Parkin is recruited into aggresomes in a stress-specific manner: over-expression of parkin reduces aggresome formation but can be dissociated from parkin’s effect on neuronal survival

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INTRODUCTION

Parkinson’s disease (PD) is characterized by loss of dopamine neurons in the substantia nigra and the presence of cytoplasmic inclusions known as Lewy bodies (LBs). Mutations in parkin cause autosomal recessive juvenile parkinsonism (AR-JP) that is distinct from sporadic PD by the general absence of LBs. Several studies have reported that parkin is present in LBs of sporadic PD but the role of parkin in LB formation is unclear. Aggresomes are perinuclear aggregates representing intracellular deposition of misfolded protein. LBs and aggresomes have been reported to share a common biogenesis. We have investigated the role of parkin in aggresome formation. In human SH-SY5Y neuroblastoma cells we observe that endogenous parkin is present in aggresomes induced by a variety of stresses including dopamine, proteosome inhibition and a pro-apoptotic stimulus. We show that vimentin is invariably collapsed around the aggresome but that the detection of ubiquitin is variable depending on the stress. We show that cells that stably over-express human wild-type parkin form fewer aggresomes upon stress compared to cells that express vector alone whereas over-expression of AR-JP causing mutants of parkin have no effect on stress-induced aggresome formation. Finally, we show that the prevention of aggresome formation by over-expression of wild-type parkin is not always associated with a beneficial effect on neuronal survival. Our findings suggest that parkin is important for aggresome formation in human neuronal cells and may lead to a better understanding of the biogenesis of LBs in sporadic PD.

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identification of disease-causing mutations in two genes that are involved in the UPS: (i) parkin, an E3 ubiquitin ligase that is mutated in autosomal recessive juvenile parkinsonism (AR-JP) (4); and (ii) ubiquitin C-terminal hydrolase (UCH-L1) that is mutated in a single family with autosomal dominant PD (5).

There have been several reports that parkin is present in LBs of brains of sporadic and familial PD cases (6–8). In contrast, AR-JP brains do not contain LBs (6,8–12) although there is one report of LBs in an AR-JP case with compound heterozygote mutations in parkin (13). The general absence of LBs in AR-JP suggests that parkin may play an important role in LB formation. Alternatively, AR-JP may have a different pathogenesis from familial and sporadic PD associated with LBs.

Previous studies in cultured cells have revealed an additional mechanism for handling misfolded proteins. When the production of misfolded proteins exceeds the cellular capacity to degrade them, proteins accumulate within single large perinuclear inclusions termed aggresomes (14). Aggresomes develop at the microtubule organizing center (MTOC) from smaller aggregates that are retrogradely transported to the MTOC along microtubules (15). Aggresomes may form when certain proteins are over-expressed (14,15), when the proteosome is inhibited (14) or are induced by certain stresses including heat shock (16). It has been suggested that aggresomes may be a way for cells to sequester toxic misfolded proteins from the cytoplasm and/or to facilitate their removal by the lysosomal autophagic pathway (17). Several recent studies have reported that parkin localizes to aggresomes or inclusions in parkin over-expressing cells in response to proteosome inhibition (18,19). Furthermore, it has recently been reported that endogenous parkin localizes to the centrosome in response to proteosome inhibition (20).

In view of the considerable interest in the role of LBs in the pathogenesis of PD and their general striking absence in AR-JP cases, we have sought to understand the cellular role of parkin in aggregate formation by studying parkin’s role in aggresome formation in neuronal cells. In contrast to previous studies (18,19), this is the first report that endogenous parkin can localize to aggresomes. Furthermore, in addition to proteosome inhibition we now report two new stresses namely dopamine and the pro-apoptotic stress, staurosporine, that are able to induce aggresome formation that suggests that dysfunction of aggresomes is not a general response to all stresses since parkin did not form aggresomes in cells incubated for 24 h with 5 μM staurosporine, an alkaloid that is a potent inducer of apoptosis and inhibitor of phospholipid/Ca2+ dependent protein kinase (PKC) (22) or for 24 h with 0.125 mM dopamine, a naturally occurring amine that is prone to auto-oxidation with resultant increase in oxidative stress (23). Both these stresses caused parkin to form aggresomes in ~35% of cells (Fig. 1C, D and F). As with MG-132 and similar to other published studies, all the aggresomes detected appeared to be juxtanuclear and to indent the nuclear membrane (Fig. 1B–D). Parkin has previously been shown to play a role in the unfolded protein response (UPR) pathway (24). However, the formation of aggresomes is not a general response to all stresses since parkin did not form aggresomes in cells incubated for 24 h with 50 μg/ml tunicamycin, a specific inhibitor of N-glycosylation in the endoplasmic reticulum and potent inducer of the UPR pathway (25) (Fig. 1E and F). Given that the concentration and duration of tunicamycin used produced a biological effect comparable with the other stresses (i.e. ~50% cell death), it is most likely that the absence of aggresome formation is specific to tunicamycin for the conditions used in this study. Moreover, the concentration of tunicamycin that we used was five times higher and of longer duration than previous studies which have also not observed significant aggregate formation with this compound in neuronal cells (19,26).

