Parkin enhances mitochondrial biogenesis in proliferating cells

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We describe a novel function of parkin, a RING protein, which is elaborately involved in mitochondrial biogenesis. Parkin was located within the mitochondrial organelle of proliferating cells. Anti-proliferative treatments released parkin from mitochondria to cytosol. Results of pharmacological treatments indicate that parkin was released from mitochondria when permeability transition pore was opened. The extra-mitochondrial localization was also observed in differentiated cells. In proliferating cells, transcription and replication of mitochondrial DNA was enhanced by parkin overexpression and attenuated by parkin suppression with siRNA. Parkin was associated with mitochondrial transcription factor A (TFAM) and enhanced TFAM-mediated mitochondrial transcription. These results indicate that parkin is involved in the regulation of mitochondrial transcription/replication other than the ubiquitin-mediated protein degradation system in proliferating cells.

INTRODUCTION

Autosomal recessive juvenile parkinsonism (AR-JP) is characterized by both homogenous clinical features and distinctive pathological findings, including the loss of dopaminergic neurons in the substantia nigra (1). Mutation in the parkin gene is known to play a major etiopathogenic role in AR-JP (2). The parkin gene contains RING-finger motifs and the gene-product functions as ubiquitin–protein ligase (E3) for protein degradation (3–5). Therefore, the reduction in ubiquitination activity seems to be associated with the development of neuronal degeneration in patients with AR-JP (3,5). In contrast, it has been well known that mitochondrial dysfunction plays an important role in the pathophysiological mechanism in sporadic Parkinson’s disease, i.e. mitochondrial complex I deficiency seems responsible for the nigral degeneration (6,7). Recently, mitochondrial dysfunction has been reported in Parkin-deficient mice (8), but the molecular insight into parkin and mitochondrial function has not been clarified.

The parkin gene is ubiquitously transcribed in both neuronal and non-neuronal tissues (2). In human and non-human brain tissues, parkin shows various intracellular localizations, including the Golgi apparatus, synaptic vesicle, endoplasmic reticulum (ER), nucleus and mitochondria (5,9–11), suggesting that parkin has as-yet-unidentified functions other than ubiquitin-mediated proteosomal degradation. In order to identify a novel function of parkin, we examined its localization in various types of cell lines. We found that parkin was localized in mitochondria of proliferating cells and moved to the extra-mitochondrial cytoplasm when cells were placed under a differentiated or quiescent condition. Furthermore, the present study indicates that parkin enhances mitochondrial biogenesis via the transcription and replication in proliferating neuronal cells.

RESULTS

Parkin is localized in mitochondria of proliferating cells

Immunofluorescent study demonstrated that signals for parkin (green) exactly corresponded to those for mitochondria (red) in a dopaminergic neuroblastoma cell line, SH-SY5Y, a rhabdomyosarcoma cell line, RD (Fig. 1A), a cerebellar medulloblastoma cell line, TE671, a monkey kidney cell line, COS-1 and a rat myocyte cell line, L6 (data not shown).
Three polyclonal anti-parkin antibodies used in this study resulted in similar mitochondrial localization. When a blocking peptide was added to the reaction mixture containing anti-parkin antibody, green signals for parkin were greatly decreased, but red signals for respiratory chain complex 3 were not changed (Fig. 1A). When anti-parkin antibody was omitted or normal rabbit serum was used instead of rabbit anti-parkin antibody, FITC signals were also diminished (not shown).

Next, we examined the intracellular expression of GFP-tagged parkin. Parkin-GFP fusion protein was localized in mitochondria stained with CMXRos, but not in the Golgi apparatus (data not shown) in all types of cells examined. As the localization of parkin-GFP exactly corresponded to that of endogenous parkin, we examined the expression of mutant parkin lacking exons 3 and 4, using GFP system. As shown in Fig. 1B, major green signals for parkin were also co-localized with those for mitochondria. On the contrary, surface 3D images demonstrated that ParkinWild-GFP signals were covered with mitochondrial signals (Fig. 1C, left panel), but parkinExon 3-4, Del-GFP signals partly put in an appearance (Fig. 1C, right panel). This indicates that parkin-GFP was localized at least inside the outer mitochondrial membrane, but the truncated parkin was partly localized outside the outer mitochondrial membrane. Intramitochondrial localization of endogenous parkin was confirmed by immunoelectron microscopic study. As shown in Figs 2A and B, gold particles corresponding to endogenous parkin were present mainly in mitochondrial cristae in SH-SY5Y cells. A total of 488 mitochondria were analyzed and the number of mitochondrion having over two gold particles was 182. In control reaction (Fig. 2C), only 19 of 633 mitochondria had over two gold particles.

