Human Molecular Genetics, 2003, Vol. 12, No. 5 **517–526**
DOI: 10.1093/hmg/ddg044

Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death

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Received October 29, 2002; Revised December 16, 2002; Accepted December 17, 2002

Parkin **gene mutations have been implicated in autosomal-recessive early-onset parkinsonism and lead to specific degeneration of dopaminergic neurons in midbrain. To investigate the role of Parkin in neuronal cell death, we overproduced this protein in PC12 cells in an inducible manner. In this cell line, neuronally differentiated by nerve growth factor, Parkin overproduction protected against cell death mediated by ceramide, but not by a variety of other cell death inducers (H_2O_2, 4-hydroxynonenal, rotenone, 6-OHDA, tunicamycin, 2-mercaptoethanol and staurosporine). Protection was abrogated by the proteasome inhibitor epoxomicin and disease-causing variants, indicating that it was mediated by the E3 ubiquitin ligase activity of Parkin. Interestingly, Parkin acted by delaying mitochondrial swelling and subsequent cytochrome c release and caspase-3 activation observed in ceramide-mediated cell death. Subcellular fractionation demonstrated enrichment of Parkin in the mitochondrial fraction and its association with the outer mitochondrial membrane. Together, these results suggest that Parkin may promote the degradation of substrates localized in mitochondria and involved in the late mitochondrial phase of ceramide-mediated cell death. Loss of this function may underlie the degeneration of nigral dopaminergic neurons in patients with Parkin mutations.**

INTRODUCTION

Parkinson's disease (PD) involves the selective degeneration of midbrain dopaminergic neurons. Recently, three genes responsible for inherited forms of PD have been identified. Mutations in the gene coding for α-synuclein and ubiquitin C-terminal hydrolase L1 (UCH-L1) cause autosomal dominant forms of PD (1,2). In contrast, mutations in the *parkin* gene are involved in an autosomal recessive, early-onset form of parkinsonism in populations of various ethnic origins (3,4). Parkin is a 465 amino acid protein with an apparent molecular weight of 52 kDa (5) containing a ubiquitin-homology domain at its N-terminus and two Ring fingers at the C-terminus. Like other Ring finger proteins (6,7), Parkin has E3 ubiquitin–ligase activity (8–10). It has been proposed that loss of Parkin function, due to disease-related mutations in its gene, may lead to the accumulation of one or more critical substrates, resulting in toxicity for dopaminergic neurons. To date, Parkin has been shown to interact with and ubiquitinate four proteins: the putative G-protein-coupled transmembrane receptor Pael-R (11), the α-synuclein interacting protein synphilin-1 (12), the synaptic vesicle protein Cdc-Rel 1 (10) and an *O*-glycosylated form of α-synuclein (13). The mechanism by which a putative accumulation of these or other as yet unknown Parkin substrates results in the death of dopaminergic neurons when Parkin is not functional is still unknown.

Numerous forms of cell death involve cascades of events that can be divided into three phases: (1) initiatory events that induce changes in mitochondrial function; (2) a decisonal stage leading to release of pro-apoptotic proteins from the intermembrane space of mitochondria such as cytochrome c; (3) activation of effector proteases and endonucleases (14). Proteins with anti-apoptotic functions can intervene at any one of these stages. Recent studies have shown that some E3 ubiquitin–ligase proteins with Ring finger domains have anti-apoptotic activity (15–17). We therefore hypothesized that

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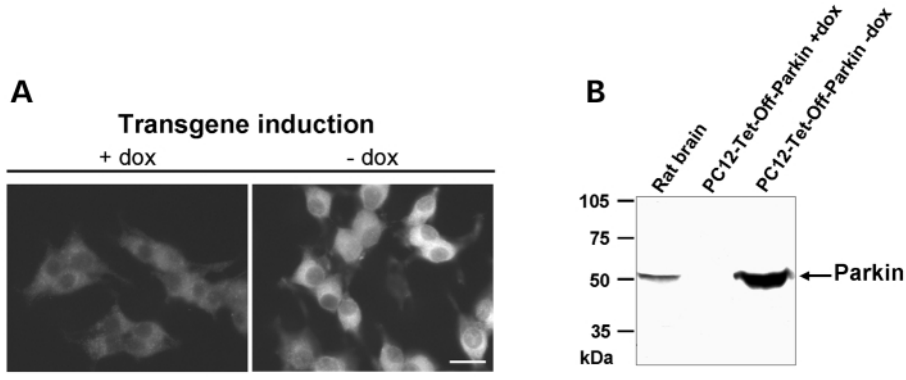

Figure 1. Characterization of clones overexpressing Parkin. (**A**) Immunocytochemical labelling of neuronally differentiated PC12-*Tet-off*-Parkin cells that overproduce Parkin in the absence of doxycycline (50 nM) with antibody ASP-5 (38) (1/200). Scale bar: 20 μM. (**B**) Western blot analysis of Parkin expression in PC12-*Tet-off*-Parkin clone HS22 compared with endogenous rat brain Parkin with the ASP-5 antibody (1/500).

Parkin may have a neuroprotective function that is abrogated by disease-related mutations. To test this hypothesis, we determined whether Parkin could prevent cell death caused by several different well-studied cell death inducers: oxidative stress, dopaminergic neurotoxins, endoplasmic reticulum stress, staurosporine toxicity and ceramide-dependent signal transduction. We report that Parkin specifically prevents cell death induced by ceramide-mediated stimuli, notably by delaying mitochondrial swelling, rupture of the outer mitochondrial membrane and the subsequent release of cytochrome c.

RESULTS AND DISCUSSION

Parkin overexpression protects against ceramide-mediated cell death

To investigate the mechanisms by which Parkin prevents dopaminergic cell death, we generated a stable PC12 cell line (PC12 *Tet-off*-Parkin) that overproduces human Parkin under the control of the tetracycline-sensitive transactivator tTA (19). PC12 *Tet-off*-Parkin cells, like untransfected PC12 cells, develop some of the phenotypic traits of dopaminergic neurons in the presence of nerve growth factor (NGF). In the absence of the tetracycline analogue doxycycline (−dox), these cells express Parkin (Fig. 1A and B).

