Hereditary Early-Onset Parkinson's Disease Caused by Mutations in *PINK1*

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by degeneration of dopaminergic neurons in the substantia nigra. We previously mapped a locus for a rare familial form of PD to chromosome 1p36 (PARK6). Here we show that mutations in *PINK1* (PTEN-induced kinase 1), are associated with PARK6. We have identified two homozygous mutations affecting the *PINK1* kinase domain in three consanguineous PARK6 families, a truncating nonsense mutation and a missense mutation at a highly conserved amino acid. Cell culture studies suggest that *PINK1* is mitochondrially located and may exert a protective effect on the cell which is abrogated by the mutations, resulting in increased susceptibility to cellular stress. These data provide a direct molecular link between mitochondria and the pathogenesis of PD.

Parkinson's disease (PD) is a common neurodegenerative disorder that is characterized by loss of dopaminergic neurons in the substantia nigra and the presence of cytoplasmic protein inclusions known as Lewy bodies. The majority of PD cases are sporadic; however, the identification of a number of genes responsible for rare familial forms of PD has provided important insights into the underlying mechanisms of the

disease. These genes, encoding α -synuclein, parkin, UCH-L1 and DJ-1 have implicated protein misfolding, impairment of the ubiquitin-proteasome system, and oxidative stress in the pathogenesis of the disease (1, 2).

We previously mapped PARK6, a locus linked to autosomal recessive, early-onset PD to a 12.5 cM region on chromosome 1p35-p36 by autozygosity mapping in a large consanguineous family from Sicily (3). Subsequent identification of two additional consanguineous families (one from Central Italy (family IT-GR) (4) and one from Spain) provided additional evidence of linkage to PARK6. A critical recombination event in the Spanish family refined the candidate region to a 3.7 cM interval between flanking markers D1S2647 and D1S1539. Fine mapping of single nucleotide polymorphisms and newly generated short tandem repeat markers in the three families defined a 2.8 Mb region of homozygosity within contig NT_004610, containing approximately 40 genes.

Candidate genes were prioritized on the basis of their putative function and expression in the central nervous system, as assessed by bioinformatic analysis and by exon amplification from a human substantia nigra cDNA library.

Sequence analysis of candidate genes in affected members from each family led to the identification of two homozygous mutations in the PTEN- induced putative kinase 1 (*PINK1*) gene. The mutations segregated with the disease phenotype in the three consanguineous families, were confirmed in the cDNA, and were absent from 400 control chromosomes including 200 chromosomes from Sicilian individuals. The Spanish family carried a G>A transition in exon 4 (nt 11185 in (NT_004610)) resulting in the substitution of a highly conserved amino acid in the putative kinase domain, G309D (fig. S1). Both Italian families carried the same G>A transitions in exon 7 (nt 15600 in (NT_004610)), which results in a W437OPA substitution, truncating the last 145 amino acids encoding the C-terminus of the kinase domain. These families shared a common haplotype, implying common ancestry (table S1).

The *PINK1* gene contains eight exons spanning ~1.8 Kb and encodes a 581 amino acid protein. The transcript is ubiquitously expressed (5) and is predicted to encode a 34-amino acid mitochondrial targeting motif (cleavage site for the mitochondrial processing peptidase between residues 34 and 35) and a highly conserved protein kinase domain (residues 156 to 509) that shows high degree of homology to the serine/threonine kinases of the Ca^{2+} /calmodulin family. To investigate the consequences of the missense mutation at the cellular level, we transiently transfected wild-type or G309D c-Myc-tagged *PINK1* cDNA constructs into monkey kidney COS-7 cells. The mutation did not alter production of mature full-length protein, which suggests it does not significantly affect protein stability (fig. S2). Both wild-type and mutant *PINK1* localized to mitochondria, as assessed by immunofluorescence microscopy of transfected COS-7 cells (Fig.1) and human neuroblastoma SH-SY5Y cells (fig. S3). Furthermore, by probing with a c-Myc antibody, the mitochondrial localization of *PINK1* was confirmed by Western blotting of mitochondrial enriched fractions obtained from COS-7 cells transiently transfected with c-Myc-tagged wild-type *PINK1* cDNA (Fig.2).

We next investigated the effect of *PINK1* mutations on mitochondrial function using a fluorescence-activated cell sorting (FACS)-based-assay of mitochondrial membrane potential ($\Delta\psi\text{m}$) by examining the distribution of tetramethylrhodamine methyl ester (TMRM), a fluorescent lipophilic cation. Mitochondrial membrane potential is central to mitochondrial biology: it defines the transport of ions, including Ca^{2+} uptake, and provides the driving force for oxidative phosphorylation (6). SH-SY5Y cells were transiently co-transfected with wild-type and mutant *PINK1* cDNA and a green fluorescent protein (GFP) reporter plasmid and then stressed with the peptide aldehyde, Cbz-leu-leu-leucinal (MG-132), which inhibits the proteasome and induces apoptosis via distinct mechanisms including

mitochondrial injury (7). Analysis of TMRM fluorescence in GFP-positive cells revealed that the *PINK1* mutation had no significant effect on $\Delta\psi\text{m}$ under basal conditions (Fig. 3A). However, following stress with MG-132 there was a significant decrease in $\Delta\psi\text{m}$ from basal levels in cells transfected with G309D *PINK1* compared with wild-type *PINK1* (G309D, $-44.1\% \pm 8.1$; versus wild-type *PINK1*, $13.0\% \pm 13.7$; $p < 0.01$; $n = 8$) (Fig. 3B; fig. S4).