In immunoblot analysis, a single protein band corresponding to endogenous parkin was detected in the mitochondrial fraction (Fig. 3A, lane 1) but not in the crude cytosol fraction (Fig. 3A, lane 2) or in the nuclear fraction (data not shown). Four anti-parkin antibodies used showed similar results.

**Figure 1.** (A) Intracellular localization of endogenous parkin and (B and C) parkin-GFP fusion protein in cultured cells. (A) Immunocytochemical study of parkin expression. Endogenous parkin (green) was exactly co-localized with mitochondria stained with respiratory chain complex 3 (red) in SH-SY5Y and RD cells. Control experiments using blocking peptide (immunogen) added to the reaction mixture containing anti-parkin antibody (Ab-1) greatly reduced green signals for parkin but not red signals for respiratory chain complex 3. Bar = 5 μm. (B) GFP signals for parkin (wild) were exactly localized in mitochondria (red) stained with CMXRos but not in Golgi apparatus (red) in COS-1 cells. GFP signals for parkin lacking exons 3 and 4 were also localized in mitochondria (red) stained with CMXRos. Bar = 5 μm. (C) Surface 3D images of signals for parkin-GFP and mitochondria show that parkinWild-GFP signals were almost covered with mitochondrial signals (left panel) but parkinExon 3-4, Del-GFP signals partly put in an appearance. Bar = 5 μm.
In this study, cellular fractionation was performed by two-step differential centrifugation and the crude cytosolic fraction contained the microsomal fraction. These were confirmed by the following results. Signals for respiratory chain complexes 1, 2, 3 and 4 were detected in the mitochondrial fraction (Fig. 3B, lane 1) but not in the cytosol fraction (Fig. 3B, lane 2). In contrast, the cytosol fraction was positive for β-COP and γ-adaptin (Fig. 3B), both of which were vesicle-associated proteins involved in membrane traffic between the rough ER and the Golgi complex. In addition, synaptophysin, a synaptic vesicle protein, was detected in the cytosol fraction but not in the mitochondrial fraction (Fig. 3B). To determine the precise localization of parkin, mitochondrial fraction was partially digested with trypsin and proteinase K. In mitochondria with both treatments, immunoreactivity pattern of parkin was similar to that of the inner membrane protein, adenine nucleotide translocator 1 (ANT1) (Fig. 3C).

**Parkin moves to the extra-mitochondrial cytoplasm under a differentiating or quiescent condition**

We employed various anti-proliferating agents to modulate the cell proliferation and cell cycle. By treatment of SH-SY5Y cells with blockers for mitochondrial energy production, CCCP (1–10 μM), ionomycin (1–10 μM) and rotenone (1–10 μM), endogenous parkin was released from the mitochondrial fraction into the crude cytosolic fraction time-dependently until 48 h after treatment (Fig. 4A). Treatment with cell-cycle blockers, butyrolactone I (10–100 μM), geldanamycin (10–100 μM), trichostatin A (10–100 μM) and oligomycin (1–5 μg/ml), resulted in the complete release of endogenous parkin from the mitochondrial fraction into the crude cytosolic fraction at 2 days after treatment (Fig. 4A). These changes were also observed in the localization of cytochrome c. In contrast, respiratory chain complexes 1, 2, 3 and 4 were not released from mitochondria. The results suggest that the mitochondrial direction does not primarily depend on the cell-cycle blockade, but on the quiescent condition that attenuates mitochondrial energy production and/or cellular proliferation. In differentiated SH-SY5Y cells, endogenous parkin was detected only in the crude cytosolic fraction (Fig. 4A). Microscopic study demonstrated that co-localization of parkin-GFP fusion protein and CMXRos was no longer observed following the treatment of L6 cells and SH-SY5Y cells with butyrolactone I, trichostatin A, geldanamycin, oligomycin CCCP, rotenone or horse serum (differentiation agent for L6 cells) (Supplementary Material).

We then examined the mechanism of parkin release from the mitochondria, using isolated mitochondria. As shown in
Fig. 4B, rotenone and atractyloside (Atr) released parkin from the mitochondria, but the major part of parkin was not released by truncated-Bid (t-Bid) treatment. This differs from results that rotenone and t-Bid released cytochrome c from the mitochondria, but not Atr. Furthermore, Atr-mediated parkin release was dose-dependently attenuated by the ANT inhibitor bongkrekic acid (BA) (Fig. 4C). These results suggest that parkin moves to the extra-mitochondrial cytoplasm via permeability transition (PT) pore under a differentiating or quiescent condition.