To screen for a mechanism of cell death that could be inhibited by Parkin, the PC12 *Tet-off*-Parkin cells were treated in both the on (−dox) and off (+dox) conditions with a series of frequently used cell death inducers (Fig. 2A). Overproduction of Parkin had no effect on basal cell death and did not promote survival when cell death was induced by oxidative stress (100 μM hydrogen peroxide, 0.5 μM 4-hydroxynonenal), dopaminergic neurotoxins (100 μM 6-hydroxydopamine, 30 μM rotenone), protein misfolding stress (10 μg/ml tunicamycin, 20 mM 2-mercaptoethanol) or staurosporine (0.3 μM; Fig. 2A). In contrast, cells overproducing Parkin were significantly more resistant to stress induced by both exogenous C2-ceramide (25 μM) and endogenous ceramide produced by NGF and serum withdrawal (20) (Fig. 2A). These results were confirmed by a more detailed kinetic analysis (Fig. 2B and C), demonstrating that death was significantly delayed by Parkin expression in both of the ceramide-dependent stress paradigms. Surprisingly, overproduction of Parkin had no effect on cell death induced by the *N*-glycosylation inhibitor tunicamycin at any time-point examined (Fig. 2D). This is in contrast to a previous report showing a protective activity of Parkin in a tunicamycin-dependent protein misfolding stress model based on the human neuroblastoma-derived cell line SH-SY5Y (11). The difference between the protective effects of Parkin in the two studies may be related to differences in the cell lines used.

The protective effect of Parkin in ceramide-mediated cell death suggests that this pathway may be involved in the pathogenesis of Parkin-related parkinsonism. Interestingly, there is evidence that this pathway may play a role in idiopathic Parkinson's disease (18,19). Dopaminergic neurons carry type I receptors for the pro-apoptotic cytokine TNF-α, which is produced by neighbouring microglial cells in parkinsonian patients (20) and can induce cell death through synthesis of endogenous ceramide (21,22). Moreover, exogenous ceramide induces the death of dopaminergic neurons in primary cultures of rat mesencephalon, where it causes free radical production and activation of the transcription factor NFκB (23). Importantly, the activation of this transcription factor has also been observed in dopaminergic neurons of the *substantia nigra* in patients with sporadic Parkinson's disease (23).

The protective action of Parkin depends on its E3 ubiquitin–protein ligase activity

Parkin has E3 ubiquitin–protein ligase activity which is dependent on its *ubiquitin-like* and *Ring-IBR-Ring* domains (9,10). Several *parkin* mutations found in patients with autosomal recessive early-onset parkinsonism impair this activity (9,11). We therefore evaluated the effect of Parkin mutations on the ability of human Parkin to protect cells from ceramide-mediated stress. The mutations studied included deletion of the *ubiquitin-like* domain (Δ^{Ubl}) and various disease-linked point mutations affecting the linker region (K161N) and the first and second *Ring* domains (R256C, C289G, C418R) (Fig. 3A). Three out of these five Parkin mutants (Δ^{Ubl}, R256C, C418R) were previously reported to abolish the E3 ubiquitin–protein ligase activity of Parkin (9,12,24).

