585 ± 0.022, \( P = 0.00395 \)), a major mitochondrial transcription factor. When His-Klokin 1 was added to the reaction mixture containing His-Parkin and mitochondria, the mitochondrial import ratio of His-Parkin was enhanced in a dose-dependent manner (lanes 2–4). These results suggest that Parkin transport is facilitated by Klokin 1. We examined the intracellular localization of Parkin in Klokin 1-silenced cells. Endogenous Parkin expression was reduced in the mitochondria and enhanced in the cytoplasm (Fig. 4C). However, the expression of β-COP and mitochondrial respiratory chain complex 3 did not change. This result is similar to that of the microscopic analysis (Fig. 3C). We determined whether overexpression of Klokin 1 enhances endogenous Parkin expression in the mitochondria of COS-1 cells. Figure 4D shows the densitometric analysis of immunoblotting signals for Parkin and β-actin. The relative mitochondrial expression of Parkin (mean ± SE) was evidently increased to 2.53 ± 0.25 in cells that overexpressed Klokin 1 (\( P = 0.00537 \)). Overexpression of Klokin 1 did not significantly enhance mitochondrial expression of β-actin and mitochondrial respiratory chain complex 3 (data not shown).

**Parkin and Klokin 1 enhance cell viability and protect cells from oxidative stress**

Because Parkin has a protective function via mitochondria (15–17), we compared the anti-apoptotic effect of Parkin with that of Klokin 1. As shown in Figure 5A, Parkin and Klokin 1 significantly enhance cell viability in cultured cells treated with H2O2 (Parkin, 1.93 ± 0.05; Klokin 1, 2.21 ± 0.03) and untreated cells (Parkin, 4.79 ± 0.04; Klokin 1, 3.61 ± 0.06). We examined whether these cyto-protective effects are independent of each other. We used siRNA to reduce endogenous Parkin expression to 10% (9). In Parkin-silenced cells, the relative cell viability decreased to 0.63 ±
When endogenous Parkin was reduced, Klokin 1 enhanced cell viability ($0.90 \pm 0.04$) (Fig. 5B). Next, we examined whether Parkin enhanced cell viability in Klokin 1-silenced cells. Parkin transfection decreased cell viability when endogenous Klokin 1 was silenced by siRNA ($0.70 \pm 0.048$, $P = 0.000778$) (Fig. 5C). However, Parkin transfection significantly increased cell viability ($3.30 \pm 0.035$, $P = 0.000778$) in scrambled RNA-transfected cells (Fig. 5D). This suggests that the protective function of Parkin is dependent on the presence of Klokin 1. We next examined the cell viability of mouse embryonic fibroblast (MEF) cells from Parkin$^{+/+}$ and Parkin$^{-/-}$ mice using the nucleofection technique for siRNA. In MEF cells from Parkin$^{+/+}$ mice, knockdown of Parkin and/or Klokin 1 significantly reduced cell viability ($P < 0.001$) (Fig. 5E). In MEF cells from Parkin$^{-/-}$ mice, knockdown of Klokin 1 significantly

Figure 3. Intracellular association between Parkin and Klokin 1. Nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI). Scale bars, 10 μm. (A) Endogenous expression of Klokin 1 in COS-1 cells with transfection of GFP-Parkin. Signals for Klokin 1 were markedly stronger in M-type cells than C-type cells or GFP-Parkin-negative cells. Signals for Tom 20 were preferentially strong in M-type cells. (B) Cotransfection of DsRed-Parkin and GFP-Klokin 1. The localization of DsRed-Parkin and GFP-Klokin 1. Strong expression of GFP-Klokin 1 was detected in the cells with M-type expression of DsRed-Parkin. The cells with C-type expression of DsRed-Parkin did not express GFP-Klokin 1. The right graph shows the expression pattern of GFP-Klokin 1 in DsRed-Parkin-positive cells. The cells that showed M-type patterns of both DsRed-Parkin and GFP-Klokin 1 were the most common (35%). The cells that showed a C-type expression pattern of DsRed-Parkin tended not to express GFP-Klokin 1. When DsRed-Parkin and GFP-Klokin were cotransfected, 64% of cells showed an M-type expression pattern of GFP-Parkin. (C) Intracellular localization of Parkin in Klokin 1-silenced cells. Strong signals of GFP-Parkin, which correspond to mitochondria, were attenuated in Klokin 1-silenced cells. The proportion of cells with M-type expression of Parkin significantly decreased from 25 to 10% after RNAi treatment ($P = 0.00029$). Conversely, RNAi treatment increased the amount of C-type cells.
reduced cell viability \( (P = 0.00815) \) but knockdown of Parkin did not \( (P = 0.597) \) (Fig. 5F).