Parkin can be imported into mitochondria
To examine the pathways for parkin insertion into mitochondria, in vitro import study was carried out. As shown in Fig. 4D, parkin-His could be imported into mitochondria with or without trypsin treatment. Trypsin-treated mitochondria showed reduced immunoreactivity of voltage-dependent anion-selective channel (VDAC) (data not shown), indicating that mitochondrial outer membrane proteins were partially removed. The present results suggest that parkin is potentially imported into the mitochondrial matrix compartment. Although free parkin was completely digested by proteinase K, parkin imported into mitochondria was resistant to proteinase treatment. On the contrary, parkin mutants were potentially imported into mitochondria, but the translocation was reduced than that of parkin wild-type. This finding was concordant with the results of intracellular localization of GFP-tagged parkin mutants.

Parkin overexpression enhances mitochondrial biogenesis
We next investigated the possibility of recombinant parkin expression modulating mitochondrial biogenesis. As shown in Fig. 5A, when wild-type parkin was expressed in SH-SY5Y cells, mitochondria-encoded mRNA levels such as ND 3–4 (complex 1), cytochrome b (complex 3), COX 1–2 (complex 4) and COX 3–ATPase 6/8 (complexes 4–5) were markedly increased compared with the levels in cells transfected with an empty vector (lane 1). However, no difference in mRNA expression levels was observed in nuclear-encoded genes, β-actin, 18s rRNA and COX 5a. Results of Southern blot study were similar to those of northern blot study (Fig. 5B). Furthermore, immunoblot analysis showed that
Figure 4. Release of endogenous parkin from mitochondrial and mitochondrial import of parkin. (A) Immunoblotting of endogenous parkin in SH-SY5Y cells. CCCP (10 μM), ionomycin (10 μM) and rotenone (10 μM) released parkin from the mitochondrial fraction into the cytosolic fraction time-dependently until 48 h after treatment. Parkin was also completely released from the mitochondrial fraction into the cytosolic fraction at 3 days after addition of butyrolactone I (100 μM), geldanamycin (100 μM), trichostatin A (100 ng/ml) or oligomycin A (5 μg/ml) to the media. These changes were also observed in the localization of cytochrome c, but respiratory chain complexes 1 and 2 were not released from mitochondria. Following differentiation treatment with retinoic acid (100 μM), parkin was detected only in the cytosolic fraction. Lane C indicates no treatment. (B) Isolated mitochondria were incubated with rotenone (10 μM), Atr (2.5 mM) or t-Bid (0.5 μM) for 3 h. Rotenone and Atr released parkin from the mitochondria but not t-Bid. Cytochrome c was released from the mitochondria by rotenone and t-Bid but not by Atr. Complex I was not released from mitochondria. (C) Isolated mitochondria were incubated with Atr (2.5 mM) and BA (0, 0.4, 2, 10 and 50 μM) for 3 h. Atr-mediated parkin release was dose-dependently attenuated by BA. Localization of complex 1 and cytochrome c was not changed. (D) Import of purified parkin into isolated mitochondria without trypsin treatment (left panel) or with trypsin treatment (right panel). Purified His-parkin (15–25 μg) could be imported into mitochondria (1 mg) without or with trypsin treatment. Although free parkin (wild-type) was completely digested by proteinase K, parkin imported into mitochondria was resistant to proteinase treatment. Parkin mutants were partially imported into mitochondria.
immunoreactivities corresponding to complexes 1–4 (Fig. 5C) were apparently increased compared with those in the case of an empty vector (lane 1). The antibody against complex 4 can only recognize a protein synthesized in mitochondria, whereas antibodies against complex 1, 2 and 3 recognize proteins synthesized in the nucleus. This suggests that proteins in mitochondrial respiratory chain complexes synthesized in mitochondria are a limiting factor for assembly of complete...
Influence of silencing of an endogenous parkin gene on mitochondrial proliferation

We found that the parkin expression level was potentially reduced up to 10% in SH-SH5Y cells by a 22-mer short interfering RNA (siRNA) duplex corresponding to parkin coding region nt 845–866, but that β-actin expression was unchanged (Figs 6A and B). This siRNA also attenuated the signals for parkin-GFP (Fig. 6A). The other targeting siRNAs did not interfere with parkin expression (data not shown). Northern blot study demonstrated that mitochondria-encoded mtRNAs were selectively decreased by the siRNA (Fig. 6C). These were compatible with the results of overexpression study.

