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Association of DJ-1 and parkin mediated by pathogenic DJ-1 mutations and oxidative stress

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The identification of rare monogenic forms of Parkinson's disease (PD) has provided tremendous insight into the molecular pathogenesis of this disorder. Heritable mutations in α-synuclein, parkin, DJ-1 and PINK1 cause familial forms of PD. In the more common sporadic form of PD, oxidative stress and derangements in mitochondrial complex-I function are considered to play a prominent role in disease pathogenesis. However, the relationship of DJ-1 with other PD-linked genes and oxidative stress has not been explored. Here, we show that pathogenic mutant forms of DJ-1 specifically but differentially associate with parkin, an E3 ubiquitin ligase. Chemical cross-linking shows that pathogenic DJ-1 mutants exhibit impairments in homo-dimer formation, suggesting that parkin may bind to monomeric DJ-1. Parkin fails to specifically ubiquitinate and enhance the degradation of L166P and M26I mutant DJ-1, but instead promotes their stability in cultured cells. The interaction of parkin with L166P DJ-1 may involve a larger protein complex that contains CHIP and Hsp70, perhaps accounting for the lack of parkin-mediated ubiquitination. Oxidative stress also promotes an interaction between DJ-1 and parkin, but this does not result in the ubiquitination or degradation of DJ-1. Parkin-mediated alterations in DJ-1 protein stability may be pathogenically relevant as DJ-1 levels are dramatically increased in the detergent-insoluble fraction from sporadic PD/DLB brains, but are reduced in the insoluble fraction from parkin-linked autosomal recessive juvenile-onset PD brains. These data potentially link DJ-1 and parkin in a common molecular pathway at multiple levels that may have important implications for understanding the pathogenesis of inherited and sporadic PD.

INTRODUCTION

Parkinson's disease (PD) is a chronic neurodegenerative disorder affecting ~1% of the population at the age of 65 rising to 4% of the population at the age of 85 (1,2). PD is a movement disorder that is mainly due to the degeneration of dopaminergic neurons in the substantia nigra pars compacta, which leads to rigidity, resting tremor, bradykinesia, postural instability and, in a subset of patients, cognitive and autonomic dysfunction (1,2). Pathologically, PD is characterized by the presence of intracytoplasmic proteinaceous inclusions termed Lewy bodies, as well as Lewy neurites, that are immunoreactive for α-synuclein (3).

The cause of PD remains unknown, and although the majority of cases appear sporadic in nature, rare monogenic forms of PD have provided tremendous insight into the pathogenesis of this disease (4,5). Four genes, α-synuclein, parkin, DJ-1 and PINK1 have been unambiguously linked to familial PD (6–9). Missense mutations (A53T, A30P and E46K) in α-synuclein, as well as whole gene multiplications, have been linked to autosomal dominant PD (6,10–13). Mutations in parkin cause autosomal recessive juvenile-onset PD (AR-JP) and are the most common cause of inherited PD, accounting for up to 50% of all recessive early-onset PD cases (7,14,15). A large number of pathogenic mutations have been identified in parkin and include exonic deletions, duplications and a variety of missense mutations and truncations. Parkin may function in the ubiquitin-proteasomal system (UPS) as an E2-dependent E3 ubiquitin ligase (16–18). A number of putative substrates for parkin exist, but their pathogenic role in PD remains elusive (16,19–26). The large number of parkin substrates may relate to the ability of

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parkin to form protein complexes with components of the UPS and chaperone systems. For example, parkin associates with the E3 ubiquitin ligase, CHIP, and the molecular chaperone, Hsp70, which together control the ubiquitination and degradation of the parkin substrate Pael-R (27).

Mutations in DJ-1 are linked with autosomal recessive early-onset PD and at least initially appear to be a rare cause of familial PD, perhaps accounting for 1–2% of all early-onset cases (28–30). A number of pathogenic mutations have been identified in DJ-1 and include exonic deletions, truncations and homozygous (L166P and M26I) and heterozygous (A104T and D149A) missense mutations (8,28–31). A rare polymorphism (R98Q) has also been identified although this is not associated with PD (32). The L166P mutant protein is impaired in its ability to form homo-dimers and exhibits markedly reduced protein stability, leading to enhanced proteasomal degradation (33–37). The biological function of DJ-1 remains obscure. DJ-1 is a highly conserved protein present in a diverse number of organisms and belongs to the DJ-1/ThiJ/PfpI protein superfamily. The crystal structure of human DJ-1 has been resolved and shows that DJ-1 exists in solution as a homodimer and the L166P mutation disrupts DJ-1 dimerization (38–42). The crystal structure of DJ-1 closely resembles that of *Escherichia coli* and yeast Hsp31 (42,43). The crystal structure of DJ-1 also highlights a Cys-106/His-126 catalytic dyad indicative of protease activity. Preliminary studies *in vitro* are consistent with the suggestion that DJ-1 may possess both chaperone activity and weak proteolytic activity (37,42). DJ-1 may also function as an anti-oxidant protein and/or as a redox sensor as it exhibits an acidic shift in p*I*-value under oxidative stress, owing mainly to oxidative modification of cysteine residues (44–47). Moreover, in cultured cells, overexpression of DJ-1 protects against oxidative injury, whereas DJ-1 knockdown enhances the susceptibility to oxidative stress (45,48).

There has been tremendous interest from researchers in attempting to link the familial-associated gene products in a common pathogenic pathway of neuronal degeneration in PD. Suggestions that there might be a converging or common molecular pathway are the observations that parkin ubiquitinates the α-synuclein-interacting protein, synphilin-1, and participates in the ubiquitination of inclusions that are formed in the presence of α-synuclein and synphilin-1 (20). Furthermore, parkin may also ubiquitinate a rare *O*-glycosylated form of α-synuclein (19). In the more common sporadic form of PD, oxidative stress and derangements in mitochondrial complex-I function are thought to play a prominent role in the demise of dopaminergic neurons (49,50). Recent observations suggest that α-synuclein and derangements in complex-I function may converge in a common pathway as inhibition of complex-I leads to α-synuclein aggregation and toxicity both *in vitro* and *in vivo* (51–56). Oxidative stress can also modify the normal function of parkin, because *S*-nitrosylation of parkin impairs its E3 ubiquitin ligase activity (57). The role of DJ-1 in such a pathway and its relationship with parkin, α-synuclein and oxidative stress has not been explored. Here, we show that parkin selectively interacts with DJ-1 harboring pathogenic mutations, as well as following various forms of oxidative stress. These interactions potentially link parkin, DJ-1 and oxidative stress in a common molecular pathway.

RESULTS

Parkin specifically but differentially interacts with pathogenic DJ-1 mutants

To investigate the relationship between DJ-1 and α-synuclein or parkin, co-immunoprecipitation experiments were performed (Fig. 1). SH-SY5Y cells were co-transfected with HA-tagged wild-type (WT) α-synuclein together with C-terminal myc-tagged DJ-1 (WT or L166P) followed by immunoprecipitation (IP) with anti-myc antibody (Fig. 1A). WT α-synuclein fails to co-immunoprecipitate with WT or L166P DJ-1 (Fig. 1A). In additional experiments, WT and L166P DJ-1 also fail to interact with α-synuclein pathogenic mutants (A30P or A53T) or UCH-L1 (data not shown). Similar experiments were performed with FLAG-tagged parkin and myc-tagged WT or mutant DJ-1 (Fig. 1B). Intriguingly, parkin specifically interacts with pathogenic mutant forms of DJ-1 (Fig. 1B). In particular, the interaction of parkin with L166P DJ-1 is particularly robust, whereas that with other pathogenic mutants (M26I, A104T and D149A) are considerably weaker, producing an interaction profile of L166P $\gg$ M26I $>$ A104T = D149A. WT DJ-1 and non-pathogenic R98Q DJ-1 fail to interact with parkin (Fig. 1B); on the other hand, parkin also fails to interact with non-pathogenic K130R DJ-1 (data not shown). As L166P DJ-1 has a number of N-terminal truncation products that may result from proteolytic processing (Fig. 1B) (33,36), we next examined whether parkin interacts with full-length or truncated forms of DJ-1 by performing co-immunoprecipitation experiments. Parkin interacts robustly with full-length L166P DJ-1 but only weakly with truncated L166P DJ-1, in addition to truncated WT DJ-1 (Fig. 1C), suggesting that full-length L166P DJ-1 primarily associates with parkin. In additional experiments, L166P DJ-1 fails to co-immunoprecipitate with various modular domains of parkin, including the RING box motif and ubiquitin-like domain (data not shown), suggesting that full-length parkin is probably required for the interaction with L166P DJ-1. To determine whether the interaction of parkin with pathogenic mutant forms of DJ-1 is associated with alterations in DJ-1 protein stability or homo-dimer formation, we examined the steady-state levels of myc-tagged WT and mutant DJ-1, as well as the capacity of myc-tagged WT and mutant DJ-1 to associate, and therefore homo-dimerize with FLAG-tagged WT DJ-1 (Fig. 1D and E). As previously reported (33), L166P DJ-1 is highly unstable, exhibiting markedly reduced steady-state levels, and fails to form homo-dimers, whereas most other DJ-1 mutants exhibit comparable steady-state levels to WT protein and share the capacity to form homo-dimers (Fig. 1D and E). A mild reduction in the steady-state levels of M26I DJ-1 is also noted, as described previously (33). Taken together, these results suggest that parkin interacts specifically with pathogenic mutant forms of DJ-1, particularly the L166P mutant, but this does not initially appear to relate to their intrinsic protein stability or their capacity to form homo-dimers.