We next determined the rate of apoptosis in MG-132-stressed SH-SY5Y cells transfected with either wild-type or G309D *PINK1* by FACS using Annexin V conjugated to the fluorochrome Phycoerythrin (Annexin V-PE). Annexin V has a high binding affinity for the membrane phospholipid, phosphatidylserine, that is exposed on the surface of apoptotic cells. Consistent with the changes in $\Delta\psi\text{m}$ following stress, we found that over-expression of wild-type *PINK1* but not mutant G309D significantly reduced the level of apoptotic cell death induced by MG-132 in GFP-positive cells (vector, $45.4\% \pm 5.0$; wild-type *PINK1*, $32.7\% \pm 4.0$; G309D, $45.8\% \pm 5.0$; $p < 0.05$; ANOVA $n = 12$) (Fig. 3C; Fig. S5). Overall these preliminary findings suggest that wild type *PINK1* may protect neurons from stress-induced mitochondrial dysfunction and stress-induced apoptosis and that this effect is abrogated by the G309D mutation. Several lines of evidence suggest that impairment of mitochondrial activity could represent an early, critical event in the pathogenesis of sporadic PD (2). Environmental toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridin (MPTP) and the pesticide rotenone induce selective death of dopaminergic neurons through inhibition of complex I activity (8, 9). Complex I deficiency and a variety of markers of oxidative stress have been demonstrated in post-mortem brains of PD patients (2, 10, 11). In addition, several reports have shown that mitochondrial dysfunction associated with oxidative stress can trigger α -synuclein aggregation and accumulation although the exact mechanisms remain unclear (12).

The *PINK1* mutations described here occur in the putative serine threonine kinase domain and thus conceivably could affect kinase activity or substrate recognition. Altered phosphorylation has been reported as a pathogenetic mechanism in other neurodegenerative diseases including Alzheimer's disease, tauopathy, and spinocerebellar ataxia (13, 14). The recent demonstration that phosphorylation of α -synuclein at serine 129 occurs in Lewy bodies in a variety of human synucleinopathy brains (15), suggests that altered phosphorylation may also play a role in PD. We hypothesize that *PINK1* may phosphorylate mitochondrial proteins in response to cellular stress, protecting against mitochondrial dysfunction. *PINK1* was originally shown to be upregulated by the tumor suppressor gene, *PTEN*, in cancer cells (5). In neurons the *PTEN* signaling pathway is involved in cell cycle

regulation and cell migration and promotes excitotoxin-induced apoptosis in the hippocampus (16). However, PINK1 has not been shown to exhibit any effects on PTEN-dependent cell phenotypes (5) and its role in the PTEN pathway therefore requires further investigation.

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18. We thank the patients and families who participated in this study; J. Sinclair for technical assistance with FACS experiments, G. Howell for bioinformatic support, M. Duchen for useful discussion and Y. Nakamura and M. Unoki for *PINK1* plasmid. Supported by grants from Telethon, Italy (E.M.V.), Italian Ministry of Health (E.M.V., B.D.), M.U.R.S.T. (B.D.), Parkinson's Disease Society, UK (N.W.W., D.S.L., R.J.H., D.G.H.), Brain Research Trust (P.M.A.S., N.W.W.), and the Deutsche Forschungsgemeinschaft (G.A., S.G.). M.M.K.M. is a Medical Research Council Clinical Research Training Fellow. GenBank accession numbers. *PINK1* genomic sequence: AL391357. *PINK1* mRNAs: AB053323, AF316873, AK075225, BC009534, BC028215. *PINK1* protein: BAB55647, AAK28062, BAC11484, AAH09534, AAH28215.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1096284/DC1

Materials and Methods

SOM Text

Figs S1 to S5

Tables S1 and S2

References

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Fig. 1. PINK1 is localized to the mitochondria in mammalian cells and its localization is not affected by the G309D mutation. (A-F) COS-7 cells transfected with wild-type (A to C) or G309D (D to F), c-myc-tagged PINK1 protein. Immunofluorescence was carried out with c-Myc antibody and mitotracker: c-myc-PINK1 [green (A, D)]; mitotracker [red (B, E)]; c-Myc-PINK1 and mitotracker merged (C, F).

Fig. 2. PINK1 is localized to the mitochondrial enriched fraction of mammalian cells. COS-7 cells were transiently transfected with c-Myc tagged wild-type *PINK1*. Cytoplasmic (lane 1) and mitochondrial enriched fractions (lane 2) were obtained and probed for c-Myc PINK1 expression by Western blot analysis using a c-Myc antibody. The same membrane was stripped and re-probed with Hsp60, complex I and GAPDH antibodies to determine the relative purity of fractions analyzed.

Fig. 3. Wild-type PINK1 but not mutant G309D PINK1 protects against stress induced mitochondrial dysfunction and apoptosis. (A,B) Effect of wild-type and mutant PINK1 on mitochondrial membrane potential ($\Delta\psi_m$) determined by FACS of GFP gated events of SH-SY5Y cells treated with vehicle (basal) or MG-132. (A) Basal TMRM values normalised to vector, mean $\pm$ SEM percentage TMRM fluorescence of vector. (B) Mean $\pm$ SEM percent change in median TMRM fluorescence from basal value for each construct after treatment with 15 μ M MG-132. * $p < 0.01$ for G309D versus wild-type PINK1; ANOVA with post-hoc Bonferroni correction; $n = 8$ from 3 independent experiments performed in duplicate or triplicate. (C) Effect of wild-type and mutant PINK1 on the amount of apoptosis determined by FACS of Annexin V-PE positive GFP gated events of SH-SY5Y cells treated with vehicle (basal) or MG-132. Mean $\pm$ SEM percent apoptotic cell death for each construct after treatment with vehicle or 15 μ M MG-132. * $p < 0.05$; ANOVA, $n = 12$ from 4 independent experiments performed in triplicate.

PINK1

Mitotracker

Merge

A

B

C

wild type

D

E

F

G309D