The composition of aggresomes is dependent on the stress

To determine whether these stress-induced parkin-positive aggresomes had the typical structure of previously reported aggresomes we sought to characterize their composition. It has previously been reported that the intermediate filament, vimentin, is invariably present in aggresomes where it forms a cage-like structure around the outside of the aggresome (17). In addition, aggresomes are enriched in chaperones such as Hsc-70 and are often reported to be ubiquitylated.

We undertook co-localization studies with an anti-parkin antibody together with anti-vimentin, anti-Hsc-70 and an anti-ubiquitin antibody that recognizes mono- and polyubiquitin. In SH-SY5Y cells treated with vehicle (DMSO or water) parkin was expressed predominantly in the cytoplasm with nuclear speckling and the staining was distinct from that of vimentin and ubiquitin although there was some cytoplasmic overlap with Hsc-70 (Fig. 2A). In cells stressed with MG-132, parkin-positive aggresomes were surrounded by vimentin. In most cells, Hsc-70 formed a ring around parkin whereas ubiquitin co-localized with parkin more centrally (Fig. 2B). These observations on MG-132 induced parkin-positive aggresome mirror those reported

RESULTS

Endogenous parkin forms perinuclear aggresomes in a stress-specific manner

Parkin has previously been detected in aggresomes (18) or inclusions (19) in neuronal cells over-expressing parkin that have been exposed to a proteosome inhibitor. To investigate the ability of endogenous parkin to localize to aggresomes, human SH-SY5Y dopaminergic neuroblasts were incubated in 10 μM of the peptide aldehyde, Cbz-leu-leu-leucinal (MG-132) that is a low molecular weight inhibitor of the proteosome (21) for 24h. Immunofluorescence studies using a human anti-parkin antibody and Hoechst stain for nuclei demonstrated that endogenous parkin formed a single large perinuclear aggresome-like structure in ~65% of cells (Fig. 1B and F) compared to no aggresomes in untreated cells (Fig. 1A and F).

To determine if parkin-positive aggresomes could form in response to other stresses, SH-SY5Y cells were incubated for 4 h with 5 μM staurosporine, an alkaloid that is a potent inducer of apoptosis and inhibitor of phospholipid/Ca2+ dependent protein kinase (PKC) (22) or for 24 h with 0.125 mM dopamine, a naturally occurring amine that is prone to auto-oxidation with resultant increase in oxidative stress (23). Both these stresses caused parkin to form aggresomes in ~35% of cells (Fig. 1C, D and F). As with MG-132 and similar to other published studies, all the aggresomes detected appeared to be juxtanuclear and to indent the nuclear membrane (Fig. 1B–D).

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Figure 1. Endogenous parkin forms perinuclear aggresomes in a stress-specific manner in SH-SY5Y cells. Sub-confluent SH-SY5Y cells were grown on glass coverslips and were treated with various agents as detailed in the Materials and Methods section. Cells were then fixed in 4% paraformaldehyde and processed for immunofluorescence with a C-terminal antibody to parkin and a rhodamine-conjugated secondary (red). Nuclei were counterstained with Hoechst (blue). Digital images were captured using a Zeiss Axiocam camera. Representative fields are displayed. (A) In control cells exposed to vehicle, endogenous parkin was detected predominantly in the cytoplasm especially within the perinuclear region. In response to 10 μM MG-132 for 24 h (B), 5 μM staurosporine for 4 h (C) or 0.125 mM dopamine for 24 h (D), endogenous parkin was detectable as a single perinuclear aggresome that indented the nucleus. (E) 50 μg/ml tunicamycin for 24 h did not induce parkin-positive aggresomes. (F) For each stress, the percentage of aggresome-containing cells was determined by counting 300 cells across 20 random fields. The data represent means ± SEM for 3–5 independent experiments. Scale bar 10 μm.
Previously (18). Furthermore, the location of parkin and ubiquitin in the aggresomes parallel one histopathological study that demonstrated that C-terminal parkin and ubiquitin immunoreactivity were strongest in the dense core of LBs (8). In contrast to MG-132-induced aggresomes, staurosporine-induced aggresomes contained vimentin but we were unable to detect the presence of Hsc-70 or ubiquitin in these aggresomes (Fig. 3A). This was not due to technical differences as the staurosporine and MG-132 co-localization studies were performed in parallel with the same antibodies. For dopamine-induced aggresomes, parkin was found to co-localize with vimentin, Hsc-70 and ubiquitin in most cells examined (Fig. 3B).