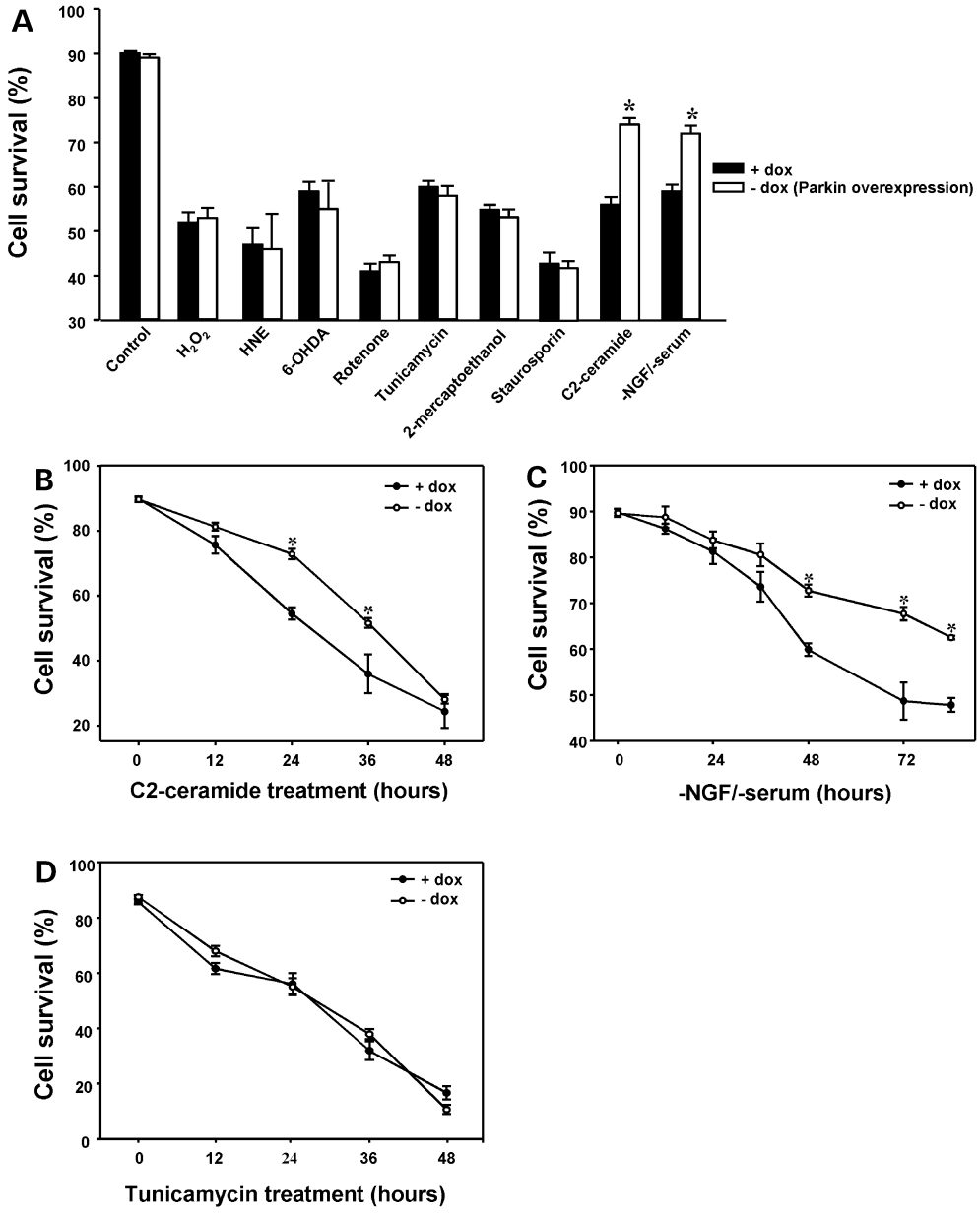

Figure 2. Parkin overproduction delayed ceramide-mediated cell death. (**A**) Protection by Parkin overproduction against cell death induced by diverse cell death stimuli used at concentrations titrated to induce 50% of cell death in 24 h. Parkin protected against the cell death induced by C2-ceramide (25 μM) or NGF and serum deprivation, a type of cell death involving the production of endogenous ceramide. In the latter paradigm, 50% cell death is observed at 48 h. Parkin did not protect against the other cell death stimuli tested (H_2O_2, 100 μM; 4-hydroxynonenal = HNE, 0.5 μM; 6-hydroxydopamine = OHDA, 100 μM; rotenone, 30 μM; tunicamycin, 10 μg/ml; 2-mercaptoethanol, 20 μM; staurosporin, 0.3 μM). The asterisk indicates significant difference from cells that did not overexpress Parkin (+dox) ($P < 0.01$). (**B** and **C**) Kinetic analysis of cell death induced by C2-ceramide (25 μM) and NGF and serum deprivation in the presence (−dox) or absence (+dox) of Parkin overexpression. Cell death is significantly delayed in Parkin-expressing cells. The asterisk indicates significant difference from cells that did not overexpress Parkin (+dox) ($P < 0.001$). (**D**) Parkin did not protect against cell death induced by tunicamycin (10 μg/ml).

Transgenes encoding normal and mutant Parkin were transiently expressed in the parent PC12 cell line. No substantial differences in the transfection efficiencies or basal survival rates were observed in cells transfected with the constructs expressing the Parkin mutants (not shown). When the transfected cells were treated with C2-ceramide or deprived of NGF and serum (Fig. 3B), normal Parkin was protective, as in the PC12 *Tet-off*-Parkin cell line. In contrast, this protective activity was abrogated by all the Parkin mutations examined, supporting the possibility that the ceramide-dependent pathway may be involved in the pathogenesis of Parkin-related parkinsonism. Interestingly, Parkin has been shown to be cleaved by caspases (25), and caspases can be activated in viable neurons in Parkinson's disease patients (26). Caspase cleavage of Parkin may therefore be a mechanism of Parkin loss of function, sensitizing dopaminergic neurons to ceramide-related stress in idiopathic Parkinson's disease.

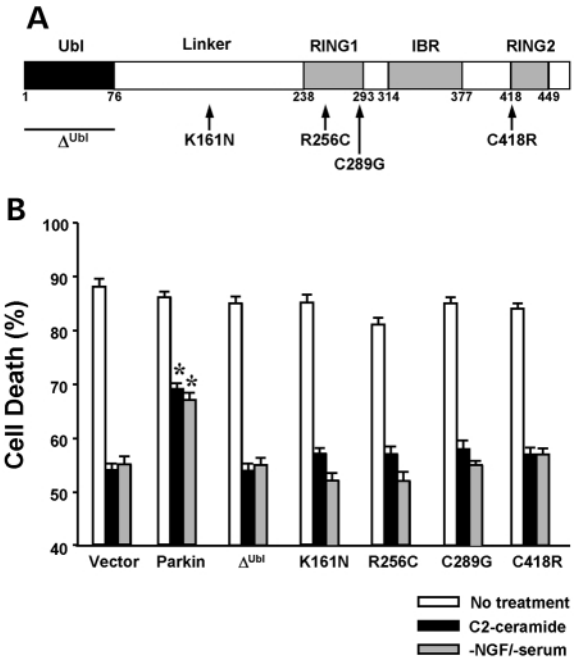

Figure 3. Mutations abolish Parkin protection against ceramide-mediated cell death. (**A**) Disease-causing Parkin mutations in the *ubiquitin-like* (Δ^{Ubl}), the linker (K161N) and *Ring-IBR-Ring* domains (R256C, C289G, C418R). (**B**) Normal Parkin, when transiently expressed in the parental PC12 cell line from which the PC12-*Tet-off*-Parkin cells were derived, was able to protect against C2-ceramide-induced cell death, measured at 24 h, or endogenous ceramide produced by NGF and serum deprivation, measured at 48 h. Transient expression of mutant forms of Parkin conferred no protection in these cell death paradigms. The asterisk indicates significant difference from cells transiently transfected with vector ($P < 0.01$).

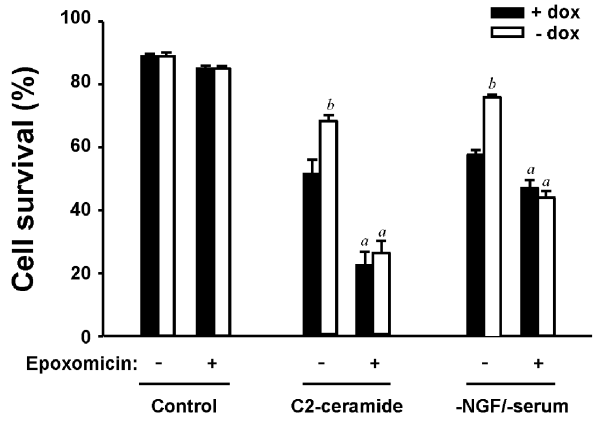

Figure 4. Proteasome inhibition abrogates Parkin protection against ceramide-mediated cell death. Epoxomicin (100 nM) exacerbated ceramide-mediated cell death and abrogated the protective effect of Parkin after C2-ceramide treatment or NGF and serum deprivation. Epoxomicin was added 2 h before the induction of cell death. [a]Significantly different from cells not treated with epoxomicin ($P < 0.05$). [b]Significantly different from cells treated with doxycycline ($P < 0.05$).