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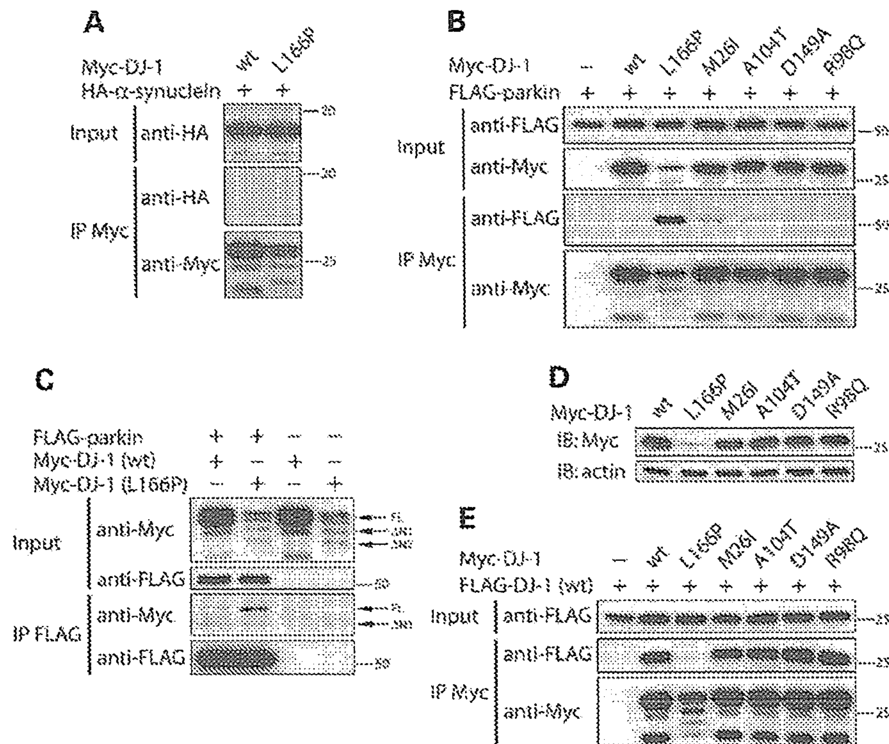

Figure 1. Parkin differentially associates with pathogenic DJ-1 mutants. (**A**) DJ-1 fails to interact with α-synuclein. Lysates from SH-SY5Y cells co-transfected with myc-tagged DJ-1 (WT or L166P) and HA-tagged WT α-synuclein were subjected to IP with anti-myc antibody. IP and input lysates (1% total soluble lysate) were analyzed by western blotting (WB) with anti-HA and anti-myc antibodies. (**B**) Interaction of parkin with pathogenic DJ-1 mutants. SH-SY5Y lysates co-expressing myc-tagged WT or mutant (L166P, M26I, A104T, D149A or R98Q) DJ-1 or control plasmid, together with FLAG-tagged parkin, were subjected to IP with anti-myc antibody, and IP and input lysates were analyzed by WB with anti-FLAG and anti-myc antibodies. (**C**) Parkin interacts primarily with full-length L166P DJ-1. SH-SY5Y lysates co-expressing myc-tagged DJ-1 (WT or L166P) together with FLAG-tagged parkin or control plasmid were subjected to IP with anti-FLAG antibody, and IP and input lysates were analyzed by WB with anti-myc and anti-FLAG antibodies. Full-length (FL) and N-terminally truncated (ΔN1 and ΔN2) myc-tagged DJ-1 species are indicated by arrows. (**D**) Analysis of steady-state protein levels of DJ-1 mutants. Lysates from SH-SY5Y cells transfected with myc-tagged WT or mutant (L166P, M26I, A104T, D149A or R98Q) DJ-1 were analyzed by WB with anti-myc antibody or with anti-actin antibody to demonstrate equal loading. (**E**) Analysis of dimerization of DJ-1 mutants. Lysates from SH-SY5Y cells co-transfected with myc-tagged WT or mutant (L166P, M26I, A104T, D149A or R98Q) DJ-1 or control plasmid, together with FLAG-tagged WT DJ-1, were subjected to IP with anti-myc antibody, and IP and input lysates were analyzed by WB with anti-FLAG and anti-myc antibodies. Molecular weight markers are indicated in kDa. All experiments were replicated three times with similar results.

Pathogenic DJ-1 mutants share a reduced capacity to form homo-dimers

Parkin interacts robustly with L166P DJ-1, however, this mutant exists largely in a monomeric form through its inability to form homo-dimers owing to protein instability (33,38). We reasoned therefore that parkin might interact preferentially with monomeric DJ-1 and that pathogenic mutations in DJ-1, other than L166P, might reduce but not completely abrogate the capacity of DJ-1 to form homo-dimers. To investigate this possibility, SH-SY5Y cells were transfected with myc-tagged DJ-1 (WT or L166P) and soluble cell lysates were treated with the covalent chemical cross-linking agent disuccinimidyl suberate (DSS). As expected, myc-tagged WT DJ-1 forms homo-dimers in a dose-dependent manner concomitant with a progressive decrease in monomeric DJ-1, whereas L166P DJ-1 fails to form homo-dimers (Fig. 2A), consistent with our co-immunoprecipitation studies (Fig. 1E). Incidentally, the level of monomeric L166P DJ-1 also decreases in a dose-dependent manner, but fails to appear at a higher molecular weight (Fig. 2A), perhaps suggesting that it becomes incorporated into an insoluble protein complex. Endogenous DJ-1 also forms robust homo-dimers, whereas hetero-dimer formation appears nominal (Fig. 2A). These data demonstrate that DJ-1 homo-dimers are amenable to chemical cross-linking and further confirm that L166P DJ-1 fails to form homo-dimers.

Next, the capacity of myc-tagged WT or mutant DJ-1 to form homo-dimers was examined using a non-saturating concentration of DSS (5 mM). With the exception of L166P DJ-1, WT DJ-1 and the other DJ-1 mutants form varying amounts of homo-dimer following cross-linking (Fig. 2B), consistent with our co-immunoprecipitation studies (Fig. 1E). To obtain a measure of dimerization capacity or efficiency, densitometry was used to generate a ratio of dimer to monomer (Fig. 2C). When compared with WT DJ-1, pathogenic mutant forms of