**Klokin 1 and ChPF overexpress in Parkin-deficient mice but not in human PARK2 brain**

Klokin 1 was widely distributed throughout the nerve cells in mouse brains, including the substantia nigra, striatum, cerebral cortex and hippocampus (Fig. 6A). Klokin 1 was detected in tyrosine hydroxylase (TH)-positive nerve cells in the substantia nigra in Parkin\(^{+/+}\) and Parkin\(^{-/-}\) mice (Fig. 6B), but its intracellular localization was different (Fig. 6A and B). Signals for Klokin 1 were observed at the interior region of cell membranes in Parkin\(^{+/+}\) mice but were diffuse in Parkin\(^{-/-}\) mice. The peripheral pattern significantly increased in Parkin\(^{+/+}\) mice \( (P = 0.036) \) and the diffuse pattern increased in Parkin\(^{-/-}\) mice \( (P = 0.001) \) (Fig. 6C). Immunoblot results demonstrated that the expression of Klokin 1,
ChPF and ChPF, but not β-actin or DJ-1, increased in the whole brain of Parkin+/+ mice (Fig. 6D). We examined the relationship with intracellular localization of Klokin 1 and the mitochondrial markers, VDAC1 and Tom 20. Signals for Klokin 1 were preferentially observed at the submembranous mitochondria of cells in Parkin+/+ mice, but those were exactly corresponded to the mitochondria in Parkin−/− mice (Supplementary Material, Fig. S4A). We then determined whether the expression of Klokin 1 was enhanced in an autopsy specimen from a human PARK2 brain. Klokin 1 was also widely expressed in the neurons from a human brain. Unlike Parkin−/− mice, their expression was not
Figure 6. Expression of Klokin 1 and ChPF in Parkin-deficient mice and human PARK2 brain. (A and B) Immunohistochemical staining of Klokin 1 in the mouse brain. (A) Klokin 1 was widely distributed in nerve cells in a mouse brain. The expression was enhanced in Parkin$^{-/-}$ mice compared with Parkin$^{+/+}$ mice. Bar: upper panel, 100 $\mu$m; lower panel 20 $\mu$m. (B) Double staining of Klokin 1 and tyrosine hydroxylase (TH). Klokin 1 were detected in TH-positive nerve cells in the substantia nigra. Signals for Klokin 1 were observed at the interior region of cell membranes in Parkin$^{+/+}$ mice but were diffuse in Parkin$^{-/-}$ mice. Scale bars, 20 $\mu$m. (C) Two distribution patterns of Klokin 1. The prevalence of the ‘peripheral pattern’ was significantly increased in Parkin$^{+/+}$ mice, and the ‘diffuse pattern’ was increased in Parkin$^{-/-}$ mice. (D) Immunoblotting for Klokin 1, ChPF$^{\Delta 996}$ and ChPF in the whole brains of mice. Signals for Klokin 1, ChPF$^{\Delta 996}$ and ChPF were enhanced in Parkin$^{-/-}$ mice compared with Parkin$^{+/+}$ mice. (E) Immunohistochemical staining of Klokin 1 in the human brain. Klokin 1 was widely distributed in the nerve cells of a human brain. Its expression was not enhanced in a PARK2 brain compared with controls ($n=2$). Scale bars, 20 $\mu$m. (F) Immunoblotting for Klokin 1, ChPF$^{\Delta 996}$ and ChPF in peripheral mononuclear cells from patients with PARK2 and control subjects. Signals for Klokin 1, ChPF$^{\Delta 996}$ and ChPF were not enhanced in patients with PARK2.
enhanced in the PARK2 brain (Fig. 6E). In the control brain, signals for klokin 1 were colocalized with those for Tom 20 and Parkin. In the PARK2 brain, signals for Klok 1 and Tom 20 were colocalized but Parkin signals were barely detected (Supplementary Material, Fig. S4B). The expression levels of Klok 1, ChPF\textsuperscript{3996} and ChPF in peripheral leukocytes were similar for patients with PARK2 mutations and control subjects but Parkin expression was greatly reduced in patients (Fig. 6F).