These results suggest that the E3 ubiquitin–protein ligase activity of Parkin and related protein degradation via the ubiquitin–proteasome pathway are essential for protection against ceramide-induced cell death. This hypothesis is supported by the observation that inhibition of proteasome activity by epoxomicin (100 nM) suppressed the protective role of Parkin in PC12 *Tet-off*-Parkin cells treated with C2-ceramide or deprived of NGF and serum (Fig. 4).

Parkin prevents ceramide-mediated cell death by delaying mitochondrial swelling, cytochrome c release and caspase-3 activation

In previous studies, we have shown that C2-ceramide or NGF and serum withdrawal induce a number of biochemical and morphological alterations at the mitochondrial level (27–30), including a rapid and prolonged increase in mitochondrial free calcium levels that is maximal 3 h after the beginning of the ceramide treatment (29,30), followed at 6 h by swelling of the mitochondrial matrix and rupture of the outer membrane (29). These events culminate in the release of cytochrome c followed by caspase-3 activation and the death of the cells (30).

As in native PC12 cells, a significant increase in mitochondrial free calcium levels was observed with the fluorescent calcium probe Rhod-2 in the PC12-*Tet-off*-Parkin cell line after 3 h of treatment with C2-ceramide. This increase was similar in cells overproducing Parkin and in cells treated with doxycycline (Fig. 5), indicating that Parkin does not affect this early event and may act further downstream in the ceramide-dependent cell death pathway. We have previously shown that the increase in mitochondrial free calcium levels is responsible for mitochondrial swelling and cytochrome c release (29,30). We therefore analysed the effects of Parkin on the alterations of mitochondrial ultrastructure.

The overexpression of Parkin did not affect the ultrastructure of mitochondria in basal conditions (Fig. 6A; control). In contrast, 6 h after the beginning of the C2-ceramide treatment, mitochondria appeared significantly better preserved in cells overexpressing Parkin compared with control cells (+dox), in which rupture of the outer mitochondrial membrane was already visible (Fig. 6A; C2-Cer, 6 h). To quantify time-related changes in mitochondrial volume in response to ceramide treatment, the area of the mitochondrial cross-sections was measured on electron micrographs at different time points (Fig. 6B). Mitochondria swelled progressively and dramatically in C2-ceramide treated control cells (+dox), but to a much lesser extent in cells overexpressing Parkin (−dox). At each time-point analysed (1, 3 and 6 h), the mean mitochondrial cross-sectional areas were significantly smaller in cells overproducing Parkin than in doxycycline-treated cells. Thus, alterations of mitochondrial morphology were considerably delayed by Parkin overproduction. Similar results were obtained by light scattering determinations of mitochondrial swelling on preparations of mitochondria isolated from ceramide-treated cells (not shown).

In agreement with the observation that Parkin delays deleterious swelling of mitochondria, less cytochrome c was released into the cytoplasm of PC12 cells overproducing Parkin than in control cells (+dox) during cell death induced by C2-ceramide or withdrawal of NGF and serum (Fig. 7). In contrast, overexpression of Parkin did not affect the amount of cytochrome c released from mitochondria of cells treated with tunicamycin, in agreement with the lack of a protective effect of Parkin (Fig. 2A) and the absence of mitochondrial swelling

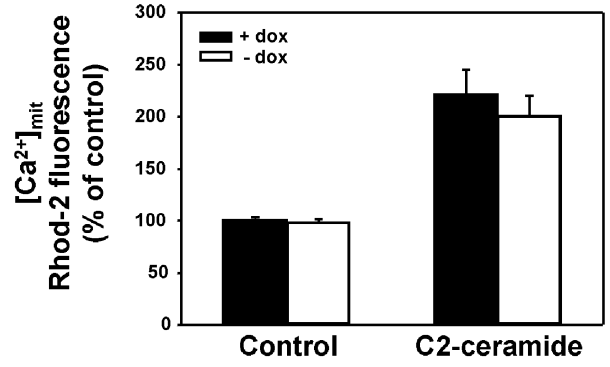

Figure 5. Parkin does not prevent the ceramide-induced increase in mitochondrial free calcium levels. C2-ceramide induced an increase in mitochondrial free calcium levels. Parkin overexpression, which prevented ceramide-mediated cell death, did not prevent the increase in mitochondrial free calcium levels in this paradigm.

(not shown) in this cell death paradigm. Similarly, Parkin overexpression prevented caspase-3 activation in C2-ceramide- and NGF and serum deprivation-induced cell death, but not in tunicamycin-induced cell death (Fig. 7). These results suggest that Parkin inhibition of mitochondrial swelling blocks the cell death pathway implicating cytochrome c release and caspase-3 activation. In addition they show that the molecular mechanism governing release of cytochrome c and activation of caspase-3 may differ in ceramide-dependent and in tunicamycin-induced cell death. We have previously shown that ceramide induces mitochondrial swelling and rupture of the outer membrane by a caspase-8- and Bid-mediated increase in mitochondrial free calcium levels (29,30). In contrast, this mechanism has not been implicated in tunicamycin-induced cell death (31,32).

Thus, Parkin may exert its protective activity by controlling mitochondrial homeostasis in models in which the molecular events that lead to cell death involve mitochondrial swelling and rupture of the outer mitochondrial membrane. In particular, in these paradigms, Parkin may act by delaying attainment of the critical volume at which the outer mitochondrial membrane ruptures, cytochrome c is released, and caspase-3 is activated.

Parkin is located on the outer mitochondrial membrane

The observation that Parkin acts downstream of the ceramide-induced increase in mitochondrial free calcium levels by reducing the extent of mitochondrial swelling and efflux of cytochrome c suggests that Parkin might act locally in the mitochondrial membrane. Previous subcellular fractionation studies have reported the presence of Parkin in the cytosol, the trans-Golgi network, the synaptic membrane compartment, postsynaptic densities and lipid rafts (33–35), and ultrastructural analyses have detected Parkin associated with cytoplasmic vesicles, endoplasmic reticulum, the outer nuclear membrane and, in a minority of the cells, the nuclear matrix (36). Interestingly, Parkin immunoreactivity has also been observed on the outer mitochondrial membrane (36), corroborating the idea that Parkin may act locally to control the levels of outer membrane protein substrates.

