RING finger 1 mutations in Parkin produce altered localization of the protein

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The Parkin gene (PRKN) encodes an E3 protein–ubiquitin ligase for which loss of function is associated with autosomal-recessive juvenile (<20 years) and early-onset Parkinsonism (<45 years). Although detailed pathological reports are scarce, brains from patients with homozygous exonic deletions demonstrate neuronal loss in the substantia nigra, albeit without the Lewy body pathology characteristic of idiopathic Parkinson’s disease. However, there are rare descriptions of more florid pathology, including Lewy bodies and tau positive astrocytes in individuals with compound heterozygous mutations. In the present study we examined whether PRKN point mutations, leading to amino acid substitutions, may alter the cellular distribution of the protein produced. Wild-type Parkin was homogeneously distributed throughout the cytoplasm with a small amount of protein in the nucleus after transfection into human embryonic kidney cells. Mutant isoforms with A82E, G328E and C431F amino acid substitutions were also normally distributed. However, two mutant isoforms, R256C and R275W, within RING finger 1 of the Parkin protein (238–293 amino acids), produced an unusual distribution of the protein, with large cytoplasmic and nuclear inclusions. We have replicated this observation in primary cultured neurons and demonstrate, by the accumulation/co-localization of cytoskeletal protein vimentin, that the inclusion bodies are aggresomes, a cellular response to misfolded protein.

INTRODUCTION

The discovery of multiple loci associated with familial forms of Parkinsonism gives an important insight into the pathways involved in pathogenesis of Parkinson’s disease (PD), and possibly related movement disorders (1). PRKN gene mutations are the predominant cause of juvenile (ARJP) and early-onset recessive Parkinsonism (2). In one study, PRKN gene mutations were found in approximately half of all families with recessive, early onset Parkinsonism, <45 years, and in 77% of apparently sporadic patients with onset before the age of 20 (3).

The range of phenotypes and inheritance patterns associated with PRKN mutations is broad. The majority of patients present with an early onset Parkinsonian syndrome, which has a relatively benign course. However, cases have been reported with many different symptoms. For example, limb onset dystonia is often seen as an early symptom (4,5) whilst other families show predominantly tremor (6). The phenotype may vary within families (7) and may vary dramatically for patients with the same mutation(s) (8). Presently, there are few examples of cases with confirmed PRKN mutations where detailed pathological information is available. Although it is often stated that Parkin cases do not contain Lewy bodies (9–11), these patients had homozygous deletions leading to complete loss of Parkin function. Patients with homozygous deletion, compound heterozygous deletion and/or point mutations have been documented with tauopathy (10,12,13) and Lewy body inclusions (14). The latter inherited an exon 3 40 bp deletion (Ex3Δ40) in trans with a 924C->T (R275W) point mutation and developed Parkinsonism at age 41 years. He came to autopsy at 52 years as a result of an automobile accident. Post-mortem exam revealed Lewy body pathology, which was consistent with idiopathic PD, rather than incidental Lewy body disease. The brain had abundant ubiquitin and α-
synuclein positive inclusions throughout the substantia nigra, locus ceruleus and basal nucleus of Meynert (14).

The Parkin protein is 465 amino acids long, having a ubiquitin-like sequence at the N-terminus and two RING fingers separated by an in-between RING (IBR) motif towards the C-terminal end of the protein. The domain motif of two RING fingers, named for Really Interesting New Gene (15), separated by an IBR domain is common to several E3 ligases, enzymes that catalyze the conjugation of activated ubiquitin to target proteins prior to their destruction via the proteasome. The E3 ligase activity of Parkin has been demonstrated (16) and several candidate substrates have now been identified in different laboratories (reviewed in 17).

The identification of Parkin as an important enzyme in the ubiquitination cycle strengthens the suggestion that proteasome dysfunction is central to neuronal loss in PD. A simple assumption about the effects of PD-linked mutations in Parkin is that they impair or ablate enzyme function. As Parkin normally protects cells against damage induced by mutant α-synuclein (18) and other agents (19), lack of Parkin activity induced by point mutations is expected to cause neuronal cell loss. However, some mutants including R275W retain ubiquitination activity (20) and hence the way in which these mutations cause disease is unclear.

There have been several studies examining the distribution of the Parkin protein. Parkin is predominantly cytoplasmic (21), but in one study a specific association with the Golgi was reported (22). Parkin has been localized to both actin-containing (23) and tubulin-containing microfilaments (24). Nuclear staining has also been reported (25), although this is dependent on the antibodies employed and antigen retrieval techniques (26). Parkin can also be associated with synaptic vesicles (27). In the present study we have examined the localization of mutant Parkin compared with wild-type protein and describe the mislocalization of two RING finger 1 mutants, R256C and R275W within aggresomes (28).