Parkin is associated with mitochondrial transcription factor A (TFAM) and enhances TFAM-mediated mitochondrial transcription

We examined in vivo association of parkin and mtDNA by chromatin immunoprecipitation (ChIP) analysis. As shown in Fig. 7A, immunoprecipitation using anti-His antibody (lane 3) revealed that four regions of displacement-loop (D-loop) were bound to parkin-His, but not other regions such as ND4L, ND3 and ATPase 8. This binding was not observed in untransfected cells (lane 4), and normal mouse serum used instead of anti-His antibody (lane 2) did not immunoprecipitate any regions of mtDNA. We then examined in vitro association of parkin and mtDNA. As D-loop is known as the major site of transcription initiation on both the heavy and light strands of mtDNA (2), we examined in vitro binding of His-parkin to D-loop region by mobility shift assay. However, significant association was not detected between parkin and mitochondrial DNA (Fig. 7D).

We hypothesized that parkin enhances mitochondrial transcription in the presence of associated protein. We then investigated whether parkin is associated with mitochondrial transcription factor A (TFAM). Co-immunoprecipitation (Co-IP) study was carried out using COS-1 cells co-transfected with His-parkin and HA-TFAM. As shown in Fig. 7B, it is evident that His-parkin is associated with HA-TFAM. This association was supported by the following results of mobility shift assay. When His-parkin (0.05–1 μg) was added to two mtDNA fragments (nt 36–352 and nt 499–741), the bands were not shifted (Fig. 7D). On the contrary, mtDNA bands were shifted when His-TFAM (0.05–0.5 μg) was added. TFAM-induced mobility shift was enhanced in the presence of His-parkin (1 μg) although the amount of parkin could not induce the mobility shift (Fig. 7D). Results of native PAGE/immunoblotting showed the direct association of His-parkin with His-TFAM (Fig. 7E). These results strongly suggest that parkin can be associated with mtDNA not directly, but via TFAM. In vitro mitochondrial DNA (mtDNA) transcription is produced from both the light-strand (LSP) and the heavy-strand (HSP) promoters (13,14) and promoter-specific initiation of transcription is induced under the presence of TFAM (14,15). We examined the effect of parkin on in vitro transcription with the partially purified TFAM fraction by using mtDNA fragments containing LSP and/or HSP regions. As shown in Fig. 7C, purified parkin dose-dependently stimulated the mitochondrial transcription containing both LSP (410 nt) and HSP (180 nt). These findings indicate that parkin enhances TFAM-mediated mitochondrial transcription.

DISCUSSION

The present study demonstrated that parkin is expressed ubiquitously in a variety of cells, localized exclusively to mitochondria in a proliferating condition. The present results are in sharp contrast to the results of previous studies, showing that parkin was not detected in mitochondria but in the cytosol, the trans-Golgi network and the synaptic vesicles in human and non-human brain tissues (11,16). This discrepancy may be due to the difference in the cellular proliferative states because neural cells are completely differentiated in adult tissues, whereas the cells used in this study were grown under a proliferating condition. In cultured cells, intracellular localization of parkin has been controversial (9,10,17). Recently, it has been reported that parkin is abundant in the mitochondrial fraction and is located on the outer mitochondrial membrane (17). However, the latter finding is not concordant with our results of proteolytic study. In mitochondria treated with trypsin and proteinase K, immunoreactivity of parkin was similar to that of the inner membrane protein ANT1. We speculate that the discrepancy depend on the unfixed localization of parkin. In fact, parkin easily left mitochondria even when some antibiotics were added to the culture medium (our observation). The present results demonstrated that parkin moved to the extra-mitochondrial cytoplasm when cells were placed under a differentiated or quiescent condition. The release of cytochrome c from mitochondria indicates that the quiescent condition induces cellular apoptosis. We then investigated the mechanism of parkin release.

The mitochondrial PT functions as a sensor for cellular stress and damage (reviewed in 18 and 19). It leads to mitochondrial swelling, outer membrane rupture and the release of apoptotic mediators including cytochrome c. ANT and VDAC play important roles in the mitochondrial PT function (18,19). Atr and t-Bid are PT pore opener agents that act on the inner membrane protein ANT and the outer membrane protein VDAC, respectively. We studied whether parkin release is related to the PT pore. Cytochrome c was released...
by t-Bid treatment, but the major part parkin was not released. In contrast, Atr released parkin but not cytochrome c. The present results seem to depend on the difference in localization between parkin and cytochrome c, i.e. cytochrome c is located in the inter-membrane space but parkin is located within the organelle. The Atr-mediated parkin release was inhibited by BA, an ANT inhibitor. These results suggest that parkin, located within the mitochondrial organelle, is potentially released through the PT pore opened. The dynamics of parkin relatively differed from that of cytochrome c. It is unlikely that parkin is associated with cytochrome c in apoptotic cells because of the results of Co-IP study (data not shown), although parkin may function as an apoptosis-related factor. The fact that parkin was released from mitochondria by the differentiating treatments, which did not induce cytochrome c release, indicates that localization of parkin is independent