To evaluate whether a significant proportion of cellular Parkin is indeed located on mitochondria, we isolated subcellular fractions corresponding to mitochondria, microsomes (essentially endoplasmic reticulum) and cytosol from PC12 *Tet-off*-Parkin cells treated or not with doxycycline (Fig. 8A). The purity of each fraction was controlled by western blot detection of specific markers. As previously reported (33,35), Parkin immunoreactivity was present in the cytosol and the microsomal fraction of cells overproducing the human protein (Fig. 8A). Importantly, Parkin was also abundant in the mitochondrial fraction (Fig. 8A). To exclude the possibility that the mitochondrial localization of overexpressed Parkin in PC12 *Tet-off*-Parkin cells reflects a non-physiological localization, we determined the distribution of endogenous Parkin by subcellular fractionation of mouse brain. A similar distribution of Parkin was observed, indicating that the results obtained in the PC12 *Tet-off*-Parkin cells were physiologically relevant.

The reliability of these results was ascertained quantitatively by measuring the enzymatic activities of markers in each fraction. As expected, the activities of lactate dehydrogenase, NADPH-cytochrome c reductase and succinate dehydrogenase were enriched in cytosol, microsomes and mitochondria, respectively (Fig. 9). Estimation of the amount of Parkin in each mouse brain fraction by optical densitometry on western blots showed that Parkin was enriched in the cytosolic fraction by a factor 1.8 and in the mitochondrial fraction by a factor 3.9 (Fig. 9). Thus, although the absolute amount of Parkin in mitochondria represents only a small proportion of the total Parkin in the cells (about 1.5%), its enrichment in this fraction suggests that it has a physiological function.

To determine whether mitochondrial Parkin was located on the outer membrane or within the organelle, the pure mitochondrial fraction prepared by subcellular fractionation was subjected to limited trypsin digestion. In agreement with a previous ultrastructural study (36), Parkin was found to be located on the cytoplasmic side of the mitochondrion, since it was digested after incubation for 10 min with trypsin, as was the voltage-dependent anion channel (VDAC), a mitochondrial outer membrane protein (Fig. 10). By contrast, cytochrome c, which is located in the intermembrane space, and ND1, a subunit of respiratory chain complex I located in the mitochondrial inner membrane, remained intact during this incubation (Fig. 10).

Altogether, these results suggest that Parkin may act directly at the mitochondrial level to prevent ceramide-mediated cell death, although they do not preclude the possibility of an indirect effect of Parkin at a pre-mitochondrial stage. Indeed, mutant α-synuclein, implicated in an autosomal dominant form of parkinsonism, indirectly sensitizes cells to mitochondria-dependent apoptosis by inhibiting proteasome activity (37). Given the reported interactions between Parkin and α-synuclein (13), Parkin may antagonize the effect of α-synuclein on proteasome activity, thus protecting cells from mitochondria-dependent cell death. However, Parkin could also regulate the turnover of one or more specific substrates associated with the outer mitochondrial membrane and implicated in the control of mitochondrial volume. Thus, the identification of such substrates of Parkin will be essential to understand the protective role of Parkin during the mitochondrial stage of cell death.