We also examined whether any putative substrates of parkin were found in aggresomes induced in our system. There are conflicting reports of α-synuclein immunoreactivity in aggresomes (18,27,28) and we were unable to detect endogenous α-synuclein in aggresomes induced by any of the above stresses (data not shown). We also could not detect the recently identified substrate, cyclin E (29), in MG-132 induced aggresomes (data not shown).

Parkin-positive aggresomes localize to the MTOC and are dependent on an intact microtubule network

It has recently been reported that parkin is recruited to the microtubule organizing centre (MTOC) in response to proteosome inhibition (20). We performed co-localization studies with an anti-parkin antibody and a γ-tubulin antibody that is a marker for the MTOC. In unstressed cells we found that parkin did not co-localize with γ-tubulin in the majority of cells (~85%) (Fig. 4A). However, we found that parkin-positive aggresomes co-localized with γ-tubulin in the presence of MG-132, staurosporine and dopamine (Fig. 4B–D) suggesting that aggresomes in our system localize to the MTOC.

Aggresome formation requires an intact microtubule system (14). We then investigated what effect the microtubule-depolymerizing drug colchicine would have on the formation of stress-induced aggresomes in our system. In cells co-incubated with colchicine (5 μg/ml) and staurosporine or dopamine we found that the formation of aggresomes was inhibited with striking alteration in the parkin and vimentin staining (Fig. 4E and F). Similar results were obtained for MG-132 treated cells (data not shown) as has been reported previously (20). In parallel wells of the same experiments with the stress alone we observed parkin-positive aggresomes with robust co-localization with vimentin (data not shown).

Stable over-expression of parkin reduces aggresome formation and protects cells against MG-132 toxicity

Since endogenous parkin has not previously been observed in aggresomes, we investigated whether altering parkin levels would affect aggresome formation. SH-SY5Y cells stably over-expressing human wild type parkin or empty vector were made and successful over-expression was demonstrated by western blotting (Fig. 5). Using an antibody to C-terminal parkin we were also able to detect a smaller ~40 kDa product in addition to parkin on western blotting (Fig. 5). This could be a partially translated parkin product or a cleaved form of parkin that has previously been reported with the same antibody in human M17 cells (30).

We found that SH-SY5Y cells over-expressing parkin formed significantly fewer aggresomes in response to MG-132 than the vector control (P < 0.05) (Fig. 6A–D). Similar results were obtained when aggresomes were stained with vimentin or parkin antibodies (Fig. 6D). Under the same conditions this reduction in aggresomes correlated with significant neuroprotection by parkin over-expression against MG-132 (10 μM) toxicity compared to vector alone (P < 0.001) (Fig. 6E). The neuroprotective effect of parkin against MG-132 toxicity is in agreement with a recent report in human M17 neuroblastoma cells (30). This was confirmed in another independently isolated set of clones (data not shown) and therefore is unlikely to be due to clonal properties acquired by the cells during the selection process.

Stable over-expression of parkin reduces staurosporine and dopamine-induced aggresomes but is not sufficient to protect against toxicity

We next determined whether altering parkin levels could affect aggresome formation in response to staurosporine or dopamine toxicity. SH-SY5Y cells stably expressing parkin formed significantly fewer aggresomes induced by either staurosporine (P < 0.01) or dopamine (P < 0.05) compared to the vector alone (Fig. 7A–C). However, in contrast to our results with MG-132, the reduction in aggresomes did not correlate with a protective effect of parkin over-expression against toxicity induced by either of these stresses (Fig. 7D and E). A previous study has reported that parkin over-expression can protect against unfolded protein stress (24). Interestingly, although tunicamycin does not form aggresomes in our system, parkin protected against this stress compared to vector alone (P < 0.05) (Fig. 7F). These data suggest that for these stresses, the formation and reduction of aggresomes are not linked to parkin's effect on neuronal survival. Again two sets of independent clones were used for these studies indicating that the reduction in aggresomes is a consequence of parkin over-expression and not simply clonal properties of the cells used.
Disease causing mutants of parkin fail to suppress aggresome formation

We next wanted to examine what effect disease causing mutants of parkin would have on aggresome formation in our system. Using a transient expression system with a GFP reporter plasmid we quantified aggresome formation with two disease causing mutants of parkin, K161N that affects the central part of the parkin protein and G328E that is an ubiquitin ligase domain mutant of parkin (Fig. 8A). The K161N...
mutation is a missense mutation that occurred in trans with a truncating deletion (202-203delAG) in one family and both mutant alleles co-segregated with disease (31). The G328E mutation is a missense mutation reported in a PD case heterozygous for a single mutation (32). Under basal conditions, transfection of parkin or the G328E mutant did not induce significant aggresome formation, however transfection of the K161N mutant resulted in a small but significant increase in aggresomes compared to the vector control ($P < 0.05$) (Fig. 8B and F). This was not due to differences in protein expression as western blotting confirmed equal wild-type and mutant parkin expression after transient transfection (Fig. 8G). By confocal microscopy we found the majority of K161N transfected cells did not localize to the MTOC but in a small percentage of transfected cells (~3%), parkin localized to aggresomes and co-localized with γ-tubulin under basal conditions (data not shown). In wild-type parkin and G328E transfected cells, we did not see parkin
co-localizing at the MTOC in the majority of cells and we did not see aggresomes under basal conditions (data not shown).