**Figure 6.** Silencing of endogenous or exogenous parkin expression in SH-SY5Y cells. (A) Synthetic siRNA (150 nm) markedly decreased parkin expression compared with that in scramble siRNA-transfected cells, but the expression of β-actin was unchanged. Signals for parkin-GFP were also attenuated when the targeting siRNA was co-transfected. Bar = 50 μm. (B) Immunoblot analysis demonstrated that 15 nm and 150 nm siRNA decreased parkin expression compared with that in 150 nm scramble siRNA-transfected cells. Below panels show the relative protein levels in cells transfected with 15 nm or 150 nm siRNA to those in cells transfected with scramble siRNA. N = 4. Error bar indicates SE. *P < 0.05. (C) Effect of parkin silencing on mitochondrial transcription. Relative mRNA or DNA levels in cells transfected with 15 nm or 150 nm siRNA to those in cells transfected with scramble siRNA. N = 4. Error bar indicates SE. *P < 0.05. Compared with 150 nm scramble siRNA transfection, 150 nm siRNA significantly decreased relative levels of mitochondria-encoded mRNAs but not those of nuclear-encoded RNAs (except parkin mRNA).
We could not clarify the precise molecular mechanism of the dynamics of parkin. However, there is at least one possibility that some proteins carry parkin to the mitochondria in a proliferating condition. We found in vitro and in vivo association of parkin with TFAM. As mitochondrial import of parkin was enhanced in the presence of TFAM or mitochondrial extract (data not shown), some proteins may play a role in the import and/or the export of parkin including TFAM. On the contrary, it is noteworthy that parkin-GFP was preferentially localized in the nuclei of some differentiated cells (Supplementary Material). It is concordant with the results of ultrastructural analysis that speckled immunodeposits corresponding to parkin has been detected in the nuclear matrix in brain tissues (11). From these findings, we speculate a possibility that parkin-associated proteins at proliferating stage are different from those at differentiating stage in vivo.

We studied the effect of parkin on the mitochondrial function. In proliferating cells, parkin enhanced mitochondrial transcription, replication, and expressions of...
mitochondrial respiratory chain complexes. These are also confirmed by the results of silencing of an endogenous parkin gene or by those of the ultrastructural study. ChIP study showed in vivo association of parkin with D-loop regions. However, mobility shift assay failed to show the direct association of parkin and mtDNA. We found that parkin could be bound to TFAM and to various D-loop regions of mtDNA through TFAM. Although parkin had no promoter-specific transcriptional activity (data not shown), parkin enhanced TFAM-mediated mitochondrial transcription. TFAM–D-loop interaction is known to be non-sequence-specific and a primary function of TFAM appears to be specific packaging of the mtDNA control region (20). It seems likely that parkin is associated with wide regions of D-loop via TFAM and enhances mitochondrial transcription. RING-finger proteins, such as parkin, play critical roles in mediating the transfer of ubiquitin to heterologous substrates by presenting a RING-finger domain as an E2 binding site (3,4,21). In contrast, RING protein not only functions as an E3 but also modulates intracellular homeostasis, including cell cycling, signaling, transcription, proliferation, apoptosis and DNA repair (22). Some RING-finger proteins directly or indirectly stimulate the transcriptional activity (23).

The present results indicate that parkin plays a role in mitochondrial biogenesis by regulating both transcription and replication of mitochondrial DNA in proliferating cells. From the results of the present study as well as previous reports, we speculate that parkin functions both as a regulator of mitochondrial biogenesis in proliferating cells and an E3 in differentiated cells. From cellular proliferation to differentiation, parkin leaves the mitochondria to the microsome or the cytosol. This dynamics seems convenient for both proliferating and differentiated cells. As parkin cannot be the only regulator of mitochondrial biogenesis, other proteins than parkin may contribute to the mitochondrial integrity in differentiated cells. On the contrary, mitochondrial dysfunction has been shown to play a major role in the development of sporadic Parkinson’s disease (24). Our results indicate that mitochondrial dysfunction is also associated with the pathophysiology of AR-JP, at least during the cell proliferating stage. Further study is needed to clarify the precise functional contribution of the RING protein to mitochondrial biogenesis.

MATERIALS AND METHODS

Cell culture and reagents
COS-1, SH-SY5Y, RD and L6 cells were cultured at 37°C (5% CO₂) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). TE671 cells were grown in a medium containing 10% heat-inactivated horse serum and 5% FBS. For differentiation, SH-SY5Y cells were grown with 100 μM retinoic acid, and L6 cells in DMEM with 2% heat-inactivated horse serum and 6 μg/ml insulin for at least for 2 weeks.