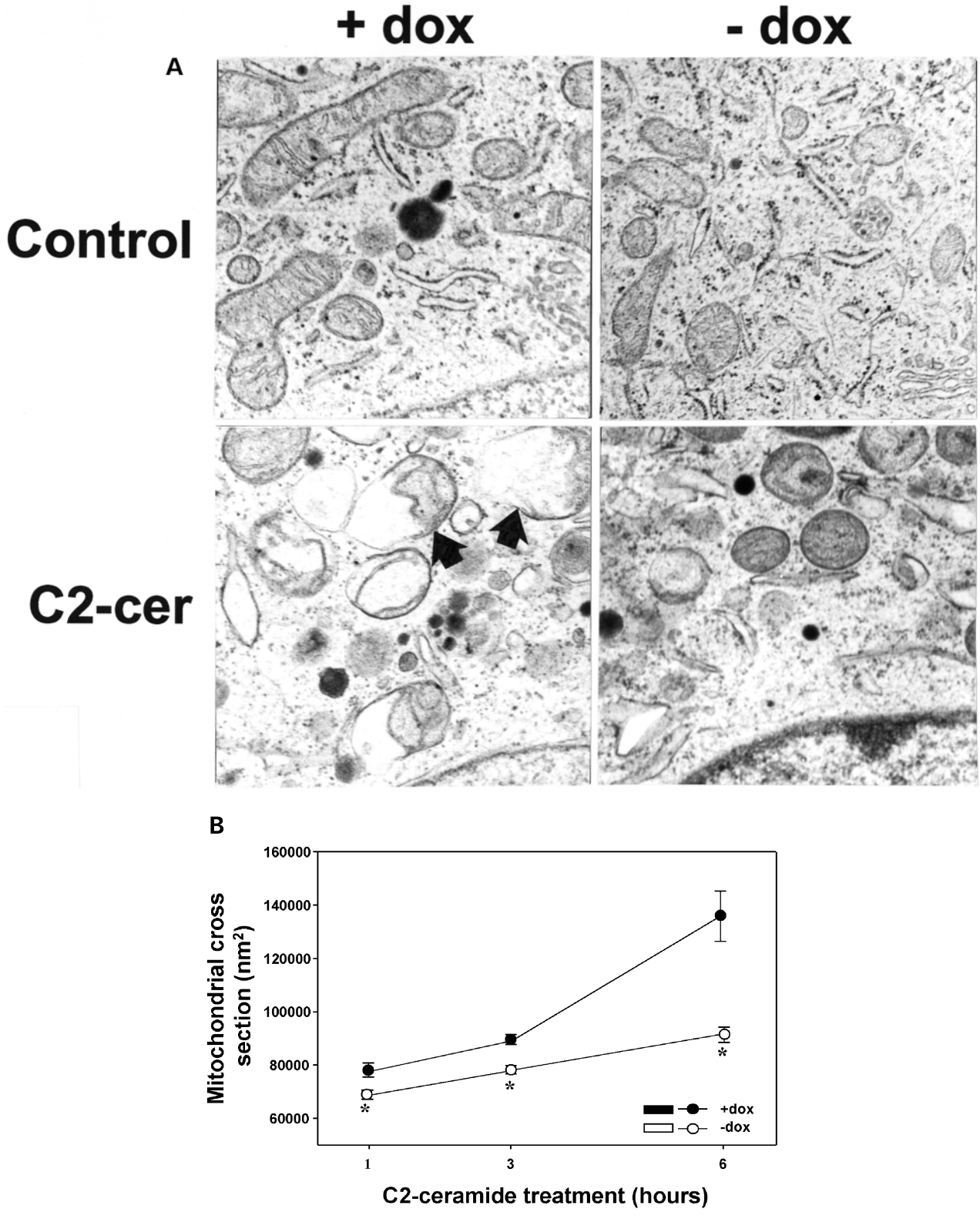

Figure 6. Parkin delays mitochondrial swelling due to ceramide. (**A**) The overproduction of Parkin in PC12-*Tet-off*-Parkin cells (−dox) did not modifiy the ultrastructure of mitochondria in basal conditions (control), but partially preserved mitochondria from swelling observed by electron microscopy after 6 h of treatment with C2-ceramide (25 μM). Arrows indicate mitochondria in which the outer membrane has ruptured. (**B**) Kinetic analysis of mitochondrial swelling during C2-ceramide treatment (25 μM), represented as the cross-sectional area of mitochondria measured on electron micrographs. The asterisk indicates significant difference from cells that did not overexpress Parkin (+dox) ($P < 0.05$).

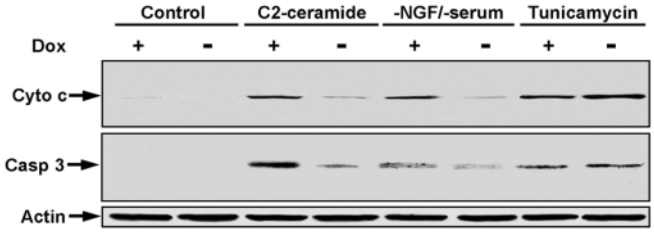

Figure 7. Parkin prevents cytochrome c release and caspase-3 activation caused by ceramide. Parkin production (−dox) prevented cytochrome c release and caspase-3 activation during cell death induced by C2-ceramide (25 μM) or serum and NGF deprivation, but not during cell death induced by tunicamycin (10 μg/ml). In all three models, cytochrome c release was assessed 12 h before 50% cell death was expected, i.e. at 12 h in C2-ceramide and tunicamycin treated cells and 36 h in NGF- and serum-deprived cells. Caspase-3 activation was assessed when 50% of cell were dead (at 24 h in C2-ceramide and tunicamycin treated cells and 48 h after NGF and serum deprivation).

MATERIALS AND METHODS

Tet-off-Parkin cell lines

We used a bidirectional pBi-L vector (Clontech) designed for concomitant tetracycline-regulated expression of a human parkin cDNA and a luciferase reporter gene (*Tet-off* system) to generate stable PC12 cell lines that conditionally express the human *parkin* cDNA (PC12 *Tet-off*-Parkin). Clones with highly inducible, low-background *parkin* expression were selected by immunocytochemical labelling of the cells [polyclonal anti-Parkin antibody (38) ASP5, 1/200] and western blot analysis [same antibody, 1/500; extraction buffer: 100 mM NaCl, 50 mM Hepes, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% Chaps, 2% Complete Mini inhibitor cocktail (Roche) and 0.2% Triton X-100].

Cell culture conditions and induction of cell death

PC12 cells, derived from a rat pheochromocytoma (39), were cultured for 7 days in L15 medium supplemented with 2% horse serum (Eurobio) and 100 ng/ml NGF 2.5S grade II (Alomone Laboratories), as previously described (29). Experiments were performed in the presence of 1% horse serum. We standardized the quantification of cell death by establishing dose–response studies for all agents used. Cells were considered viable when they excluded the non-permeable intercalating agent propidium iodide (1 μM). Experiments were routinely performed with doses that induced 50% of the cell death at 24 h in the absence of Parkin overexpression (+dox). When serum and NGF withdrawal was used to induce cell death, 50% of cell death occurred at 48 h.

Transient production of mutant forms of human Parkin

Human Parkin cDNAs harbouring a deletion of the ubiquitin-like domain (Parkin Δ^{Ubl}) or the point mutations K161N, R256C, C289G and C418R found in patients with Parkin-induced parkinsonism (Fig. 2A), were inserted into pcDNA3. These plasmids were used to transfect the parental PC12 cells from which the PC12 *Tet-off*-Parkin clones were derived with the DMRIE-C liposome (Invitrogen), according to the manufacturer's instructions. Co-transfection with a pcDNA3 vector containing the cDNA for green fluorescent protein (GFP) allowed the identification of transfected cells (more than 90% of GFP-positive cells also expressed Parkin). Cell death was induced 48 h after transfection.

Mitochondrial free calcium levels

Mitochondrial free calcium levels were quantified in viable cells with the positively charged rhodamine derivative Rhod-2 (10 μM, Molecular Probes, Eugene, OR, USA), which becomes fluorescent when it fixes a calcium ion. The cells were incubated for 30 min in L15 medium containing the probe, then rinsed twice with culture medium. Fluorescence images of randomly chosen fields (six to eight cultures per well; three wells per condition) were acquired (excitation, 525–580 nm; emission, 615–645 nm) with a 60× objective at regular intervals during the cell death process using an inverted fluorescence microscope equipped with a video camera and an image-analysis system (Imstar, Paris). Fluorescence was quantified over the whole surface of each cell body (nucleus excluded) in the selected fields. The mean intensity of fluorescence/pixel was then calculated for each cell.

Mitochondrial swelling

To analyse mitochondrial volume in whole cells, electron microscopy was performed as previously described (29). We quantified the cross-sectional area of mitochondria on electron micrographs (10 000× magnification) with Mercator software (Explora Nova, France). At least 150 mitochondria were measured in six to ten cells in each condition.