Over-expression of wild type parkin significantly reduced aggresome formation in response to MG-132 compared to vector \( (P < 0.05) \) (Fig. 8C and F), in response to staurosporine \( (P < 0.05) \) (Fig. 8D and F) and in response to dopamine \( (P < 0.01) \) (Fig. 8E and F). In contrast, the K161N and G328E mutants were not significantly different from the vector for any stress suggesting that parkin’s ability to suppress aggresome formation is dependent on an intact central domain and ubiquitin ligase domain (Fig. 8A–F).
DISCUSSION

Two observations suggest that parkin plays a key role in LB biogenesis in vivo. Firstly, there have been several reports demonstrating parkin in Lewy bodies of sporadic and familial PD (6–8) and further studies performed at the Queen Square Brain Bank within our institution have confirmed this (R. Bandopadhyay, personal communication). Secondly, AR-JP brains, in all but one case lack LBs. This suggests that parkin plays an important role in LB formation either directly or indirectly via ubiquitylation of specific substrates that in turn promote LB formation. Therefore, to address the role of parkin in LB formation, we have studied parkin’s role in aggresome formation since LBs share many of the characteristics of aggresomes and are postulated to form in the presence of excess protein misfolding (33).

Our study demonstrates that endogenous parkin is detectable in aggresomes following stress. Two recent studies have demonstrated that parkin is detectable in aggresomes or inclusions in cells over-expressing parkin exposed to a variety
of proteosome inhibitors (18,19). However, these studies used tagged forms of parkin and have not addressed the role of endogenous parkin in their systems. Another study published while this manuscript was in preparation reported that endogenous parkin localizes to the centrosome in the presence of proteosome inhibition (20).

Our study is the first to show that a pro-apoptotic agent, such as staurosporine, and the oxidant, dopamine, can induce parkin-containing aggresomes. There is accumulating evidence that protein misfolding and UPS activation occur during apoptosis (34). Furthermore, two recent reports suggest that parkin-related neurodegeneration may be mediated through the apoptotic cascade (35,36). The relative specificity of the neuronal loss to dopamine neurons suggests that dopamine dysfunction itself may be important in the pathogenesis of PD (37). Previous studies have shown that oxidative stress in neurons leads to increased ubiquitin-protein conjugates (38) and that the UPS is involved in the removal of oxidized proteins (39). The most compelling evidence linking oxidative damage with abnormal proteolysis has been the in vivo demonstration that chronic exposure of the mitochondrial complex I inhibitor, rotenone, was able to induce the formation of LB-like aggregates as well as striatal neurodegeneration in rats (40). Our observations that dopamine in our system is able to induce the formation of parkin-positive aggresomes leads us to postulate that, like staurosporine, both these stresses may result in increased levels of misfolded proteins that exceed the cell’s degradative capacity.

The finding of dopamine-induced aggresomes is particularly interesting as it may explain why LBs are found relatively specifically within substantia nigral neurons in PD.

Figure 4. Parkin-positive aggresomes localize to the MTOC and are sensitive to microtubule disruption. Sub-confluent SH-SYSY cells were grown on glass coverslips and treated with vehicle (A), MG-132 (B), staurosporine (C) or dopamine (D) as detailed in the Materials and Methods section. Cells were then fixed in 4% paraformaldehyde and processed for confocal laser microscopy by double labelling cells with a C-terminal antibody to parkin and a rhodamine-conjugated secondary (red) and an anti-γ-tubulin antibody with a FITC-conjugated secondary (green). Digital images were obtained by confocal laser scanning. Representative fields from two independent experiments are displayed. (A) In vehicle treated cells parkin did not co-localize with γ-tubulin in the majority of cells. (B) In MG-132 treated cells parkin-positive aggresomes co-localized with γ-tubulin. (C) In staurosporine treated cells parkin-positive aggresomes co-localized with γ-tubulin. (D) In dopamine treated cells parkin-positive aggresomes co-localized with γ-tubulin. Note that the area of γ-tubulin staining in the stressed cells was generally increased compared to unstressed cells. (E, F) Sub-confluent SH-SYSY cells were grown on glass cover-slips and treated with staurosporine (E) or dopamine (F) in the presence of 5μg/ml colchicine for the duration of the stress as detailed in the Materials and Methods section. Cells were then fixed in 4% paraformaldehyde and processed for confocal laser microscopy by double labelling cells with a C-terminal antibody to parkin and a rhodamine-conjugated secondary (red) and an anti-vimentin antibody with a FITC-conjugated secondary. Colchicine blocked aggresome formation to all stresses with severely altered parkin staining and vimentin staining. Aggresomes were seen in parallel wells without colchicine in the same experiment (data not shown). Scale bar 8 μm.
Tunicamycin did not induce aggresomes. A recent study in SH-SY5Y cells using a concentration of tunicamycin five times lower than the dose used in our study also did not find that tunicamycin induced parkin-positive inclusion formation (19). A previous study in primary hippocampal neurons and glia did not find tunicamycin-induced aggregates but did find altered distribution of parkin in glia (26). It may be that while tunicamycin can induce ER stress and protein unfolding, these proteins may remain within the ER and are not transported into the cytoplasm for degradation by the UPS (41).