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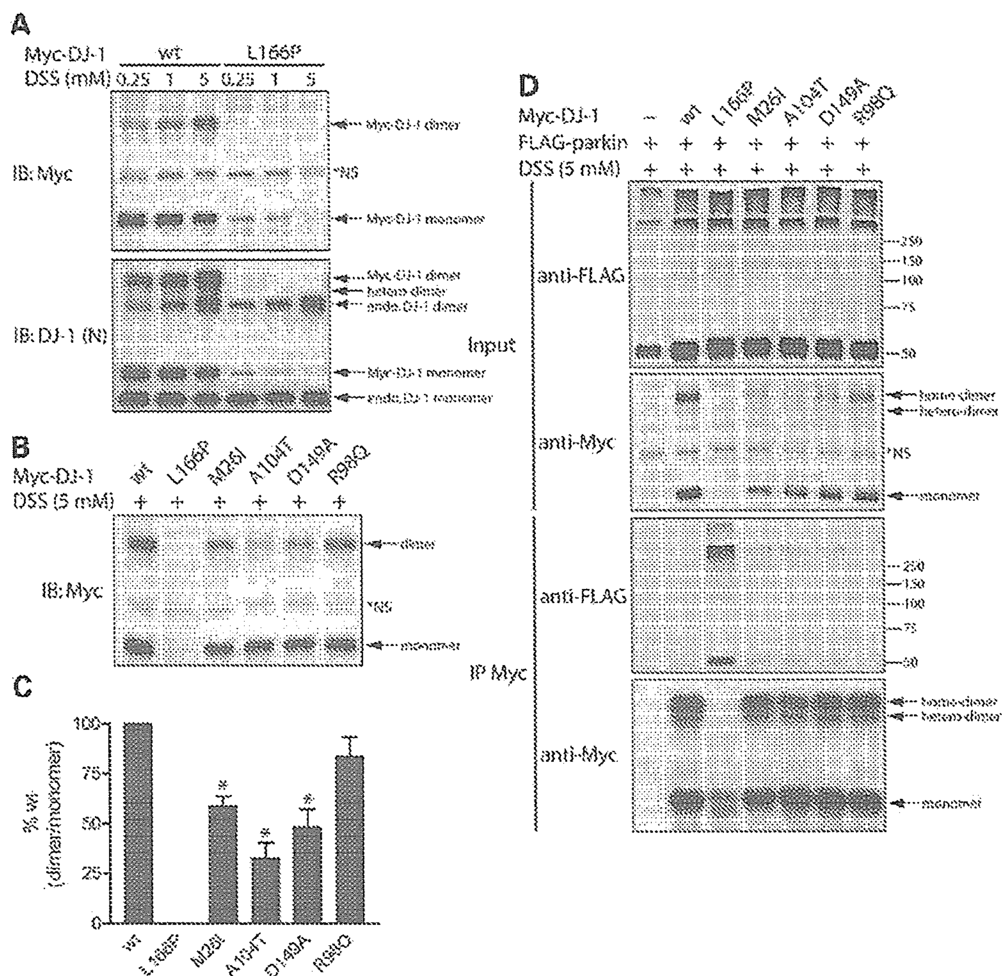

Figure 2. Reduced homo-dimerization of pathogenic DJ-1 mutants. (**A**) Cross-linking of DJ-1 homo-dimers. Equivalent soluble lysates from SH-SY5Y cells transfected with myc-tagged DJ-1 (WT or L166P) were treated with increasing concentrations of DSS and myc-tagged or endogenous DJ-1 was detected by WB with anti-myc (upper panel) or anti-DJ-1 (N) (lower panel) antibodies, respectively. The corresponding position of myc-tagged (*Myc*) or endogenous (*endo*) DJ-1 monomers and homo-dimers are indicated. (**B**) Reduced cross-linking of pathogenic DJ-1 mutants. Equivalent lysates from SH-SY5Y cells transfected with myc-tagged WT or mutant (L166P, M26I, A104T, D149A or R98Q) DJ-1 were treated with 5 mM DSS and homo-dimer formation was assessed by WB with anti-myc antibody. (**C**) Quantification of homo-dimer levels and normalization to monomer levels from (B) by densitometry reveals a significant reduction in the homo-dimerization of pathogenic DJ-1 mutants. DJ-1 dimer–monomer ratios are expressed as a percentage (%) of WT levels, and bars represent the mean ± SE of three independent experiments, $^*P < 0.005$ when compared with WT levels (Student's t-test). (**D**) Parkin fails to cross-link to pathogenic DJ-1 mutants. SH-SY5Y cells were co-transfected with myc-tagged WT or mutant (L166P, M26I, A104T, D149A or R98Q) DJ-1 or control plasmid, together with FLAG-tagged parkin. Equivalent lysates were treated with 5 mM DSS and then subjected to IP with anti-myc antibody. IP and input lysates were analyzed by WB with anti-FLAG and anti-myc antibodies. *NS denotes non-specific band detected with anti-myc antibody. Molecular weight markers are indicated in kDa. All experiments were replicated three times with similar results.

DJ-1 exhibit a significantly reduced capacity to form homo-dimers, i.e. a reduced ratio of dimer to monomer, whereas dimerization of non-pathogenic R98Q DJ-1 is comparable with WT protein (Fig. 2C). Next, to determine whether parkin interacts preferentially with monomeric or dimeric forms of mutant DJ-1, co-immunoprecipitation experiments were performed with DSS cross-linked SH-SY5Y lysates co-transfected with FLAG-tagged parkin and myc-tagged DJ-1 (WT or mutant) (Fig. 2D). Parkin fails to covalently cross-link to either monomeric or dimeric forms of mutant DJ-1 (Fig. 2D), as suggested by the absence of modified forms of DJ-1 or parkin with increased molecular weight. These data suggest either an indirect interaction between parkin and DJ-1 mutants, or that particular amino acid side chain residues (e.g. primary amines) are not available for cross-linking at the protein interaction interface. However, pathogenic DJ-1 mutants, especially the L166P mutant, retain the ability to co-immunoprecipitate with full-length parkin in addition to a substantial proportion of high molecular weight (HMW) parkin (>250 kDa; Fig. 2D), suggesting that (i) a proportion of parkin may exist as part of a large protein complex or is extensively modified, i.e. by auto-ubiquitination and (ii) DJ-1 mutants can differentially associate

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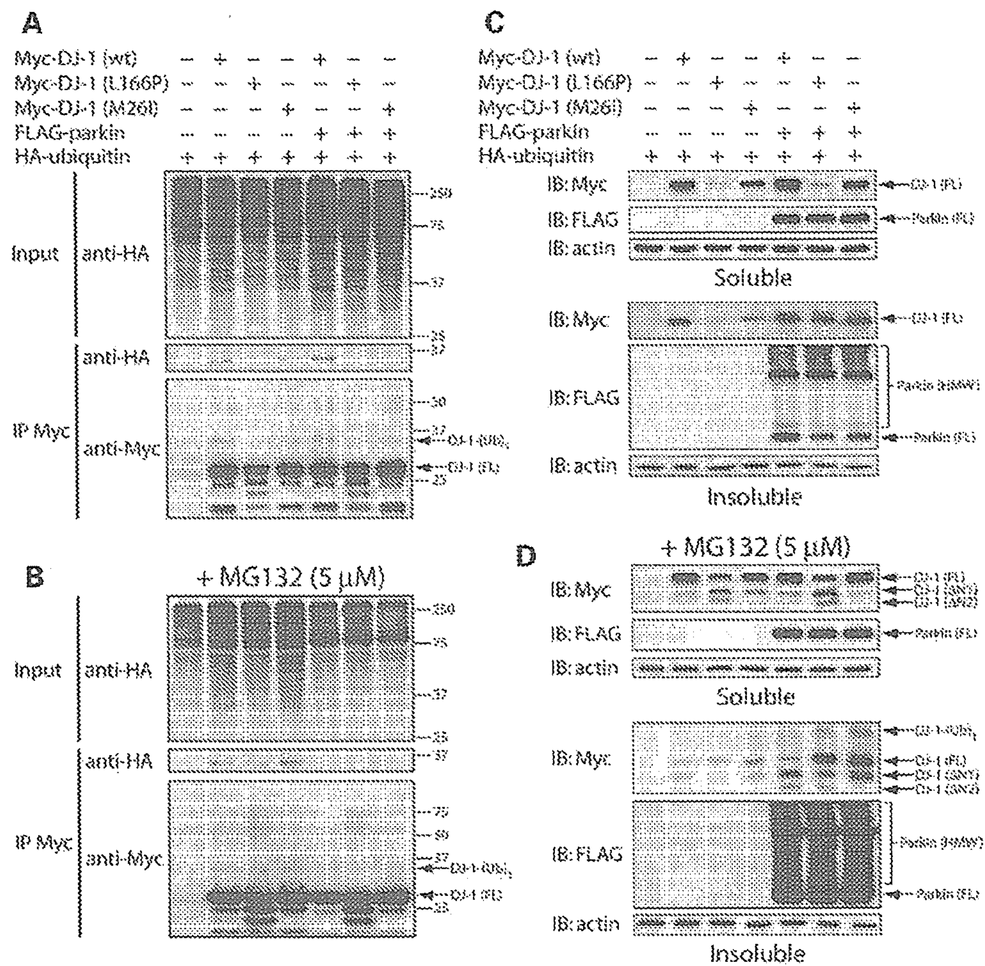

Figure 3. Parkin fails to ubiquitinate and enhance the degradation of DJ-1. (A and B) Parkin fails to ubiquitinate DJ-1. (A) SH-SY5Y cells were co-transfected with myc-tagged WT, L166P or M26I DJ-1 or control plasmid, together with HA-tagged ubiquitin, with or without FLAG-tagged parkin. Equivalent soluble lysates were subjected to IP with anti-myc antibody, and IP and input lysates were analyzed by WB with anti-HA and anti-myc antibodies. (B) Similar ubiquitination experiments were performed using transfected cells treated with the proteasome inhibitor MG132 (5 μM) for 24 h, as described earlier. (C and D) Effect of parkin on DJ-1 steady-state protein levels. (C) SH-SY5Y cells co-transfected as detailed earlier were fractionated into 1% Triton X-100-soluble or -insoluble fractions, and equivalent fractions analyzed by WB with anti-myc and anti-FLAG antibodies or with anti-actin antibody to demonstrate equal loading. (D) Similar steady-state experiments were performed using transfected cells treated with the proteasome inhibitor MG132 (5 μM) for 24 h, as described earlier. Full-length (FL), N-terminally truncated (ΔN1 and ΔN2) and mono-ubiquitinated (DJ-1-$(Ub)_1$) myc-tagged DJ-1 species or HMW FLAG-tagged parkin species are indicated by arrows. Molecular weight markers are indicated in kDa. All experiments were replicated at least three times with similar results.

with both full-length and HMW forms of parkin. Importantly, using chemical cross-linking, we are unable to determine whether parkin associates preferentially with monomeric or dimeric forms of mutant DJ-1. Collectively, these findings suggest that pathogenic mutant forms of DJ-1 share a reduced capacity to form homo-dimers that strongly correlates with their propensity to interact with parkin, perhaps suggestive of parkin binding preferentially to monomeric DJ-1.