**DISCUSSION**

Several biochemical abnormalities have been implicated in the pathogenesis of PD, including mitochondrial dysfunction, free radical-mediated damage, excitotoxicity and inflammatory changes (18). Mitochondrial dysfunction has been implicated in the pathological feature associated with PD, i.e. mitochondrial complex I deficiency appears to be responsible for nigral degeneration (19,20). Significant insight into the cellular processes that rely on Parkin function has been obtained from studies of Parkin loss-of-function mutations, which have demonstrated a protective role for Parkin within mitochondria. Characterization of Parkin mutant animal models has revealed prominent mitochondrial defects (21). In human and non-human brain tissues, Parkin shows various intracellular localizations, including the Golgi apparatus, synaptic vesicle, endoplasmic reticulum, nucleus and mitochondria (22–25). Recently, Parkin was found to associate with functionally impaired and depolarized mitochondria (4,26,27). Several studies have revealed Parkin-mediated mitochondrial autophagy and that PINK1 is required to recruit Parkin to the mitochondria (4,12,28,29).

In several cellular models, Parkin is predominately located in the cytoplasm under basal conditions and translocates to the mitochondria upon reduction in mitochondrial membrane potentials by CCCP or in response to reactive oxygen species (4,12,13,28). In contrast, we and others have observed that Parkin is mainly detected in the mitochondrial fraction in cultured cells under basal conditions (9,10). More recently, Parkin has been found to be localized to the mitochondria in mammalian and Drosophila cells (5,30). In rat cortical neurons, 20–25\% of cells showed evidence of Parkin localized to their mitochondria under basal culture conditions. Mitochondrial depolarization by CCCP does not induce Parkin translocation to the mitochondria or mitophagy (31). The Parkin mitophagy pathway may be heavily dependent on the cellular bioenergetic state (31).

In the present study, we aimed to first clarify the localization of Parkin. We found that the intracellular localization of Parkin changed with the cellular growth phase. Parkin was mainly detected in the cytoplasm during the early culture period but was present in the mitochondria of cells in the maturity period. GFP-Parkin signals were exceptionally strong in the mitochondria of COS-1, HeLa, HEK293, L-6, RD and SH-SY5Y cells. We observed that CCCP treatment recruited cytosolic Parkin to the mitochondria, consistent with previous reports (4,12,13,28) and the mitochondria to which Parkin was translocated showed relatively weak signals for GFP-Parkin. However, the mitochondria in which Parkin was initially present showed strong GFP-Parkin signals. High mitochondrial expression of Parkin seemed to maintain membrane potential and prevent CCCP-induced mitochondrial depolarization. We and others have found that Parkin is directly linked with the mitochondrial genome and enhances mitochondrial replication or transcription (9,10). These results indicate that there is an active pathway to guide Parkin to the inside of mitochondria.

Most mitochondrial proteins have a specific N-terminal pre-sequence that allows recognition by the mitochondrial import apparatus and subsequent import into mitochondria (14). Because Parkin lacks an MTS, we hypothesized that an unidentified protein transports Parkin to the mitochondria. We identified this protein, which we named Klok 1. It is a splice variant of ChPF, but it differs from ChPF in its intracellular localization and function. Klok 1 has an MTS and can be easily imported into mitochondria. In the presence of Klok 1, Parkin moves into the mitochondria. Klok 1 also enhances the mitochondrial localization of Parkin. Mitochondrial proteins with MTSs are usually imported into mitochondria by a general import pathway (32). However, some mitochondrial proteins do not follow this mechanism. For example, MTF1, the yeast mitochondrial transcription factor, is located in the mitochondrial matrix but lacks a typical MTS even in the precursor form. Import of MTF1 occurs through an unknown pathway that does not require the receptor on the outer mitochondrial membrane, the membrane potential across the inner membrane or ATP hydrolysis (33,34). Further studies are necessary to clarify the pathway through which Parkin and Klok 1 are transported into the mitochondria.