In addition to their localization to the MTOC and sensitivity to microtubule disruption, aggresomes are characterized by their constituents. All stress-induced aggresomes that we observed were surrounded by vimentin that has been reported to be an invariant feature (17). Only MG-132 and dopamine-induced aggresomes also contained Hsc70 and ubiquitin. We were unable to detect ubiquitin or Hsc-70 immunoreactivity in staurosporine-induced aggresomes. Previously, other types of aggresomes formed by over-expression of GFP-250 (15) and superoxide dismutase (SOD) (42) have been reported to be negative for ubiquitin immunoreactivity. It may be that the structure of these aggresomes is distinct from those induced by MG-132 and dopamine or that the ubiquitin epitope is not accessible to the ubiquitin antibody used. Alternatively, the incorporation of ubiquitin and Hsc-70 may occur after parkin is recruited to aggresomes and after the 4 h duration that we used to study the effects of staurosporine (in contrast to the longer duration for the other stresses). One could delineate between these possibilities by purifying the aggresomes induced by staurosporine and examining the components more directly.

While endogenous parkin appears to be recruited to aggresomes, we found that aggresome formation is reduced in cell lines over-expressing human wild type parkin. Moreover, this property of parkin appears to be dependent on an intact central and ubiquitin ligase domain as the disease-causing mutants K161N and the G328E prevented this effect. Interestingly the K161N mutant promoted aggresome formation under basal conditions albeit at low levels that suggests that its pathogenicity may be mediated in part by altered conformational changes. In the presence of stress, we did not observe any difference between either mutant and vector alone. This suggests that the mutations have an inactivating effect on parkin activity of the mutant protein rather than a dominant negative effect on endogenous parkin. A recent report also

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**Figure 5.** Over-expression of parkin in human SH-SY5Y neuroblastoma cell lines. Parkin protein expression was determined in SH-SY5Y clonal lines transfected with vector (lane 1) or expression plasmid for human parkin (lane 2) by western blot analysis using a C-terminal antibody to human parkin. Full-length parkin is detected as a band running at 52kDa (arrow). A C-terminal fragment (~40kDa) in the parkin-transfected clonal lines (arrow) is also noted that has been previously reported with this antibody in human M17 lines (30). 1 μg of protein lysate was loaded in each lane. Protein loading was determined by re-probing the same membrane with GAPDH antibody. Inset: prolonged exposure of the same membrane allowed detection of the endogenous parkin protein in the vector lane 1 (•).
Over-expression of parkin reduces MG-132-induced aggresome formation and protects cells against MG-132 toxicity. Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown on glass coverslips and treated with 10 μM MG-132 for 24 h. Adherent cells were fixed with 4% paraformaldehyde and processed for immunofluorescence with an anti-vimentin antibody and a FITC-conjugated secondary (green) (A, B) or with a C-terminal parkin antibody and rhodamine-conjugated secondary (red) in independent experiments (C). Digital images were captured using a Zeiss Axiocam camera. Arrows point to aggresomes in (B) and (C). Representative fields at low (A) and high (B) magnification from the same experiment demonstrate that parkin-expressing stable cell lines (right) form fewer vimentin-positive aggresomes compared to vector (left). Note that vimentin-stained aggresomes appear solid at these magnifications. Identical exposure times were used within each experiment. (C) Representative fields demonstrating that parkin-expressing stable cell lines (right) form fewer parkin-positive aggresomes compared to vector (left). Exposure times for vector were three times longer than parkin-expressing cells. (D) The percentage of aggresomes induced by MG-132 in either vector or parkin-stable cell lines was determined by counting 300 cells across 20 random fields for each clone. There was no significant difference in the number of aggresomes detected using either vimentin or parkin antibody to stain aggresomes. Parkin expressing stable cell lines formed significantly fewer aggresomes than vector alone at *P < 0.05* (**) by Student’s paired t-test. Similar results were obtained with different clones. Data are expressed as means ± SEM for three independent experiments for each antibody used. (E) Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown in tissue culture plates and treated with 10 μM MG-132 for 24 h. All cells (floating and adherent) were harvested and cell viability was determined by trypan blue dye exclusion. Cells stably expressing parkin were significantly protected against MG-132 toxicity compared to vector at *P < 0.001* (***) by Student’s paired t-test. Data are expressed as means ± SEM for three independent experiments. Similar results were obtained with different clones. Scale bar 60 μm in (A) and 30 μm in (B) and (C).
found that several other parkin mutants (point mutations and truncations) had the potential to aggregate under basal conditions (19). In contrast to our study, they did not find any difference in aggresome frequency between wild type and mutant parkin in MG-132 treated cells. This may be due to differences in methodology. The latter study used tagged proteins and analyzed inclusion formation in non-neuronal COS-7 cell cultures (19). Furthermore, another recent study found that a parkin missense mutation that retains ligase activity, R275W, formed aggresomes under basal conditions but not wild type parkin (45). Of particular interest is that this mutation occurred in trans with an exonic deletion in an ARJP case associated with LBs at post-mortem (13).