Parkin fails to ubiquitinate DJ-1 but instead enhances DJ-1 protein stability

As parkin specifically and differentially interacts with pathogenic mutant forms of DJ-1, the ability of parkin to ubiquitinate and to enhance the degradation of these mutants was explored (Fig. 3). We chose to study further the L166P and M26I DJ-1 mutants, because these show the strongest interaction with parkin (Fig. 1B). To ascertain whether parkin ubiquitinates DJ-1, SH-SY5Y cells were co-transfected with HA-tagged ubiquitin and myc-tagged DJ-1 (WT, L166P or M26I) with and without FLAG-tagged parkin. This was followed by IP with anti-myc antibody and probing with anti-HA antibody to monitor ubiquitination of DJ-1, or with anti-myc antibody to monitor the formation of DJ-1-ubiquitin conjugates (Fig. 3A). In the absence of parkin, a small proportion of WT and M26I DJ-1 is mono-ubiquitinated, whereas mono-ubiquitination of L166P DJ-1 is not detected probably secondary to its reduced protein stability (Fig. 3A).

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In the presence of parkin, we fail to observe enhanced ubiquitination of WT and mutant DJ-1 (Fig. 3A). Moreover, with the exception of mono-ubiquitinated DJ-1, we fail to observe any HMW DJ-1-ubiquitin conjugates (Fig. 3A), suggesting the absence of poly-ubiquitinated forms of DJ-1. To determine whether the failure to observe enhanced ubiquitination of DJ-1 by parkin was potentially due to the proteasomal degradation of poly-ubiquitinated forms of DJ-1, similar experiments were performed in the presence of the proteasome inhibitor MG132 (Fig. 3B). Following proteasome inhibition, we fail to detect the formation of HMW DJ-1-ubiquitin conjugates but instead consistently detect mono-ubiquitinated DJ-1 species, including L166P DJ-1 (Fig. 3B). However, we also observe a marked accumulation of full-length and truncated forms of L166P DJ-1 to near WT levels, further suggesting that this mutant is not subject to poly-ubiquitination. Parkin overexpression does not influence the overall levels of mono-ubiquitinated DJ-1 following proteasome inhibition, but rather redistributes them from a detergent-soluble to an insoluble fraction (Fig. 3B and D). Taken together, these findings demonstrate that L166P and M26I mutant DJ-1 are not ubiquitinated by parkin despite their interaction, whereas DJ-1 can exist in a mono-ubiquitinated form independent of parkin overexpression.

The failure to observe ubiquitination of L166P and M26I mutant DJ-1 in the presence of parkin was surprising. To explore whether the absence of HMW DJ-1-ubiquitin conjugates in the detergent-soluble fraction was secondary to these conjugates residing or being sequestered into the detergent-insoluble fraction, we examined and compared the 1% Triton X-100-soluble and -insoluble fractions from the earlier mentioned ubiquitination experiments (Fig. 3C and D). Unexpectedly, parkin dramatically increases the amount of full-length L166P and M26I mutant DJ-1 in the detergent-insoluble fraction, and also has a smaller effect on WT DJ-1 (Fig. 3C). In the detergent-soluble fraction, parkin also facilitates a small increase in the steady-state levels of WT, L166P and M26I DJ-1 (Fig. 3C), suggesting that parkin may generally enhance the stability of DJ-1. Although full-length and HMW forms of parkin are present in the insoluble fraction, only full-length parkin is detected in the soluble fraction (Fig. 3C). In the detergent-insoluble fraction, the stabilizing effect of parkin on full-length L166P and M26I mutant DJ-1 steady-state levels is markedly enhanced following treatment with the proteasome inhibitor MG132, having only a small effect on WT DJ-1 (Fig. 3D). In addition, parkin also enhances the levels of N-terminally truncated forms of both WT L166P and M26I DJ-1 following proteasome inhibition (Fig. 3D), and parkin also promotes the redistribution of mono-ubiquitinated DJ-1 from the soluble into the insoluble fraction (Fig. 3B and D). The enhanced levels of DJ-1 in the insoluble fraction following proteasome inhibition also correlate with the dramatic accumulation of full-length and HMW forms of parkin in this fraction (Fig. 3D), whereas in the soluble fraction only full-length parkin is detected. These HMW forms of parkin likely represent poly-ubiquitinated species that accumulate following proteasome inhibition. In the detergent-soluble fraction, the small stabilizing effect of parkin on DJ-1 levels is still observed following proteasome inhibition (Fig. 3D). In these experiments, we fail to observe detergent-insoluble HMW DJ-1-ubiquitin conjugates in the absence or presence of parkin (Fig. 3C and D), further suggesting that parkin does not poly-ubiquitinate mutant DJ-1. Collectively, these findings demonstrate that parkin can increase the steady-state levels of L166P and M26I mutant DJ-1, primarily of detergent-insoluble species, and this effect is enhanced by proteasome inhibition. Taken together, these results indicate that although parkin interacts with L166P and M26I mutant DJ-1, parkin enhances neither their ubiquitination nor their degradation or turnover. Instead, parkin may promote the stability of L166P and M26I mutant DJ-1. This may suggest that the interaction of parkin with these mutants is either indirect or subserves an alternative as yet undetermined biological function.

Oxidative stress promotes the association of parkin and DJ-1

As parkin interacts selectively with pathogenic DJ-1 mutants but not with WT protein, the possibility that parkin may only interact with DJ-1 under pathogenic or stressful conditions was explored. To this end, the ability of WT DJ-1 and parkin to associate under conditions of oxidative stress was examined by co-immunoprecipitation experiments (Fig. 4A–C). First, the effects of hydrogen peroxide on the ability of parkin and WT DJ-1 to interact were monitored. Hydrogen peroxide oxidatively modifies cysteine residues in DJ-1, particularly Cys-106, resulting in an acidic shift in p*I*-value (44,45,58). SH-SY5Y cells were co-transfected with FLAG-tagged parkin and myc-tagged WT DJ-1, followed by treatment with hydrogen peroxide for 24 h, and IP with anti-myc antibody. Hydrogen peroxide treatment results in a dose-dependent increase in the interaction of WT DJ-1 with parkin (Fig. 4A). However, WT DJ-1 and parkin fail to interact under control conditions. To determine whether parkin interacts with WT DJ-1 under other forms of oxidative stress, the effects of the mitochondrial complex-I inhibitor, 1-methyl-4-phenylpyridinium ion (MPP^+), and the nitric oxide (NO) donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP) were examined. Treatment with both MPP^+ and SNAP leads to a dose-dependent increase in the interaction of parkin with WT DJ-1 comparable with that observed with hydrogen peroxide (Fig. 4B and C). Under all three oxidative conditions, a dose-dependent decrease in cell viability is observed (data not shown). Taken together, these results suggest that parkin and DJ-1 can be linked together under conditions of oxidative stress.