RESULTS

Transfection of human cell lines with a myc-tagged Parkin construct produced a protein of approximately 50 kDa (Fig. 1), both N- and C-terminal antibodies showing similar results. In these SDS-soluble fractions of whole cell extracts, Parkin was present as a monomeric species with little evidence of aggregated higher-molecular-weight oligomeric species as reported in the insoluble fraction of cells treated with proteasome inhibitors (29). We used a series of point mutations (A82E, R256C, R275W, G328E and C431F) distributed throughout the domain structure of Parkin (see below), all of which were expressed at similar levels in cells. We also used one deletion mutation, Ex3A40, which results in a C-terminally truncated protein (14). The truncated protein could be readily detected using antibodies to the myc tag and N-terminal anti-parkin (Fig. 1).

We next determined the distribution of wild-type and mutant forms of Parkin. In preliminary experiments we found that distribution of Parkin was similar whether we used untagged constructs and anti-Parkin antibodies or constructs fused to either HA or myc tags (data not shown). Figure 2 shows results of experiments using myc-tagged constructs, allowing us to determine the distribution of the deletion construct as well as full-length proteins. Similar to previous studies, we found that the majority of wild-type Parkin was homogeneously localized to the cytoplasm. Unlike previous reports, we saw no specific staining in the Golgi (21,22). We noted that a portion of Parkin was present in the nucleus. This was fixation-dependent and was not seen with some types of permeabilization. A similar distribution was noted using polyclonal antibodies to the C-terminus of Parkin or using N-terminally tagged constructs (data not shown).

Several mutant Parkin molecules were distributed in the cytoplasm and nucleus in a manner indistinguishable from that of the wild-type protein. These included the point mutations A82E, G328E and C431F as well as Ex3A40 (Fig. 2). However, two point mutations, both located in RING finger 1, showed a different localization, with Parkin inclusions in the cytoplasm and nucleus of the majority of transfected cells. Nuclear inclusions were particularly prominent with the R256C mutation. Parkin-positive inclusions did not strictly overlap with DNA, as evidenced by YOYO-1 staining, suggesting that these do not interact with nuclear material directly.

In many cells (Fig. 2I and J) a single, large perinuclear accumulation of mutant Parkin was seen, reminiscent of centriole associated structures termed aggresomes (28,30). To address this possibility, we co-stained cells for Parkin and vimentin, the latter previously reported to be redistributed to the outside of aggresomes (28,31). Figure 3 shows that this phenomenon is also seen with R275W Parkin inclusions in cells, but not with wild-type or C431F Parkin, which do not form aggresome-like structures. Furthermore, perinuclear inclusions contained ubiquitin (Fig. 3J–L). Immunoreactivity with monoclonal anti-ubiquitin varied in intensity between cells, and was more prominent after exposure to proteasome inhibitors (data not shown).

Other mutant proteins such as expanded huntingtin form aggresomes in cells that are also positive for α-synuclein (31), which is a major component of Lewy bodies (32). We compared the distribution of wild-type and R275W Parkin in primary hippocampal neurons, which express detectable levels of endogenous α-synuclein. Hippocampal neurons were transduced with viral particles engineered to express wild-type or mutant Parkin, and neurons were identified by staining with neuronal markers (18). Myc tagged constructs were used to avoid the contribution of endogenous mouse Parkin to the observed signal. As in transfected cell lines, Parkin was distributed throughout the cytoplasm of neurons and was also present in the nucleus (Fig. 4). Neuritic processes were readily labeled in these experiments. R275W Parkin, however, formed cytoplasmic and nuclear inclusions, similar to results in human cell lines. Mutant Parkin did not fill the neurites of the cells and instead remained localized to a few juxtanuclear areas. These inclusions overlapped with the distribution of endogenous α-synuclein (Fig. 4), although there was no specific recruitment of α-synuclein into aggresomes.

We considered whether mislocalization of RING finger 1 mutants might affect localization of the wild type protein. Figure 5 shows cells co-transfected with untagged R275W Parkin and myc-tagged wild-type Parkin. Wild-type Parkin remained normally distributed, outside of the inclusions. There...
was no additional effect of addition of the proteasome inhibitor MG132 at 5 μM for 6 h (Fig. 5B and D).