The last couple of years has seen an explosion in reports of substrates for parkin including a glycosylated form of α-synuclein (α-Sp22), cyclin E, CDCrel-1, synphilin-1, Pael-R, alpha/beta tubulin, p38 subunit of the aminoacyl-tRNA synthetase complex and synaptogamin (7,29,46–51) that suggests that parkin may have a general role in the ubiquitylation and degradation of misfolded proteins.

Figure 7. Over-expression of parkin reduces staurosporine- and dopamine-induced aggresome formation but is not sufficient to protect cells from toxicity. Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown on glass coverslips and treated with 5 μM staurosporine for 4 h (A) or 0.125 mM dopamine for 24 h (B). Adherent cells were fixed with 4% paraformaldehyde and processed for immunofluorescence with an anti-vimentin antibody and the appropriate secondary. Digital images were captured using a Zeiss Axiocam camera. Arrows point to aggresomes. (A) Representative fields from the same experiment demonstrating that parkin-expressing stable cell lines (right) form fewer staurosporine-induced aggresomes than vector (left). Note that staurosporine resulted in the cells appearing smaller. (B) Representative fields from the same experiment demonstrating that parkin-expressing stable cell lines (right) form fewer dopamine-induced aggresomes than vector (left). (C) The percentage of aggresomes induced by staurosporine or dopamine in either vector or parkin-expressing stable cell lines was determined by counting 300 cells across 20 random fields for each clone. Parkin expressing cell lines formed significantly fewer staurosporine-induced aggresomes at \( P < 0.01 \) (**) and dopamine-induced aggresomes at \( P < 0.05 \) (*) by Student’s paired t-test. (D, E, F) Sub-confluent SH-SY5Y cell lines stably expressing vector or human parkin were grown in tissue culture plates and treated with 5 μM staurosporine for 4 h (D), 0.125 mM dopamine for 24 h (E) or 50 μg/ml tunicamycin for 24 h (F). All cells (floating and adherent) were harvested and cell viability was determined by trypan blue dye exclusion. There was no significant difference in cell death by staurosporine or dopamine between parkin-expressing cells and vector. However, we did observe that parkin over-expression significantly protected cells against tunicamycin at \( P < 0.05 \) (*). Data are expressed as means ± SEM for three independent experiments. Similar results were obtained with a different set of clones. Scale bar 30 μm.
It has recently been shown in vivo that parkin over-expression in transgenic α-synuclein-expressing Drosophila was able to reduce the number of α-synuclein-positive grain-like structures and ubiquitin positive LB-like neurites (52). Over-expression of parkin in our cell lines may result in increased ubiquitylation and degradation of misfolded proteins thus abrogating the formation of aggresomes. Furthermore, the formation of aggresomes may be dependent on the accumulation of only a few ‘key’ misfolded proteins such as tubulin or α-Sp22 and their ubiquitylation and degradation by excess parkin may be sufficient to prevent aggresome formation. In this regard a recent study showed that the ubiquitin ligase, Chip-1, but not a mutant lacking ubiquitin ligase activity reduced aggresomes formed by the αB-crystallin mutant, αBR120G (53).

An alternative mechanism may be related to parkin’s effect on proteosome activity. Protein misfolding and aggregation itself can inhibit the proteosome (54,55). It has recently been reported that over-expression of parkin preserved proteosome function in response to an expanded polyglutamine polypeptide and also reduced its aggregation (56). Furthermore, it has been demonstrated that over-expression of wild-type but not a disease causing-mutant of parkin is able to rescue cells from α-synuclein-induced proteosome inhibition and toxicity (30). Parkin has also been shown to bind directly to the Rpn10 subunit of 26S proteosomes (57). Therefore excess parkin may reduce or confer resistance to proteosome impairment caused directly by a proteosome inhibitor (MG-132) or indirectly by misfolded proteins generated by stresses such as staurosporine or dopamine. Whilst our observations on parkin over-expression on aggresome formation do shed significant light on the cellular role of parkin, it would also be interesting to examine the effects of suppression of parkin expression on aggresome formation but this is beyond the scope of the current paper.