To determine the consequences of the interaction of parkin and WT DJ-1 following oxidative stress, we examined the ability of parkin to ubiquitinate DJ-1, as well as the effect of parkin on DJ-1 steady-state levels, under oxidative conditions. First, ubiquitination experiments were performed as described earlier with myc-tagged WT DJ-1 and FLAG-tagged parkin under similar conditions of oxidative stress that promote the maximal interaction of both proteins (Fig. 4D). We fail to detect HMW DJ-1-ubiquitin conjugates in the absence or presence of parkin following oxidative stress, but we continue to consistently observe mono-ubiquitinated DJ-1 irrespective of the presence of parkin (Fig. 4D). Parkin does not modify the actual level of mono-ubiquitinated DJ-1 observed following

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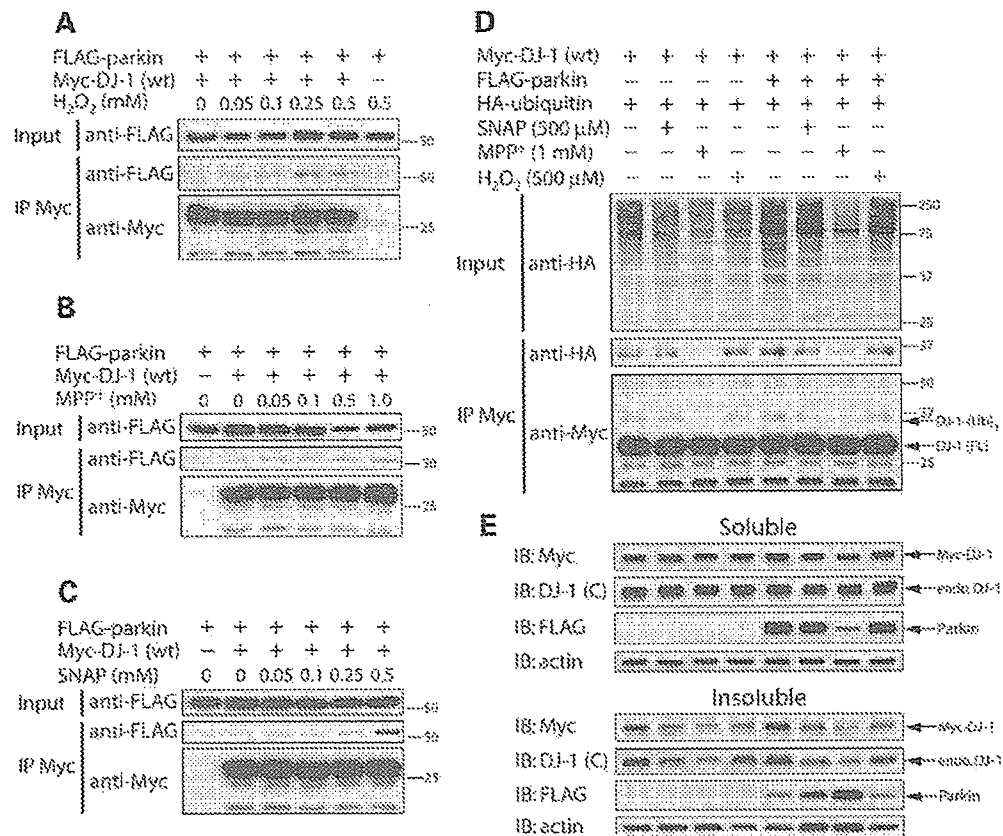

Figure 4. Oxidative stress promotes the association of parkin and DJ-1. (**A**) Hydrogen peroxide treatment promotes the interaction of parkin and DJ-1. SH-SY5Y cells were co-transfected with myc-tagged WT DJ-1 or control plasmid, together with FLAG-tagged parkin, followed by treatment with increasing concentrations of hydrogen peroxide (H_2O_2) for 24 h. Lysates were subjected to IP with anti-myc antibody, and IP and input lysates were analyzed by WB with anti-FLAG and anti-myc antibodies. Similar experiments were performed using transfected cells treated with increasing concentrations of (**B**) MPP^+ and (**C**) SNAP for 24 h, as described earlier. (**D**) Parkin fails to ubiquitinate DJ-1 following oxidative stress. SH-SY5Y cells were co-transfected with myc-tagged WT DJ-1 and HA-tagged ubiquitin, with or without FLAG-tagged parkin, followed by treatment with SNAP (500 μM), MPP^+ (1 mM) or H_2O_2 (500 μM) for 24 h. Equivalent soluble lysates (2.5 mg protein) were subjected to IP with anti-myc antibody, and IP and input lysates were analyzed by WB with anti-HA and anti-myc antibodies. Full-length (FL) and mono-ubiquitinated [DJ-1-(Ub)$_1$] myc-tagged DJ-1 species are indicated by arrows. (**E**) Effect of parkin on DJ-1 steady-state protein levels following oxidative stress. SH-SY5Y cells co-transfected and treated as in (D) were fractionated into 1% Triton X-100-soluble or -insoluble fractions, and equivalent fractions analyzed by WB with anti-myc, anti-DJ-1 (C) and anti-FLAG antibodies or with anti-actin antibody to demonstrate equal loading. Myc-tagged (*Myc*) or endogenous (*endo*) DJ-1 are indicated. Molecular weight markers are indicated in kDa. All experiments were replicated at least three times with similar results.

oxidative stress, but instead marginally enhances the steady-state levels of soluble DJ-1 (Fig. 4D and E), as demonstrated earlier (Fig. 3C), thus leading to IP of greater quantities of full-length and mono-ubiquitinated DJ-1 in this experiment. This is particularly apparent under control conditions (Fig. 4D and E). It was not possible to perform this experiment in the presence of proteasome inhibitors secondary to excessive cell death (data not shown). These results suggest that WT DJ-1 is not ubiquitinated by parkin under conditions of oxidative stress.

To determine the effects of parkin on the steady-state levels of WT DJ-1 following oxidative stress, we examined and compared the detergent-soluble and -insoluble fractions from the earlier mentioned ubiquitination experiment (Fig. 4E). Under control conditions, parkin marginally enhances the levels of WT DJ-1 in both the soluble and insoluble fractions (Fig. 4E), as observed earlier (Fig. 3C). This stabilizing effect of parkin on WT DJ-1 is abrogated under oxidative conditions (Fig. 4E). Furthermore, these oxidative conditions lead to a marked reduction in the steady-state levels of WT DJ-1 in the detergent-insoluble fraction irrespective of parkin overexpression, with no obvious changes in the soluble fraction. Oxidative stress also has a similar effect on the steady-state levels of detergent-insoluble endogenous DJ-1 in these cells (Fig. 4E). These results suggest that oxidative stress may reduce the stability of detergent-insoluble forms of DJ-1. In contrast, treatment with MPP^+, and to a lesser extent SNAP, results in a marked redistribution of full-length parkin from the detergent-soluble to the insoluble fraction (Fig. 4E). This redistribution may reflect movement of parkin between different cellular compartments or organelles, or a change in the biochemical properties of parkin. Additional

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experiments using quantitative DSS cross-linking show that oxidative stress has no effect on homo-dimer formation of detergent-soluble WT DJ-1 (data not shown). Collectively, these results indicate that although parkin interacts with WT DJ-1 under oxidative stress, this does not promote the parkin-mediated ubiquitination or degradation of DJ-1. Instead, the small stabilizing effect of parkin on WT DJ-1 may be impaired by oxidative stress. Intriguingly, oxidative stress can reduce the stability of insoluble DJ-1, as well as reducing the solubility of parkin. Our findings support the idea that the interaction of DJ-1 and parkin under oxidative stress may be an indirect association or may serve an alternative biological role.

L166P mutant DJ-1 associates with parkin, CHIP and Hsp70

Parkin exists in a macromolecular protein complex with CHIP and Hsp70, where this complex participates in the ubiquitination and degradation of parkin substrates such as Pael-R (27). To ascertain whether DJ-1 mutants could additionally interact with components of this parkin complex, co-immunoprecipitation experiments were performed (Fig. 5). We chose to study only L166P DJ-1 in these experiments because this mutant displays the most robust interaction with parkin. SH-SY5Y cells were co-transfected with myc-tagged DJ-1 (WT or L166P) together with HA-tagged CHIP, FLAG-tagged parkin or both proteins, followed by treatment with the proteasome inhibitor MG132 for 24 h to restore L166P DJ-1 levels to those of WT, and IP with anti-myc antibody. L166P DJ-1 specifically interacts with CHIP in the presence or absence of parkin, whereas WT DJ-1 fails to interact with CHIP (Fig. 5A). Parkin fails to appreciably alter the interaction of CHIP with L166P DJ-1. In a similar manner, CHIP fails to alter the interaction of parkin with L166P DJ-1, suggesting that parkin and CHIP may interact with L166P DJ-1 independently of each other. In similar co-immunoprecipitation experiments, we also monitored the ability of L166P DJ-1 to interact with Hsp70. SH-SY5Y cells were co-transfected with myc-tagged DJ-1 (WT or L166P) together with V5-tagged Hsp70 or control plasmid, followed by treatment with or without MG132 for 24 h, and IP with anti-V5 antibody. We find that only full-length L166P DJ-1 interacts with Hsp70, on the other hand, Hsp70 also interacts with N-terminally truncated forms of WT and L166P DJ-1 (Fig. 5B). In the presence of the proteasome inhibitor MG132 to restore L166P DJ-1 levels, the interaction of Hsp70 with full-length and truncated forms of L166P DJ-1 is enhanced and we additionally observe a weak interaction of full-length WT DJ-1 with Hsp70 (Fig. 5B). The interaction of full-length WT DJ-1 with Hsp70 following proteasome inhibition may relate to the putative chaperone function of DJ-1. To determine whether Hsp70 can promote the stability of L166P DJ-1, similar to the effect of parkin (Fig. 3D), we examined the steady-state levels of WT or L166P DJ-1 in the detergent-insoluble fraction in the absence or presence of Hsp70 following proteasome inhibition (Fig. 5C). Hsp70 markedly enhances the level of full-length L166P DJ-1, as well as N-terminally truncated forms of WT and L166P DJ-1, with smaller effects on full-length WT DJ-1 (Fig. 5C). This finding suggests that Hsp70 can promote the stability of detergent-insoluble DJ-1, primarily L166P DJ-1.