**DISCUSSION**

The association of *PRKN* mutations with recessive forms of familial Parkinsonism facilitates analysis of the mechanism(s) leading to neurodegeneration and nigral neuronal loss. The discovery that Parkin was an E3 ubiquitin–protein ligase has renewed interest in the ubiquitin–proteasome system as a central pathway perturbed in idiopathic PD (33). It is widely accepted that Parkin cases lack Lewy bodies, and that Parkin expression may be a prerequisite for Lewy body formation (34). However, there are few detailed pathology reports of cases with defined Parkin mutations. Reports of tau pathology in the majority of cases (10,12,13) and Lewy bodies in another (14) suggests there may be a closer relationship between the mechanism of nigral neuronal death in Parkin cases and other causes of PD.

In the current study we have described the distribution of Parkin protein and shown that some point mutations form intracellular inclusions. Notably, two RING finger 1 substitutions, R256C and R275W, produced striking cytoplasmic and nuclear inclusions, which may contribute to the pathogenic mechanism for these specific mutations. These are somewhat reminiscent of the types of inclusions seen with expanded polyglutamine proteins such as ataxin-1 (35), huntingtin (31,36) or androgen receptors (37), all of which produce cytoplasmic and nuclear inclusions when the mutant forms are transfected into cell lines. In these cases, it is thought that misfolding of the expanded polyglutamine protein largely drives its accumulation into aggregated, insoluble intracellular inclusions. The inclusions of mutant huntingtin and androgen receptors have been characterized as aggresomes and our results indicate that RING finger 1 Parkin mutations also form aggresomes. It is likely that the formation of intracellular and intranuclear inclusions by these RING finger 1 Parkin mutations is a function of misfolding of the parent molecule. Whether these observations are limited to mutations in RING finger 1 is not yet clear, but of the mutations we have screened to date these are the only two that form aggresomes. One caveat is that wild-type Parkin can accumulate into aggresomes under conditions where proteasome activity is diminished (29,38). It is also known that Parkin can be degraded by the proteasome
(39,40), presumably a result of its autocatalytic ubiquitylation activity (41). Therefore, R275W and R256C promote a function of the wild-type protein that can also be seen at high expression levels of the wild-type protein and is likely to be an intrinsic property of Parkin. We did not see aggresome-like inclusions with wild-type Parkin, perhaps reflecting a relatively more modest level of overexpression and not exposing cells to proteasome inhibitors. However, exposure to MG132 did not noticeably increase the number of inclusions in our hands, suggesting that expression levels may be more important. We also did not see any effect of R275W Parkin on the wild-type distribution, suggesting that these mutations have dominant but not dominant-negative effects.

One of these mutations, R275W, has been shown previously to retain activity towards at least one of its putative substrates, synphilin-1 (20). Therefore, it is likely this mutation does not produce simple loss of function. Our observation of inclusion formation suggests R256C and R275W RING finger 1 mutations produce disease by an alternative mechanism. It has been suggested that Lewy bodies may be related to aggresome formation (42). The fact that a R275W mutation (in trans with an Ex3Δ40bp) has been found associated with Lewy body pathology suggests transgenic models over-expressing RING finger 1 Parkin isoforms will provide insight into Parkin function, Lewy body formation and the relationship between ARJP and idiopathic PD.

In summary, we demonstrate that ‘not all Parkin mutations are created equal’; Parkin mutations may confer recessive loss-of-function or dominant gain-of-function, depending on the protein domain affected. We show that one way in which RING finger 1 mutations may exert a toxic effect is by altered protein localization/aggregation, within aggresomes. We postulate that
these observations help explain Lewy body pathology in some patients with Parkin mutations, including carriers, who may as a consequence be at increased risk of developing idiopathic PD.