It remains unclear what role LBs play in the pathogenesis of PD. They may be toxic, protective or an epiphenomenon (58). The nature of aggresomes is also uncertain with reports that they are toxic (55) or protective (59). In our system the correlation between parkin’s role in aggresome formation and reduction and parkin’s role in neuroprotection appear to be stress-specific. With MG-132, the majority of cells contained aggresomes and their reduction in number with over-expression of parkin correlated with enhanced survival. This was not the case for staurosporine or dopamine-induced aggresomes. For these stresses it may be that the aggresomes remain independent from the main cascades mediating death such as apoptotic pathways that are induced by dopamine as well as staurosporine (60,61). Furthermore, our observations with tunicamycin further demonstrate that neurodegeneration and parkin’s effect on neuronal survival can occur indepen-
dently of aggresome formation. Although the presence of visible aggregates appears to be dissociated from the neurotoxic effect of the stressors used, this does not rule out the significance of aggregation in the disease pathogenesis of PD. Moreover, the key pathological changes that occur in aggregation diseases are often associated with a temporal delay that is difficult to address in short-term cell culture systems.

In summary, the studies reported here demonstrate that parkin is important for aggresome formation. However, over-expression of parkin leads to fewer aggresomes, an effect that is dependent on intact central and ubiquitin ligase domains. Finally and perhaps most significantly, these cell studies demonstrate that one can use different stresses to separate parkin’s role in aggresome formation from its effect on neuronal survival.
MATERIALS AND METHODS

Expression plasmids
For stable parkin expression, wild type human parkin cloned into the expression vector pcDNA3 (Invitrogen) was a kind gift from Dr Rohan de Silva (University College, London). For transient expression studies, human parkin was cloned into pcDNA3.1/Zeo-(-) (Invitrogen) and site-directed mutagenesis was employed to produce the K161N and G328E parkin mutants as detailed previously (61).

Cell culture
Human dopaminergic neuroblastoma SH-SY5Y cells (62) were obtained from the European Collection for Animal Cell Culture (ECACC) at passage 13. Cells were maintained in 85 cm² flasks in Ham’s F12 and minimum essential medium (MEM) with Earles salts (1:1) (Gibco BRL, Paisley, UK) supplemented with 15% fetal calf serum (PAA Laboratories, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 x non-essential amino acids (all from Gibco BRL) and incubated in a humidified 5% CO₂ atmosphere at 37 °C. The medium was changed every 3 days and cells split 1:5 every 5 days.

For generating stable parkin expressing cell lines, 6-well plates of sub-confluent SH-SY5Y cells of passage #16 were transfected with 4 μg of cDNA and 3 μl of Lipofectamine 2000 (Invitrogen Life Technologies) in OPTIMEM (Gibco BRL). After 2 days, transfected cells were selected for in media containing 0.4 mg/ml G418 (Invitrogen). Sixteen clonal colonies were picked for each cDNA construct and screened. Cells were harvested for western blot analysis when they reached 70% confluency.

For transient parkin expression studies SH-SY5Y cells (passage 19–25) were seeded onto coverslips at 2 x 10⁴ cells per well in 24-well plates and left overnight to reach 30–40% confluency. Cells were co-transfected with a GFP reporter plasmid and wild-type or mutant parkin cDNA in a ratio of 2:3, respectively, to a total amount of 1 μg cDNA per well and 1 μl Lipofectamine 2000 per well in OPTIMEM. Immunostaining with a parkin antibody on wild-type parkin-transfected cells showed that all GFP positive cells over-expressed parkin and non-GFP cells had normal levels of parkin (data not shown) validating this system for examining the effects of transient expression. After transfection cells were left overnight before being exposed to a particular stress.