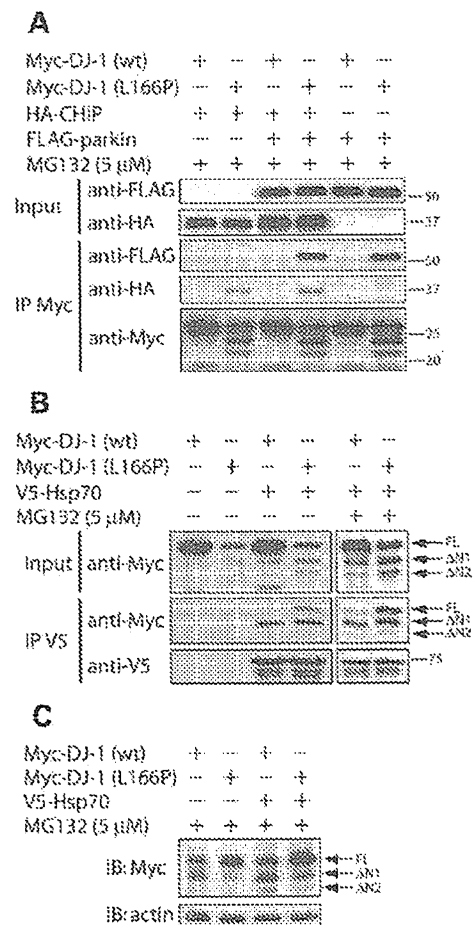

Figure 5. Association of L166P DJ-1 with parkin, CHIP and Hsp70. (**A**) Interaction of L166P DJ-1 with CHIP and parkin. SH-SY5Y cells were co-transfected with myc-tagged WT or L166P DJ-1, together with HA-tagged CHIP, FLAG-tagged parkin or both proteins, followed by treatment with the proteasome inhibitor MG132 (5 μM) for 24 h. Lysates were subjected to IP with anti-myc antibody, and IP and input lysates were analyzed by WB with anti-HA, anti-FLAG and anti-myc antibodies. (**B**) Interaction of L166P DJ-1 with Hsp70. SH-SY5Y cells were co-transfected with myc-tagged WT or L166P DJ-1, together with V5-tagged Hsp70 or control plasmid, followed by treatment with or without the proteasome inhibitor MG132 (5 μM) for 24 h. Lysates were subjected to IP with anti-V5 antibody, and IP and input lysates were analyzed by WB with anti-myc and anti-V5 antibodies. (**C**) Effect of Hsp70 on DJ-1 steady-state protein levels. The 1% Triton X-100-insoluble fraction from cells co-transfected with myc-tagged WT or L166P DJ-1 with or without V5-tagged Hsp70, followed by treatment with MG132 (5 μM) for 24 h, was analyzed by WB with anti-myc and anti-actin antibodies. Full-length (FL) and N-terminally truncated (ΔN1 and ΔN2) myc-tagged DJ-1 species are indicated by arrows. Molecular weight markers are indicated in kDa. All experiments were replicated with similar results.

This stabilizing effect reflects the interaction profile of Hsp70 with WT and L166P DJ-1 (Fig. 5B). Taken together, these results indicate that both CHIP and Hsp70 are able to interact with L166P DJ-1, and suggest that parkin may associate with L166P DJ-1, and possibly other DJ-1 mutants, as part of a larger protein complex containing CHIP and Hsp70. This observation may explain the failure of parkin to directly ubiquitinate L166P and M26I mutant DJ-1 (Fig. 3A and B).

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Alterations of DJ-1 protein levels in Parkinson's disease brains

Parkin enhances the steady-state levels of DJ-1 primarily in the detergent-insoluble fraction in cultured cells, and this may relate to increased protein stability. To explore whether this finding has potential pathophysiological relevance, we monitored the levels and detergent solubility of DJ-1 in human post-mortem cingulate cortex tissue from aged-matched normal control, Alzheimer's disease (AD) and PD/dementia with Lewy bodies (DLB) patients (Table 1; Fig. 6). Although the relative amount of DJ-1 does not change in the detergent-soluble fraction of all cases, a dramatic increase of DJ-1 levels in the detergent-insoluble fraction is observed in PD/DLB cases when compared with control or AD cases (Fig. 6A). Quantification and normalization of DJ-1 protein levels reveals a >5-fold increase of DJ-1 in the insoluble fraction of PD/DLB cases when compared with combined control and AD cases, whereas there is no apparent difference in the soluble fraction (Fig. 6A). Parkin resides only in the detergent-insoluble fraction in the aged human brain, but we fail to observe any difference in parkin levels in PD/DLB cases compared with control or AD cases (Fig. 6A). To determine whether this observation is specifically related to pathologically affected regions of PD/DLB brains, we examined DJ-1 levels in similar protein fractions derived from cerebellum tissue, a region not affected by the pathogenesis of PD/DLB. We observe no apparent differences of DJ-1 levels in the detergent-soluble or insoluble fractions of PD/DLB cases compared with control or AD cases (Fig. 6B), suggesting that increased DJ-1 levels observed in PD/DLB cingulate cortex are directly related to the pathogenesis of PD/DLB.

To determine whether the absence of parkin influences the levels of DJ-1 protein *in vivo*, we examined brain tissue from the frontal cortex of patients with parkin-linked AR-JP (due to parkin exonic deletions) and aged-matched normal controls (Fig. 6C). We find that the absence of parkin in the frontal cortex of AR-JP brains leads to a marked reduction of DJ-1 in the detergent-insoluble fraction in three out of four AR-JP cases (cases 1, 3 and 4) when compared with control cases (Fig. 6C), as revealed by densitometry. We observe no consistent change of DJ-1 levels in the detergent-soluble fraction of control and AR-JP cases, although two out of four AR-JP cases (cases 3 and 4) display noticeably reduced levels of DJ-1 when compared with all control cases (Fig. 6C). As expected, parkin is detected in the detergent-insoluble fraction of control brains, but is absent from AR-JP brains, as previously described (19,59). Consistent with the stabilizing effect of parkin on DJ-1 in cultured cells, these data might suggest that parkin is able to influence the stability of DJ-1 *in vivo*; in particular, the absence of parkin leads to a reduction of DJ-1 primarily in the detergent-insoluble fraction.

DISCUSSION

The major findings of this study are that parkin selectively but differentially interacts with pathogenic mutant forms of DJ-1. Pathogenic DJ-1 mutants exhibit impairments in homo-dimer formation. Parkin fails to ubiquitinate and enhance the degradation of L166P and M26I mutant DJ-1, but instead promotes their stability. Furthermore, L166P DJ-1 may be capable of forming a protein complex with parkin, CHIP and Hsp70. Oxidative stress promotes an interaction between parkin and WT DJ-1, although this does not lead to the ubiquitination or degradation of DJ-1. Parkin-mediated alterations in DJ-1 stability may be pathogenically relevant as DJ-1 levels are dramatically increased in the detergent-insoluble fraction from sporadic PD/DLB brains, but are reduced in the insoluble fraction from parkin-linked AR-JP brains. This study links together, at multiple levels, two gene products associated with familial forms of PD that may have important implications for understanding the molecular pathogenesis of this disease.