Cytochrome c release

Cytochrome c release was analysed by western blotting. Proteins were extracted 12 h after treatment with C2-ceramide or tunicamycin and 36 h after NGF and serum withdrawal (i.e. 12 h before the time point at which cell death reaches 50%). Cells were lysed in buffer containing 250 mM Sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EGTA, 1 mM DTT and protease inhibitor set (Roche diagnostic). Homogenates were centrifuged twice at 750*g* for 10 min at 4°C, and supernatants were centrifuged at 10 000*g* for 15 min at 4°C. The resulting supernatants were further centrifuged at 100 000*g* for 1 h at 4°C. The remaining supernatants contained cytosolic proteins that were separated by 15% SDS–PAGE. The protein blots (15 μg/lane) were incubated with a monoclonal anti-cytochrome c antibody (1/250, PharMingen International), then with a secondary antibody conjugated with horseradish peroxidase (1/50 000). Immunoreactivity was detected with the Super Signal chemiluminescence detection kit (Pierce).

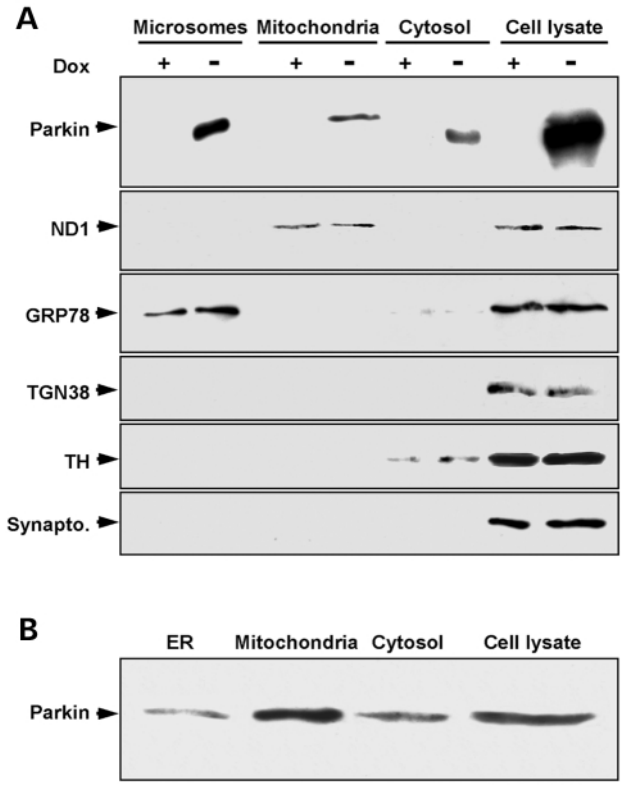

Figure 8. Parkin is present in the mitochondria. (**A**) Subcellular fractionation (see Methods) of PC12 *Tet-off*-Parkin cells that overproduce Parkin (−dox) or not (+dox). Ten micrograms of protein were loaded in each lane. Parkin, labelled with the ASP-5 antibody (38), is present in the mitochondrial, microsomal and cytosolic fractions. The purity of the mitochondrial fraction is shown by the presence of the mitochondrial ND1 gene product and the absence of markers for the ER (GRP78), trans-Golgi network (TGN38), cytosol (tyrosine hydroxylase) and synaptic vesicles (synaptophysin). (**B**) The distribution of Parkin was similar in subcellular fractions of mouse brain analyzed with an antibody directed against mouse Parkin (36).

Caspase-3 activation

Cells were treated for 24 h with C2-ceramide or tunicamycin and deprived of NGF and serum for 48 h. Proteins were extracted in buffer containing 50 mM Pipes / NaOH (pH 6.5), 2 mM EDTA, 0.1% Chaps, 0.1% Triton X100, 5 mM DTT, and protease inhibitor set (Roche diagnostic). Lysates were centrifuged at 10 000*g* for 15 min at 4°C, and 30 µg of supernatant proteins were separated by 10% SDS–PAGE. The remaining steps were performed as described above, with a rabbit polyclonal antibody raised against cleaved caspase-3 (Asp175 Antibody, Cell Signalling).

Subcellular fractionation

Quantitative subcellular fractionation was carried out as previously described (40). Briefly, cells were resuspended in 10 ml of buffer A (0.25 M sucrose, 10 mM Hepes/NaOH, pH 7.5, 1 mM DTT, protease inhibitor) and lysed mechanically. The lysate was clarified by centrifugation at 1000*g* for 10 min at 4°C to remove unlysed cells, large debris and nuclei. The