We used the following anti-proliferating agents: CCCP (0.1–10 μM, an uncoupler that inhibits mitochondrial oxidative phosphorylation), ionomycin (0.1–10 μM, a calcium ionophore that releases Ca²⁺ from mitochondria), rotenone (0.1–10 μM, an inhibitor of respiratory chain complex I), oligomycin (0.1–5 μg/ml, an inhibitor of mitochondrial F₁F₀-ATPase), butyrolactone I (1–100 μM, a cyclin-dependent protein kinase inhibitor), geldanamycin (1–100 μM, a cell cycle blocker at G₂ stage) and trichostatin A (1–100 μM, a cell cycle blocker at G₁ stage). Isolated mitochondria were treated with rotenone (10 μM), Atr, a PT pore-opener agent that acts on ANT (2.5 mM), t-Bid, a PT pore-opener that acts on VDAC (0.5 μM) and BA, an ANT inhibitor (0.4–50 μM).

Intracellular localization was analyzed using BODIPY TR ceramide (5 μM, for staining of the Golgi complex, Molecular Probes) and Mitotracker Red CMXRos (100 nM, for staining of mitochondria, Molecular Probes).

Mutagenesis and cell transfection
Full-length parkin cDNA and a deletion mutant lacking exons 3 and 4 were constructed as previously described (25). In addition, we cloned mutants by using appropriate primer pairs or the QuikChange site-directed mutagenesis kit (Stratagene). Full-length or deletion-mutant parkin cDNA was subcloned into pEGFP-C2 (Clontech, CA, USA), pcDNA3.1(+) (Invitrogen Corp., CA, USA) or pcDNA4/HisMax (Invitrogen). We amplified the cDNA of full-length TFAM by PCR and ligated it into pcCMV-HA vector (Clontech) or pcDNA4/HisMax. Cells were transfected using Effectene™ Transfection Reagent (Qiagen, CA, USA) or Lipofectamine™ 2000 (Invitrogen Corp.). Morphological study and Southern-blot and immuno-blot analyses were performed at 48 h after transfection. Northern blot analysis was performed at 24 h after transfection. For creation of stable transfectants, full-length parkin cDNA was subcloned into pcDNA4/HisMax (Invitrogen). A stable cell line of RD cells expressing 6 × His-tagged parkin was successfully generated by Zeocin (300 μg/ml) selection.

Silencing of parkin gene using siRNA
Synthesized 21-mer dsRNAs with a two-base (dTdT) overhang corresponding to the parkin coding region nt 79–100, nt 847–865, nt 926–947 (GenBank accession no. AB009973) were purchased from Dharmacon Research (Lafayette, CO, USA). SH-SY5Y cells were cultured in six-well plates, and the targeting siRNA duplex and scramble siRNA duplex (Dharmacon Research) were transfected with Lipofectamine™ 2000. To test the silencing effect of siRNA on exogenous parkin expression, parkin-GFP was co-transfected with the targeting siRNA or scramble siRNA duplex. The effects of siRNA were evaluated 12–30 h after transfection of siRNA duplex, 0.2 μg/well (15 nm) and 2.0 μg/well (150 nm).

Immunological analysis
We used three kinds of rabbit antibodies against parkin: Ab-1 (Oncogene, CA, USA), Ab-293 (produced in our laboratory) and Ab-2132 (Cell Signaling Technology, MA, USA) produced against synthetic peptides corresponding to amino-acid residues 81–98, 293–306 and around 400 of human parkin, respectively. In addition, monoclonal mouse anti-parkin antibody, MoAb-5A1 was purchased from IBL (Gunma, Japan). Monoclonal antibodies against respiratory chain...
complexes 1 (20C11–B11–B11), 2 (21A11–AE7), 3 (13G12–AF12–BB11) and 4 (12C4–F12) were purchased from Molecular Probes. Monoclonal antibodies against β-actin (AC-15), β-COP, γ-adapin and synaptophasin, and goat polyclonal antibodies against VDAC1 and ANT1 were purchased from Sigma and Santa Cruz Biotech, respectively.

Double-immunostaining was performed as reported elsewhere (26). In brief, cells were fixed with 4% PFA in PBS for 15 min followed and treated with 0.5% Triton X-100 in PBS for 5 min. After blocking with 5% bovine serum albumin (BSA) in PBS, cells were incubated with primary antibodies including anti-parkin polyclonal antibodies and anti-respiratory chain complexes 1–4 monoclonal antibodies for an hour. The cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG absorbed mouse, rat and human sera, and then with tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG absorbed rabbit, human, goat, sheep and bovine sera. For double-immunostainings for parkin and β-actin, FITC-conjugated anti-mouse IgG absorbed rabbit, rat, sheep, bovine, goat and human sera and TRITC-conjugated anti-rabbit IgG absorbed mouse, bovine, goat, human, rat and sheep sera were used. Control experiments were performed by using normal rabbit serum instead of rabbit anti-parkin antibody, by omitting primary antibody and by adding a synthetic peptide (4 μg/ml) to the reaction mixture containing anti-parkin antibody, Ab-1. Fluorescent signals were analyzed by a confocal laser microscope, TCS NT (Leica, Heiderberg, Germany).