ChPF was discovered as a necessary factor to polymerize chondroitin (35). Although Klok 1 is a splice variant of ChPF, there are structural and localization differences. Parkin is localized inside mitochondria, but ChPF/ChPF\textsuperscript{Δ996} is mainly localized outside mitochondria. Mitochondrial localization of Parkin was accompanied by endogenous enhancement of Klok 1 expression and Klok 1 increased mitochondrial Parkin expression, indicating that Klok 1 is essential to mitochondrial localization of Parkin. We found that Klok 1 had anti-apoptotic effects on cultured cells even under oxidative stress. The function of Parkin was inhibited in Klok 1-deficient cells. This function probably depends on the presence of Klok 1. However, Klok 1 enhanced cell viability in Parkin-deficient cells. This suggests that Klok 1 protects cells against apoptotic stimuli even in the absence of Parkin. Therefore, Klok 1 appears to directly protect mitochondria in addition to its indirect effects through Parkin. Klok 1 appears to compensate for the cellular dysfunction induced by Parkin deficiency. We determined whether expression of Klok 1 was altered in the brain tissues of Parkin null mice and humans. Klok 1 and ChPF/ChPF\textsuperscript{Δ996} were overexpressed in the brains of Parkin-deficient mice, but their expression was not enhanced in the human PARK2 brain. Klok 1 is likely overexpressed to compensate for neuronal loss in Parkin-deficient mice. This may be why Parkin-deficient mice have milder symptoms and pathological changes than humans and are not a robust model of familial PD (36,37). Taken together, the loss of compensatory expression of Klok 1 and ChPF may be related to neuronal loss in brains of PARK2 patients.
In conclusion, our results suggest that mitochondrial Parkin prevents mitochondrial depolarization. Klokin 1 may import Parkin into the mitochondria to facilitate mitochondrial biogenesis and compensates for Parkin deficiency.

**MATERIALS AND METHODS**

**Cell culture and reagents**

COS-1, HeLa, HEK293, SH-SY5Y, RD and L6 cells were cultured at 37°C (5% CO₂) in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-incubated fetal bovine serum. MEF cells were cultured in the rat fibroblast growth medium (Cell Application, San Diego, CA, USA). Intracellular localization was analyzed using MitoTracker Red, CMXRsos (100 nM for staining of mitochondria, Molecular Probes) and 4,6-diamino-2-phenylindole (DAPI) (1 μg/ml for staining of nuclei, Dojindo, Japan). For mitochondrial depolarization, CCCP (10 μM) was added to the culture medium for 6 h at 37°C. For live cell imaging, cells were treated with 30 nM TMRM (Invitrogen Corp., CA, USA) for 30 min to evaluate mitochondrial membrane potential.

**Mouse and human samples**

Materials included brain samples from mice and humans. Mice used as experimental tests and controls were littermates. MEF cells were generated from Parkin⁻/⁻ mice, which have been characterized previously (38). The brain autopsy tissue was obtained from a patient with PARK2 lacking exon 3 (39,40). Peripheral leukocytes were obtained from two patients with PARK2 lacking exons 3–4 (41) and from 10 normal controls.

**Yeast two-hybrid screen**

A yeast two-hybrid analysis was performed with a Matchmaker Two-Hybrid System 3. A pre-transformed cDNA library of human fetal brain and human skeletal muscle was constructed in a Saccharomyces cerevisiae host strain, Y187 (Clontech Laboratories, Inc., CA, USA). A cDNA library of SH-SY5Y cells transiently transfected with Parkin was constructed using a Matchmaker Library Construction & Screening Kit (Clontech). The bait for library screening was full-length human Parkin cDNA fused to a GAL4-DNA-binding domain (GAL4 BD) in the pAS2 vector. A total of 118-positive colonies were identified following the instructions provided by the manufacturer.