The selective association of parkin with pathogenic mutant forms of DJ-1 suggests that these mutants might share common properties. However, the L166P mutant clearly differs from other DJ-1 mutants in that it interacts with parkin most robustly. L166P DJ-1 is a highly unstable, unfolded protein that fails to dimerize and is rapidly degraded by the 20/26S proteasome (33–35). Unfolding of the L166P mutant might conceivably promote its association with a variety of proteins or protein complexes, including components of the UPS or chaperone systems. Furthermore, L166P DJ-1 may be incorporated into higher-order protein complexes in cultured cells (35). Parkin or a parkin-associated complex does not appear to be involved in the ubiquitination or degradation of L166P DJ-1. Instead, parkin can stabilize L166P or M26I mutant DJ-1, particularly detergent-insoluble forms, through a mechanism that does not involve ubiquitination, thus arguing in favour of a protective or stabilizing role for parkin. The mechanism of parkin's unexpected stabilizing effect is unclear, but might result from delaying or reducing the normal turnover of exogenous DJ-1. Although it is possible that parkin or Hsp70 might stabilize DJ-1 by sequestration or redistribution into distinct inclusion

Table 1. Clinical details of human subject's tissue used for western blotting

Subjects	Diagnosis	Brain region	PMD (h)	Age (year)
Control 1	—	CING, CB	10	66
Control 2	—	CING, CB	4	74
Control 3	—	FRNTL	15	61
Control 4	—	FRNTL	6	67
Control 5	—	FRNTL	10	68
Control 6	—	FRNTL	15	49
AD 1	AD	CING, CB	14	88
AD 2	AD	CING, CB	9	80
PD/DLB 1	PD/DLB/AD	CING, CB	5	82
PD/DLB 2	PD/cortical LB	CING, CB	9	75
PD/DLB 3	PD	CING, CB	16	77
PD/DLB 4	PD/DLB/AD	CING, CB	12	84
PD/DLB 5	PD/AD	CING, CB	21	64
AR-JP 1	PD	FRNTL	15	62
AR-JP 2	PD	FRNTL	4	65
AR-JP 3	PD	FRNTL	18	52
AR-JP 4	PD	FRNTL	10	68

AD, Alzheimer's disease; PD, Parkinson's disease; DLB, dementia with Lewy bodies; LB, Lewy bodies; AR-JP, autosomal recessive juvenile-onset PD; PMD, post-mortem delay; CING, cingulate cortex; CB, cerebellum; FRNTL, frontal cortex.

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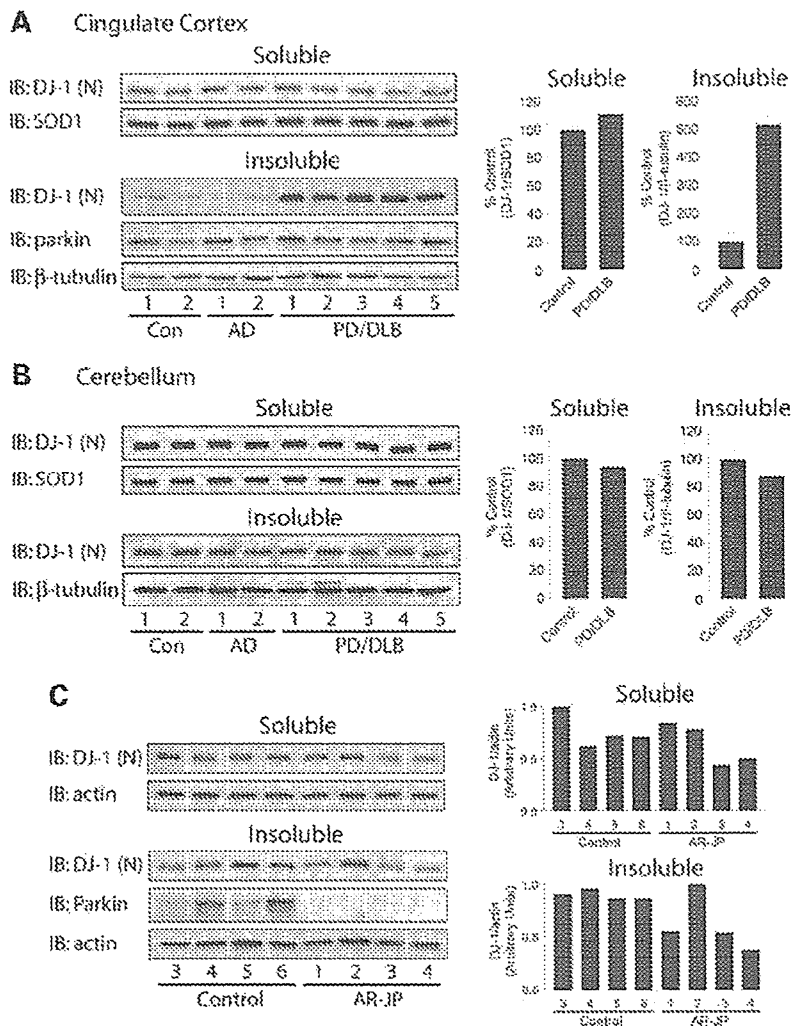

Figure 6. Alterations in DJ-1 protein levels in sporadic and parkin-linked PD brain. (**A**) Increased levels of insoluble DJ-1 in PD/dementia with Lewy bodies (DLB) brains. Equivalent detergent-soluble and -insoluble fractions extracted from cingulate cortex tissue of control (Con), AD and PD/DLB brains were analyzed by WB with anti-DJ-1 (N), anti-parkin, anti-SOD1 or anti-β-tubulin antibodies, as indicated. DJ-1 protein levels were quantified and normalized in the detergent-soluble and -insoluble fraction of control (control + AD; $n = 4$) and PD/DLB ($n = 5$) brains. Data are expressed as a percentage (%) of control levels as the ratio of DJ-1 to SOD1 (soluble fraction) or β-tubulin (insoluble fraction), and bars represent the mean ± SE. (**B**) Similar experiments were performed on detergent-soluble and -insoluble fractions extracted from cerebellum tissue of control, AD and PD/DLB brains. (**C**) Reduced levels of insoluble DJ-1 in parkin-linked AR-JP brains. Detergent-soluble and -insoluble fractions extracted from frontal cortex tissue of control and parkin-deficient AR-JP brains were analyzed by WB with anti-DJ-1 (N), anti-actin or anti-parkin antibodies, as indicated. DJ-1 protein levels were quantified and normalized in the detergent-soluble and -insoluble fraction of control and AR-JP brains. Data from individual cases are expressed in arbitrary units as the ratio of DJ-1 to actin. All experiments were replicated with similar results.

bodies or cellular organelles, this is perhaps doubtful given that this stabilizing effect is observed in both detergent-soluble and insoluble fractions. Such soluble and insoluble fractions are unlikely to simultaneously contain either inclusion bodies or organelles due to their intrinsic solubility in detergent. Thus, parkin's stabilizing effect is likely related to a more generalized effect, such that parkin binding might reduce the availability of DJ-1 for degradation. It will be of interest to determine whether parkin's stabilizing effect extends to DJ-1 harboring other pathogenic mutations, such as A104T and D149A, or the recently identified homozygous E64D mutation (60).

As L166P DJ-1 exists entirely in a non-dimeric form, a large proportion of which is monomeric (37), this might suggest that parkin selectively associates with DJ-1 monomers. Other pathogenic DJ-1 mutants demonstrate impairments in homo-dimer formation, yet still retain the capacity to form a significant population of dimers, which may lead to the availability of much smaller levels of monomeric DJ-1. This may explain the relatively weaker interaction of these DJ-1 mutants with parkin compared with the non-dimeric L166P mutant. The association of parkin with DJ-1 mutants may not be directly relevant to the pathogenesis of

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PD as not all cases of DJ-1-linked PD result from missense mutations. Indeed, exonic deletions appear to be a prominent cause of DJ-1-linked PD, some of which result in a complete loss of DJ-1 protein (61). Therefore, the interaction of parkin and DJ-1 may instead link both proteins in a common molecular pathway but only under selective conditions, including pathogenic mutations or oxidative stress.

The failure of parkin to ubiquitinate or to enhance the degradation of L166P or M26I mutant DJ-1 is suggestive of an indirect interaction, perhaps as part of a larger protein complex that contains CHIP and Hsp70 together with other proteins. The function of such a protein complex is unclear. Lack of a direct parkin-mediated effect on L166P DJ-1 highlights the distinct possibility that parkin may serve subsidiary roles other than ubiquitination that might include protein stabilization or refolding. We find no evidence for polyubiquitination of L166P DJ-1 in our study, suggesting that ubiquitination does not play a major role in the proteasomal degradation of L166P DJ-1. Previous studies have similarly failed to demonstrate the formation of HMW L166P DJ-1-ubiquitin conjugates, but have instead observed the accumulation of full-length and truncated L166P DJ-1 following proteasome inhibition (33,34,36,37). The unfolded nature of L166P DJ-1 may therefore be sufficient for degradation in an ubiquitin-independent manner by the 20/26S proteasomal complex, similar to other natively unfolded proteins including α-synuclein and tau (62,63). Intriguingly, a small proportion of DJ-1 can be mono-ubiquitinated independent of parkin overexpression, raising the possibility that DJ-1 function may be regulated, in part, by non-degradative mono-ubiquitination.