Immunostaining and microscopy
At the times indicated, wild-type SH-SY5Y cells or stable SH-SY5Y cells were fixed and prepared for immunofluorescence and standard fluorescence or confocal microscopy. Briefly, cells were washed three times in PBS and fixed in 4% paraformaldehyde for 10 min. Cells were washed three times with PBS and permeabilized for 5 min in 0.1% Triton X-100 in TBS (TBST). Coverslips were then incubated in block buffer (20% goat serum, 0.1% TBST) for 20 min. Cells were incubated overnight at 4°C with one of the following primary antibodies in antibody diluting buffer (1% goat serum, 0.1% TBST): rabbit polyclonal anti-parkin (C-terminal) antibody (1:100; Cell Signalling Technologies); mouse monoclonal anti-vimentin (clone V9) antibody (1:100; Dako); mouse monoclonal anti-ubiquitin (clone FK2) antibody (1:100; Affiniti Research Products Ltd, UK); mouse monoclonal anti-Hsc-70 (B-6) antibody (1:100; Santa Cruz Biotechnology Inc.) and mouse monoclonal anti-γ-tubulin antibody (1:100; Sigma). Coverslips were washed three times in 0.1% TBST and incubated for 30 min at room temperature with one of the following secondary antibodies in antibody diluting buffer: Alexa Fluor 568 goat anti-rabbit IgG conjugates (1:2500; Molecular Probes); Alexa Fluor 488 goat anti-mouse IgG conjugates (1:1000; Molecular Probes) or for 60 min with TRITC donkey anti-mouse IgG (H + L) (1:300; Jackson ImmunoResearch Laboratories Inc). For nuclear staining, Hoechst 33258 (2 μg/ml) was added to the secondary antibodies in buffer. For double labelling studies coverslips were incubated with primary antibodies sequentially at 4°C for 12 h each and after washes were incubated with a mixture of both secondaries. Coverslips were washed three times with TBS and mounted on glass slides (Fisher) with anti-fade mounting medium (Dako). Standard immunofluorescence was performed using a Zeiss Axioskop 2 plus microscope and digital images were captured using a Zeiss AxioCam camera. Confocal images were obtained using a Leica TCS SP II Confocal Scanner and images were processed in Adobe Photoshop (v.VII). Negative controls omitting each primary antibody separately or in combination were performed in each case and no significant staining was seen (data not shown).

Western blot assays
For stable cell lines, cells were grown in 6-well plates until 70–80% confluent and then harvested. For transient parkin and mutant expression studies, sub-confluent wells were transfected as described above. Cells were harvested 48 h after transfection. Cells were then washed twice in Hank’s Balanced Salt Solution (HBSS; Gibco BRL) and then re-suspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing protease inhibitors (complete, mini protease inhibitor cocktail, Roche, Germany) at a concentration of 1 x 10⁶ cells/100 μl buffer. Cells were then incubated on ice for 10–15 min and centrifuged at 13 000 g for 10 min and stored at −80°C. Protein concentrations in all lysates were determined using BCA assay (Pierce, USA). Lysates were diluted in 2 x SDS sample buffer and run on a 10% SDS–PAGE gel. Gels were transferred to nitrocellulose membranes and blocked with 5% dried milk/0.1%Tween 20 (Sigma) in PBS (PBST) for 1 h. Membranes were probed with an anti-parkin antibody overnight at 4°C (1:1000; Cell Signalling Technologies). Membranes were then washed three times in 0.1%PBST and probed with HRP-conjugated anti-rabbit IgG (1:2000; Dako) for 1 h at room temperature. Membranes were developed using ECL reagent (Bio-Rad, UK). Equal loading of samples was confirmed by stripping membranes and reprobing with a mouse monoclonal anti-GAPDH antibody (1:1000; Chemicon) and HRP-conjugated anti-mouse IgGs (1:2000; Dako). Membranes were developed as above.
Cell viability and aggresome studies

For viability studies SH-SYSY cells were plated in 6-well plates at a density of $1 \times 10^5$ cells/well and left overnight to reach 40% confluency. Kill curves were established on cells for all stresses used except MG-132 (data not shown) and drug concentrations causing ~50% death were chosen for viability studies. The concentration and duration of MG-132 treatment was similar to that used in a previous study (63). Stable SH-SYSY cell lines expressing either the empty vector pCDNA3 or over-expressing parkin (parkin-pCDNA3) were plated at $1 \times 10^5$ cells/well and treated with the following compounds: 10 µM MG-132 (Z-Leu-Leu-Leu-H; Sigma) for 24 h; 5 µM staurosporine (Sigma) for 4 h; 0.125 mM dopamine (3-hydroxytyramine hydrochloride; Sigma) for 24 h and 50 µg/ml tunicamycin (Sigma) for 24 h. Control cells were treated with the appropriate vehicle namely DMSO or water. Following treatment, wells were washed twice in HBSS and all cells (floating and adherent) harvested. Cells were maintained on ice and death was assayed using trypan blue exclusion. For all stresses, experiments were performed in duplicate and the results reported are an average of three independent experiments. Two independent sets of clones of the empty vector and parkin over-expressing cell lines were examined with similar results. Results for one set of clones is displayed.

For aggresome studies, SH-SYSY cells were plated on glass coverslips in 24-well plates. For endogenous parkin studies and parkin stable cell lines, aggresome frequency was quantified in 100–250 GFP-positive cells per coverslip in three independent experiments for each stress. Two independent sets of clones of the empty vector and parkin over-expressing cell lines were examined with similar results. Results for one set of clones is displayed.

For transient parkin expression studies, aggresomes were quantified in 100–250 GFP-positive cells per coverslip in three independent experiments for each stress. For experiments with colchicine (Sigma), cells were co-incubated with each stress in independent experiments for each stress. For experiments with staurosporine and 24 h with MG-132 or dopamine).

Statistical analysis

Statistical significance was determined using Student’s paired t-test for all comparisons.

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