**Cell transfection and nucleofection**

cDNA was subcloned into pEGFP-C2 (Clontech), pcDNA3.1(+) (Invitrogen), pDsRed1-C1-Vector (Clontech), pcDNA4/HisMax (Invitrogen) or pCMV-HA (Clontech). Cells were transfected using Lipofectamine™ 2000 or Lipofectamine™ LTX Reagent (Invitrogen). For MEFs, nucleofection was performed using the Nucleofector Kit 1 (Amaxa, Gaithersburg, MD, USA) according to the manufacturer’s protocol. Morphological and immunoblot analyses were performed 48 h after transfection.

**RNAi treatment**

For Klokin 1 interference, three pairs of 25 nucleotide sense and antisense RNA oligomers were designed and chemically synthesized by Invitrogen Japan. The oligonucleotides were as follows: RNA1: sense, 5′-AAACGCUAGCCUCCGUGA AGUAGU-3′ and antisense, 5′-ACUACUUCACOGACAGCAC GCUUU-3′; RNA2: sense, 5′-AUCAAAAGCGCUAGU GAACUACC-3′ and antisense, 5′-UGUGAAUGGGCUACC GACCUUUGAU-3′; and RNA3: sense, 5′-AAUGUAGGA GAAAGAGGCUGCC-3′ and antisense, 5′-GGCAGGCC UUCUUUCCUGCAUU-3′. Cells were cultured in six-well plates, and the targeting siRNA duplex and scrambled siRNA duplex were transfected with Lipofectamine™ 2000. To test the silencing effect of siRNA on exogenous Klokin expression, GFP-Klokin was cotransfected with either the targeting siRNA or scrambled siRNA duplex. The effects of siRNA were evaluated 48 h after transfection with the siRNA duplex at 0.2 mg/well (15 nM) and 1.0 mg/well (75 nM). For the silencing of Parkin, we used a 22-mer siRNA duplex corresponding to nts 845–866 of the Parkin coding region, as previously reported (9).

**Immunological analysis**

We used three rabbit antibodies against Parkin. Ab-1 (Oncogene, San Diego, CA, USA), Ab-293 (from our laboratory) and Ab-2132 (Cell Signaling Technology, MA, USA) were produced against synthetic peptides corresponding to amino acid residues 81–98, 293–306 and ~400 of human Parkin, respectively. Monoclonal antibodies against Parkin (PRK8), Tom 20 (Clone 29) and respiratory chain complex 3 (13G12-AF12-BB11) were purchased from Sigma-Aldrich, Santa Cruz, USA, and Molecular Probes, respectively. Monoclonal antibodies against β-COP and γ-adaptin and goat polyclonal antibodies against VDAC1 and ANT1 were purchased from Sigma. Monoclonal anti-histone H2B antibody was purchased from GenWay Biotech (CA, USA). To generate anti-Klokin 1 antibody, polyclonal antiserum against synthetic polypeptide GPGPPELGRDTRFDRGC were raised in New Zealand white rabbits and affinity purified.

For immunocytochemical analysis, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 12 min and treated with 0.5% Triton X-100 in PBS for 5 min. After blocking with 5% bovine serum albumin (BSA) in PBS for 20 min, cells were incubated with primary antibodies. Control experiments were performed using normal rabbit or mouse serum instead of primary antibodies. Secondary antibodies included fluorescein isothiocyanate-conjugated anti-mouse IgG and TRITC-conjugated anti-rabbit IgG.

For immunohistochemical analysis, 25 μm thick sections were incubated in PBS containing 3% BSA and rabbit anti-Klokin 1 antisera. We used the Histofine Simple Stain Kit (Nichirei Co., Tokyo, Japan) and the tyramide signal amplification (TSA) system with Cyanine 3 (NEL744; PerkinElmer Inc., Shelton, CT, USA). Staining with the TSA-Cyanine 3 system was performed according to the manufacturer’s instructions. For the double immunostaining, the sections were first labeled for Klokin with the TSA-Fluorescein system (NEL741001; PerkinElmer) and then labeled with
Coimmunoprecipitation analysis