**MATERIALS AND METHODS**

**Cloning of Parkin and mutagenesis**

The full-length cDNA of Parkin (GenBank accession number NM004562) was amplified from adult human brain using RT-PCR and cloned into pCDNA3.1 mycHis (Invitrogen). The forward primer (5'- GCTAGAATTCTACCCAGCCACCATGA-3') included an EcoRI site and a 2 bp alteration to convert the native translation initiation consensus sequence GTG ACC ATG to an optimal Kozak consensus sequence GCC ACC ATG, promoting translation. For constructs lacking a C-terminal tag the reverse primer (5'- GCTTCTAGACCCTGGCTACACGTCGAACCA-3') included an XbaI site and the native termination codon. Some constructs were produced which contained a myc/his tag at the C-terminus of the recombinant construct using an alternate reverse primer which (5'- GCTTCTAGACCCTGGCCCCACGTCGAACCA-3') included an XbaI site and a 3 bp alteration to remove the native termination codon. Mutagenesis to introduce point mutations was performed using the Transformer Site-Directed Mutagenesis Kit (Stratagene).
Kit (Clontech) according to the manufacturer’s protocols (primer sequences used for mutagenesis are available on request from the authors). For cloning into the HSV1 amplicon, the tagged constructs were digested with HindIII/Pmel and cloned into the HindIII/EcoRV sites of a modified pHSVPrpUC (the kind gift of Dr R.L. Neve, Harvard Medical School). All constructs were fully sequenced to verify integrity utilizing the BigDye Terminator Reagent mix and an AB1377 automated sequencer (PE Applied Biosystems). DNA for transfection was purified using EndoFree maxiprep kits (Qiagen) according to the manufacturer’s instructions.

**Transient transfections**

HEK293 cells were maintained in OPTIMEM supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin and streptomycin (all from Gibco BRL). Cells were transiently transfected using Fugene (Roche). For western blots, transfections were performed

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**Figure 4.** Localisation of wild type and R275W Parkin in primary neurons and overlap with α-synuclein. Primary hippocampal neurons were transduced with viral vectors expressing wild-type (A–C and G–I) or R275W (D–F and J–L) mutant Parkin. Parkin expression from the viral vectors was identified using myc staining on the red channel (B, E, G, I) and compared to MAP2 (A, D) or α-synuclein (H, J) staining on the green channel. A higher power example of a Parkin-transduced cell with large perinuclear accumulations (arrows) and nuclear inclusions (arrowheads) is shown in the insert in (E). Merged images (C, F, I, L) show overall localisation of Parkin in the cell and overlap with α-synuclein Scale bars in (F) and (L) represent 20 μm and apply to all images in each set (A–F and G–L, respectively). Representative images from duplicate experiments from different preparations of primary neurons.
in 6-well plates with 2 μg of DNA. Protein extraction, electrophoresis and blotting were performed as described previously (43) and developed using rabbit polyclonal antibodies to the C-terminus of Parkin (Cell Signaling Technology), to the N-terminus of Parkin (34,44) or monoclonal antibodies to c-myc (clone 9E10, Roche), each diluted 1:1000 in blocking buffer and applied overnight at 4°C.

For localization of Parkin, cells were fixed in 4% paraformaldehyde in PBS, and permeabilized with 0.1% (w/v) TritonX100 in PBS and blocked with PBS plus 5% goat serum, 5% FBS and 0.1% Tween 20. Primary antibodies were applied overnight at 40°C, and were either monoclonal anti-myc (1:1000) or polyclonal antibodies to c-myc (clone 9E10, Roche), each diluted 1:1000 in blocking buffer and applied overnight at 4°C.

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**Primary cultures and viral transduction**

Primary hippocampal neurons were prepared by a modification of methods developed for culturing midbrain neurons (18,45). Briefly, hippocampi were dissected from 2-day postnatal mouse pups and dissociated with papain and plated on top of pre-established cortical glia cell monolayers at a density of 80,000 cells per well. Neuronal growth medium (47% minimal essential medium, 40% Dulbecco’s modified Eagle’s medium, 10% Ham’s F12 medium, 3.4 g/l glucose, 0.25% albumin, 0.5 mM glutamine, 100 mg/ml transferrin, 15 mM putrescine, 30 nM triiodothyronine, 25 mg/ml insulin, 200 nM progesterone, 115 nM corticosterone, 5 mg/ml superoxide dismutase, 432 U/ml catalase and 500 μM kynurenate) was preconditioned by adding to glial feeder layers 24 h prior to plating neurons. After 24 h, mitotic activity was inhibited by adding 11 mM 5-fluorodeoxyuridine and 55 mM uridine. Neurons from 3–4 week cultures were used for subsequent experiments. The pHSVPrpUC construct containing Parkin cDNA (wild-type or R275W) was packaged into recombinant viral particles using 5d11.2 helper virus and 2–2 cells as a packaging cell line as described (46) and purified using sucrose gradients. Recombinant viruses were titered on human neuroblastoma cell lines using staining for Parkin to score positive transduction. Primary neurons were plated exposed to viral particles for 48 h prior to fixing and staining as above. For these experiments we used multiplicities of infection (MOIs) of less than 1.0 to generate both transduced and non-transduced cells in the same cultures. To identify neurons transduced with human Parkin, cells were fixed as above and co-stained for myc and the neuronal marker microtubule associated protein-2 (MAP2).

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