CO-IP was carried out by using mouse monoclonal anti-His antibody (Amersham Pharmacia Biotech, NJ, USA) and rabbit polyclonal anti-HA antibody (Molecular Probes). We prepared IP-parkin/TFAM bound to anti-His/HA-linked protein G beads from extracts (2 mg protein) of COS-I cells co-transfected with pcDNA4/HisMax-parkin and pCMV-HA-TFAM. Normal mouse or rabbit immunoglobulin-linked protein G beads was used for negative controls. Co-IP samples were separated by 4–20% SDS–PAGE, and processed for immunoblotting followed by visualization by the ECL method (27).

Cellular fractionation and treatment of mitochondria
Cells were homogenized in a sucrose buffer containing 20 mM Tris–HCl (pH 7.6), 50 mM KCl, 2 mM MgCl$_2$, 0.25 m sucrose and the proteinase 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 after 7000g for 10 min) and cytosolic (non-mitochondrial) fraction (supernatant of the second centrifugation). Sucrose sedimentation was repeated twice in the mitochondrial fraction. This cytosolic fraction therefore contains the microsomal fraction. Protein samples (50 μg/lane) were processed for immunoblotting (27). To clarify the precise localization of parkin in mitochondria, two proteolytic treatments were carried out. Isolated mitochondria incubated with a sucrose buffer containing 0.1 mg/ml trypsin or 0.05 mg/ml proteinase K for 5–120 min at 25°C, were centrifuged at 7000g for 15 min. Resulting pellets were processed for immunoblotting.

Electron and immunoelectron microscopy
SH-SY5Y cells were fixed in cacodylate buffer containing 2.5% glutaraldehyde (GA), embedded in epoxy-resin and processed for routine procedures. For immunoelectron microscopy, SH-SY5Y cells were fixed with 4% PFA and 0.2% GA and embedded in LR White Resin (Polyscience, PA, USA) as previously described (28). Ultrathin sections were incubated with rabbit anti-parkin antibody at 4°C overnight. The sections were incubated with 20 nm gold-labeled goat anti-rabbit IgG, and then stained with uranyl acetate and lead citrate. Control experiment was performed by using normal rabbit serum instead of rabbit anti-parkin antibody. For quantitative analysis, sections were observed by an electron microscope, JEOL JEM-1200 EX II, at a magnification of 30,000×, and micrographs were taken randomly in regions of sound structural integrity. The total area analyzed was about 500 μm$^2$ in test and control experiments.

Northern and Southern blot analyses
We prepared four mitochondrial cDNA probes for ND 3–4 (complex, nt 10125-11332), cytochrome b (complex 3, nt 14744–15678), COX 1–2 (complex 4, nt 7059–8190) and COX 3–ATPase 6/8 (complex 4–5, nt 8290–9550) and three nuclear cDNA probes for COX 5a (24)(complex 4), β-actin and 18s rRNA. Five micrograms of DNA digested with Pvu II and 10 μg of RNA were applied per lane in Southern (29) and northern (30) blotting, respectively. Blotting study was carried out at least four times. Blot data were quantified and statistically analyzed by the Mann–Whitney test.

Purification of proteins
The full-length parkin and TFAM were cloned into pET15b and expressed in E.coli BL21. Protein production was induced for 4 h with 1 mM IPTG in BL21 cells at 37°C, which were lysed with a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1% Triton X-100, 6 μg guanidine hydrochloride, 500 mM NaCl, 10 mM imidazole and proteinase inhibitor cocktail set III (Calbiochem) diluted to 1:250. RD cells transfected with parkin wild-type and mutants were also solubilized with a lysis buffer lacking guanidine hydrochloride. The samples were applied to Ni$^{2+}$-Chelating Sepharose FF, washed with 100 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. An extract of mitochondria containing both mtRNA polymerase and TFAM was prepared from human RD cells transfected with TFAM by the procedures of Topper and Clayton (31).

In vitro import experiments
Import of purified protein into the isolated mitochondria (32) was carried out with modification. In some experiments, mitochondrial protein was treated with trypsin (60 μg/ml) to partially remove mitochondrial outer membrane proteins (32). His-tagged parkin wild-type and mutants were used in this study. Typically, 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. Reaction mixture was partly treated with proteinase K (2 mg/ml) at 0°C for 15 min to digest proteins that was not imported. Mitochondrial extract was prepared as follows. Mitochondrial pellet was suspended in a buffer containing 10 mM Tris–HCl (pH 8.0), 0.4 M KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT and proteinase inhibitors for 30 min at 4°C. After centrifugation at 9000g, the supernatant was concentrated and stored at –80°C.