COS-1 cells were transfected with 3 μg of pcDNA4/His-Max-Parkin and pCMV-HA Klokin 1. After 48 h, the cells were washed with cold PBS and harvested in immunoprecipitation (IP) buffer (1.0% Triton X-100 and 1 x protease inhibitor cocktail [Roche] in PBS). Lysates were rotated at 4°C for 2 h, followed by centrifugation at 17500 g for 15 min. The supernatant fractions were combined with 25 μl protein A/G sepharose and pre-incubated with 5 μg mouse monoclonal anti-HA or anti-His antibody, followed by overnight rotation at 4°C. The protein A/G sepharose complex was pelleted and washed once with IP buffer supplemented with 500 mM NaCl, twice with IP buffer alone and three times with PBS. Immunoprecipitates or inputs (1% total lysate) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to western blot analysis. Normal mouse or rabbit immunoglobulin-linked protein G beads were used for negative controls. IP analysis was also performed using mouse brains and human muscle tissues to examine the endogenous association between Parkin and Klokin 1. For this study, monoclonal anti-Parkin antibody and polyclonal anti-Klopin 1 antibody were used.

Image analysis

Cells were imaged using a Leica TCS-NT (Leica Microsystems GmbH, Wetzlar, Germany) or an ECLIPSE 80i (Nikon, Tokyo, Japan). Digital images were acquired with MetaMorph software (Molecular Devices, Tokyo, Japan) and imported into Adobe Photoshop CS4. The intensity of GFP signal/cell was quantified using an image analysis system, NIS Elements (Nikon).

Statistical analysis

Experimental results were reproduced in at least three independent experiments. We used the Mann–Whitney U-test, one-factor analysis of variance or Wilcoxon signed test for statistical analyses.

Cellular fractionation

Cells were homogenized in a sucrose buffer containing 20 mM Tris–HCl (pH 7.6), 50 mM KCl, 2 mM MgCl2, 0.25 mM sucrose and protease inhibitor cocktail Set III (Calbiochem, Darmstadt, Germany). The homogenate was processed for two-step differential centrifugation to obtain the following fractions: nuclear fraction (pellets obtained after 800 g for 10 min), mitochondrial fraction (pellets obtained after 7000 g for 10 min) and cytosolic (non-mitochondrial) fraction (supernatant of the second centrifugation), as previously reported (9). The cytosolic fraction contained the microsomal fraction. Protein samples (50 μg/lane) were separated by 12.5% SDS–PAGE and processed for immunoblotting, followed by visualization using electrochemiluminescence (9). To clarify intramitochondrial localization, isolated mitochondria were incubated with a sucrose buffer containing 0.05 mg/ml proteinase K for 5–120 min at 25°C and centrifuged at 7000 g for 15 min. The resulting pellets were processed for immunoblotting.

Purification of recombinant proteins

Parkin, Klokin 1 and TFAM cDNAs were cloned into pET15b (Novagen) and expressed in E. coli BL21 (Novagen), as previously described (9). Protein production was induced for 4 h with 1 mM IPTG in BL21 cells at 37°C, which were then lysed with a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1% Triton X-100, 6 M guanidine hydrochloride, 500 mM NaCl, 10 mM imidazole and a protease inhibitor cocktail. The samples were applied to Ni2+-chelating Sepharose FF, washed with 50 mM imidazole and eluted with 200 mM imidazole. The eluent was concentrated and applied to Q-Sepharose, washed extensively and eluted with appropriate NaCl concentrations.

In vitro import experiments

Purified protein was imported into isolated mitochondria as previously described (33) with some modification (9). His-tagged Parkin and Klokin-1 were used in this study. Typically, a mitochondrial suspension containing 1 mg of mitochondrial protein was incubated at 25°C for 20 min with 15–25 μg of purified His-tagged proteins. The reaction mixture was partially digested with proteinase K (2 mg/ml) at 0°C for 15 min to degrade proteins.

Cell viability assay

Cell viability assay was assessed by the CellQuanti-Blue Cell Viability Assay Kit (BioAssay Systems, Hayward, CA, USA) using 96-well plates. The fluorescence intensity (excitation 530–570 nm, emission 590–620 nm) was measured using an Infinite 200 (Tecan Ltd., Männedorf, Switzerland).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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