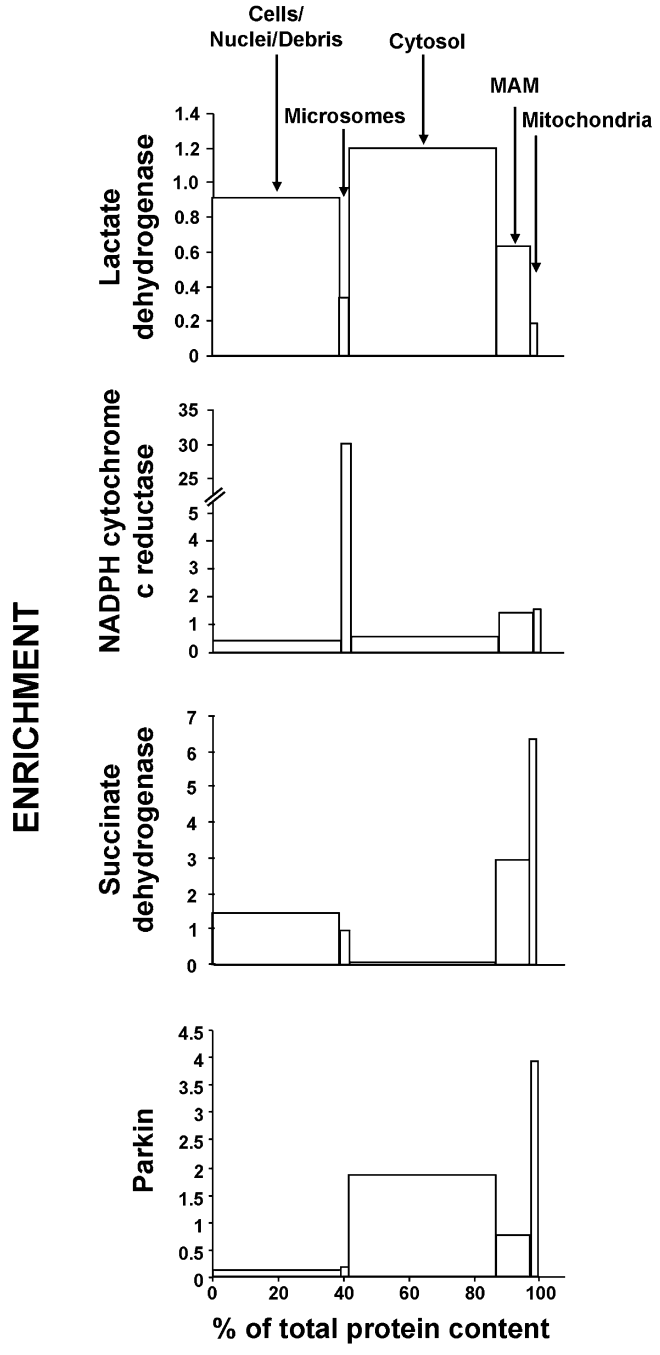

Figure 9. Parkin is enriched in the mitochondrial fraction. The enzymatic activity of lactate dehydrogenase, NADPH-cytochrome c reductase and succinate dehydrogenase were quantified in each fraction. The enrichment (percentage of recovered enzyme activity/percentage of protein content in each fraction) of these enzymes was calculated in each fraction and represented on the ordinates. Lactate dehydrogenase, NADPH-cytochrome c reductase and succinate dehydrogenase were enriched in the cytosolic, microsomal and mitochondrial fractions, respectively, showing that the fractionation procedure was successful. The quantity of Parkin in each fraction was estimated by optical densitometry on western blot and enrichment (percentage of recovered Parkin content/percentage of protein content in each fraction) was calculated. Parkin is enriched in cytosol (1.8 times) and in mitochondria (3.9 times). The sum of Parkin content in each fraction represents 105.9% of total amount in the initial homogenate, showing that no material was lost during the purification procedure.

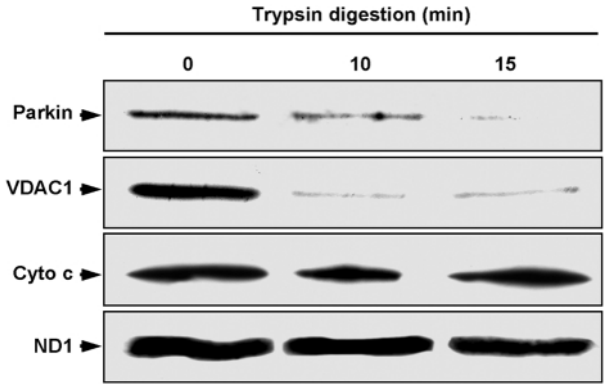

Figure 10. Parkin is localized on the cytoplasmic side of mitochondria. Limited digestion of mitochondria (prepared as in Fig. 8) with trypsin-EDTA (0.05%, 37°C) shows that Parkin is accessible to the protease in a similar manner as the outer membrane protein VDAC, suggesting that it is localized on the cytoplasmic surface of the mitochondrion.

resulting supernatant was centrifuged at 10 000*g* for 10 min at 4°C. The 10 000*g* supernatant (S10) was centrifuged at 100 000*g* for 1 h. The microsomal pellet (P100, containing endoplasmic reticulum and other membranes) was resuspended in buffer A. The 100 000*g* supernatant (S100) represented the cytosolic fraction. The P10 pellet was resuspended in 2 ml isolation buffer (250 mM mannitol, 25 mM Hepes / NaOH, pH 7.5, 0.5 mM EGTA), layered on top of 20 ml of a 30% (v/v) Percoll solution (prepared in 225 mM mannitol, 25 mM Hepes/NaOH, pH 7.5, 1 mM EGTA) and centrifuged for 30 min at 95 000*g*. Two dense bands appeared: the lower band corresponded to pure mitochondria and the upper band represented mitochondria associated with membranes (MAM). Both fractions were recovered, washed with buffer A and resuspended in isolation buffer.

Purity of fractions and localization of Parkin

Mitochondria were identified with an antibody against ND1 (NADH dehydrogenase 1), a mitochondrion-encoded subunit of respiratory chain complex I (1/250, generously provided by Dr Anne Lombes). Endoplasmic reticulum, trans-Golgi network and cytosol were identified with antibodies against the ER lumen protein GRP78 (1/500, Stressgen), TGN38 (Transduction Laboratories) and tyrosine hydroxylase (1/500, Diasorin), respectively. We assessed possible contamination with synaptic vesicles by incubation with an antibody directed against synaptophysin (1/3000, Sigma). Parkin was detected with the ASP-5 antibody when the fractionation was carried out in PC12 *Tet-off*-Parkin cells. For confirmation, the subcellular fractionation was repeated with mouse brain, and Parkin was detected with an antibody directed against residues 71–91 of the murine protein (36).

To analyse the fractionation procedure, the enrichment of specific markers in each fraction (percentage of recovered enzyme activity in a fraction/percentage of total recovered proteins in this fraction) was calculated as previously described (41). The enzymes assayed were lactate dehydrogenase (42), NADPH-cytochrome c reductase (43) and succinate dehydrogenase (42), marker of cytosol, microsomes and mitochondria, respectively. The enrichment of Parkin in each fraction (percentage of recovered Parkin in a fraction/percentage of total recovered proteins in this fraction) was determined by optical densitometry on western blot with Mercator software (Explora Nova). A fraction is considered to be enriched in a marker or in Parkin when the ratio is greater than 1.

The precise localization of Parkin in mitochondria was assessed by carrying out limited proteolysis. Samples were incubated with 0.05% trypsin-EDTA at 37°C during 10 min. The reaction was stopped by adding Laemmli buffer and the samples were western blotted to test for the presence of Parkin.

Statistical analysis

Statistical analysis was performed by ANOVA. Differences between groups were analysed with the Student–Newman–Keuls test. Results were considered to be statistically significant at $P < 0.05$. Data are expressed as mean ± SEM. Experiments were performed in triplicate and repeated at least three times.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Anne Lombes (INSERM U506, Paris) for the ND1 antibody. This work was supported by Ministère de la recherche et de la technologie (F.D., C.H.) and Aventis Pharma (O.C., W.J.G.).

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