ChIP analysis
A stable cell line of RD cells (1 x 10⁶) expressing His-parkin was collected and fixed with PBS containing 1% formaldehyde for 10 min. Cross-linking was added by the addition of 0.125 M glycine for 5 min at room temperature. Cells were washed twice in ice-cold PBS and solubilized for 1 h at 4°C with lysis buffer containing 50 mM Tris–HCl (pH 8.0), 1% sodium dodecyl sulfate (SDS), 10 mM EDTA and proteinase inhibitors for 30 min at 4°C. After centrifugation at 13,000g, the supernatant was diluted 10-fold with ChIP solution (10 mM Tris–HCl, pH 8, 200 mM NaCl, 1% Triton X-100 and proteinase inhibitor cocktail set III). Samples were precleared by incubating them with 30 μl of protein G-Agarose also precleared with sheared salmon sperm DNA. After centrifugation for 10 min at 13,000g, the supernatant was combined with 30 μl of protein G-Agarose preincubated with anti-parkin antibody (5 μl) followed by rotating 4°C for 8 h. Immunoprecipitates were washed three times with ChIP buffer, ChIP buffer containing 500 mM NaCl and TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), respectively. Immunocomplexes were eluted off the beads by incubation with 200 μl of 1% SDS and 50 mM NaHCO₃. The eluent was incubated at 65°C for 6 h to reverse the crosslinks. The DNA was extracted with the QIAamp DNA mini kits (Qiagen) and then analyzed by PCR. The following regions of mitochondrial DNA were amplified with appropriate primer pairs: nt 1–352, nt 316–477, nt 316–600, nt 499–741, nt 8366–8572 (ATPase 8), nt 10059–10403 (ND3), nt 10470–10766 (ATPase 8), nt 10059–10403 (ND3), nt 10470–10766 (ND4L). Normal mouse serum was used instead of anti-parkin antibody to check non-specific binding. Cross-linked and sheared genomic DNA (input) was used as positive controls. ChIP assays were performed on three independent occasions.

In vitro transcription analysis
We modified the methods of in vitro transcription reported previously (15,31). Transcription reaction was carried out at 37°C for 2 h in reaction mixtures (20 μl) containing 10 mM Tris–HCl (pH 8.0), 10 mM MgCl₂, 1 mM DTT, 10% glycerol, 50 mM KCl, 400 μM ATP, 400 μM CTP, 400 μM GTP, 300 μM UTP, 100 μM Texas Red-5-UTP (Molecular Probes), 10 U of RNasein, 200 ng of mtDNA fragment (nt 1–741), partially purified TFAM (1 μg) and purified His-parkin (0.1, 0.5 and 2 μg). The reaction was stopped by adding 200 μl volumes containing 10 mM Tris–HCl (pH 8.0), 0.2 M NaCl, 1 mM EDTA, 150 μg/ml proteinase K, 1% SDS and 4 U of RNasein and incubated for 45 min at 37°C. After ethanol precipitation, pellet was dissolved in 40 μl volumes containing 20 U of DNase I, 40 mM Tris–HCl (pH 7.5), 8 mM MgCl₂, 5 mM DTT, 10 U of RNasein, incubated 15 min at 37°C. Then, labeled nucleic acids were separated from unincorporated nucleotides by using a Microspin G-25 column. After the second ethanol precipitation, pellet was dissolved and loaded onto 5% polyacrylamide–urea gel. Signals for Texas Red-5-UTP were visualized with a fluorescent image analyzer, FMBIO II Multi-View (Takara, Tokyo, Japan).

Mobility shift analysis
We cloned DNA fragments corresponding nt 36–352 and 499–741 of human mtDNA and mtDNA fragments were 5’-end-labeled with fluorescein (Amersham Biosciences Corp., NJ, USA). Each reaction mixture (15 μl) contained 300 ng of DNA fragments, 20 mM Tris–HCl (pH 7.4), 5 mM MgCl₂, 50 mM KCl, 10% glycerol, 100 μg of BSA and 1 mM DTT. Purified parkin (0.05, 0.2, and 1 μg) and/or TFAM (0.05, 0.15 and 0.5 μg) were incubated in the reaction mixture for 15 min at 37°C and loaded onto 5% polyacrylamide in 50 mM Tris, 380 mM glycine, 1 mM EDTA and 1 mM MgCl₂.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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REFERENCES