The oxidative stress-induced interaction of parkin and DJ-1 failed to result in the ubiquitination or enhanced degradation of DJ-1, perhaps suggestive of an indirect interaction and/or that this association may serve an alternative biological function. Current evidence suggests that DJ-1 may function as an anti-oxidant protein and/or as a sensor of oxidative stress (44–48). Furthermore, DJ-1 may be functional under oxidative conditions because its reported chaperone and protease activities are not apparently sensitive to hydrogen peroxide treatment (37,42). As oxidative conditions do not apparently impair DJ-1, the oxidative stress-induced association of parkin and DJ-1 is unlikely the result of damaged DJ-1 protein, in contrast to the effects of pathogenic mutations, but instead may serve an alternative role. One possibility is that parkin and DJ-1 might converge in a common molecular pathway in response to oxidative stress. At present, however, the significance of the oxidative stress-induced association of parkin and DJ-1 and its relevance to the pathogenesis of PD is not understood but clearly warrants further attention.

DJ-1 levels are dramatically increased in the insoluble fraction from sporadic PD/DLB brains. The significance of this finding is not understood but might relate to increased stability of the DJ-1 protein and/or up-regulation of DJ-1 mRNA as a consequence of disease pathogenesis. Oxidative stress is one obvious candidate for enhancing DJ-1 protein levels. Parkin is primarily detergent-insoluble in the aged human brain (64). Increased levels of oxidative stress that are commonly observed in the brains of sporadic PD patients (49,50) may promote the association of parkin and DJ-1, subsequently leading to stabilization of DJ-1 exclusively in the insoluble fraction. Although this might provide an attractive putative mechanism, it is difficult to reconcile this notion with the observation that oxidative stress may actually reduce the stability of insoluble DJ-1, at least in human SH-SY5Y cells. The generally reduced levels of DJ-1 in the insoluble fraction from parkin-deficient AR-JP brains might suggest that loss of insoluble parkin may primarily destabilize DJ-1 in this fraction, consistent with the stabilizing effect of parkin in cultured cells. However, more cases need to be examined to determine whether the reduction in DJ-1 levels is a consistent feature of parkin-linked AR-JP brains. Increased DJ-1 levels in PD/DLB brains might alternatively be related to the presence of Lewy bodies or insoluble forms of α-synuclein. However, this appears unlikely given that DJ-1 fails to interact with α-synuclein and does not localize to Lewy bodies or other inclusions in sporadic PD (35,65,66). Increased levels of detergent-insoluble DJ-1 appear to be a common feature of many neurodegenerative diseases (65,66). The tendency of DJ-1 to co-localize with hallmark neuronal and glial tau inclusions in a number of neurodegenerative tauopathies likely accounts for these increased levels of insoluble DJ-1. As DJ-1 is absent from Lewy bodies in PD and DLB brains (65,66), the increased levels of insoluble DJ-1 observed in PD/DLB brains may represent a distinct pathological species with altered biochemical properties that specifically results from disease pathogenesis.

In conclusion, we report an association between the PD-linked gene products parkin and DJ-1 mediated by pathogenic DJ-1 mutations and oxidative stress. Pathogenic mutations in DJ-1 tend to impair homo-dimer formation, and parkin might be involved in stabilizing these mutants rather than targeting them for proteasomal degradation. In contrast, the oxidative stress-induced association of parkin and DJ-1 may link both proteins in a common pathway related to cellular stress. The stabilizing effect of parkin on DJ-1 appears to be one functional outcome from the association of both proteins, which may be relevant both in cultured cells and *in vivo* in PD. The association of parkin and DJ-1 at multiple levels may potentially link both proteins in a common molecular pathway, and this may have important implications for understanding the pathogenesis of PD.

MATERIALS AND METHODS

Expression plasmids, cell culture and antibodies

Full-length human DJ-1 cDNA was cloned into the mammalian expression plasmid pcDNA3.1-Myc-His (Invitrogen), and point mutations were introduced by PCR-mediated site-directed mutagenesis using the QuickChange kit (Stratagene). Human α-synuclein and ubiquitin cDNAs were cloned into pRK5-HA vector as described previously (20). Expression plasmids for FLAG-tagged human parkin and V5-tagged human Hsp70 were kindly provided by R. Takahashi (RIKEN Brain Science Institute, Japan), HA-tagged mouse CHIP was kindly provided by S. Hatakeyama (Kyushu University, Japan) and FLAG-tagged human DJ-1 was kindly provided by H. Ariga (Hokkaido University,

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Japan). A plasmid containing β-galactosidase cDNA was used as a control in all experiments. The integrity of all constructs was confirmed by sequencing.

Human SH-SY5Y neuroblastoma cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were transfected with plasmids using Lipofectamine Plus reagent (Invitrogen) according to the manufacturers recommendations.

Affinity-purified rabbit polyclonal anti-DJ-1 antibodies, DJ-1 (N) and DJ-1 (C), raised to N- and C-terminal epitopes, respectively, have been described previously (33); mouse monoclonal anti-Myc (clone 9E10), anti-Myc-HRP and anti-HA-HRP antibodies were obtained from Roche; mouse monoclonal anti-FLAG (M2), anti-FLAG-HRP, anti-β-tubulin and rabbit polyclonal anti-actin antibodies were obtained from Sigma; mouse monoclonal anti-V5 and anti-V5-HRP antibodies were obtained from Invitrogen; mouse monoclonal anti-parkin (clone PRK8) antibody has been described elsewhere (64); rabbit polyclonal anti-SOD1 antibody was kindly provided by D. Borchelt (Johns Hopkins University); HRP-coupled anti-mouse and anti-rabbit antibodies were obtained from Pierce.

Co-immunoprecipitation and western blotting

For co-immunoprecipitation from cell cultures, SH-SY5Y cells were transfected with 2 μg of each plasmid. After 48 h, cells were washed with cold PBS and harvested in IP buffer [0.5% Triton X-100, 1× Complete mini protease inhibitor cocktail (Roche), in PBS] Lysates were then rotated at 4°C for 1 h followed by centrifugation at 17 500g for 15 min. The supernatant fractions were then combined with 50 μl protein G sepharose 4 fast flow (Amersham), pre-incubated with 5 μg mouse monoclonal anti-myc, anti-FLAG or anti-V5 antibody followed by rotating overnight at 4°C. The protein G sepharose complex was pelleted and washed once with IP buffer supplemented with 500 mM NaCl, twice with IP buffer alone and three times with PBS. Immunoprecipitates or inputs (1% total lysate) were resolved by SDS–PAGE and subjected to western blot analysis. Bands were visualized by enhanced chemiluminescence (Amersham). Quantitation of protein expression was performed using densitometry analysis software (AlphaImager, Alpha Innotech Corp.) and data were analyzed by Student's t-test to determine differences between groups.

Where indicated, SH-SY5Y cells were treated for 24 h with MG132 (5 μM; Affiniti Research), hydrogen peroxide (Sigma), MPP^+ (Sigma), SNAP (Calbiochem) or DMSO-PBS as a control where appropriate, prior to harvesting in IP buffer and submitting to IP, as described earlier.

Cross-linking assay

Soluble cell lysates were quantitated using the BCA kit (Pierce) with BSA standards. Equal quantities of lysate were incubated with DSS (Pierce) for 2 h at 4°C. The reaction was quenched by incubation with 50 mM Tris–HCl, pH 7.5 for 15 min at room temperature. Lysates were analyzed by western blotting with anti-myc or anti-DJ-1 (N) antibody. For co-immunoprecipitation experiments, cells were transfected with 2 μg of pcDNA3.1-Myc-DJ-1 and pcDNA3-FLAG-parkin plasmids, and after 48 h, cells were harvested in IP buffer (1% Triton X-100). Equivalent soluble lysates (3 mg total protein) were incubated with 5 mM DSS for 2 h, quenched with 100 mM Tris–HCl, pH 7.5, and IP was performed with an anti-myc antibody. Immunoprecipitates were stringently washed five times with IP buffer supplemented with 500 mM NaCl and once with PBS, and analyzed by western blotting.

Ubiquitination assay

SH-SY5Y cells were transfected with 2 μg of pcDNA3.1-Myc-DJ-1, pRK5-HA-ubiquitin or pcDNA3-FLAG-parkin plasmids. After 48 h, cells were harvested in IP buffer (1% Triton X-100, 1× Complete protease inhibitor cocktail, in PBS) and IP was performed with an anti-myc antibody. Immunoprecipitates were stringently washed five times in IP buffer supplemented with 500 mM NaCl and once with PBS, and submitted to western blotting with anti-HA and anti-myc antibodies. For preparation of detergent-insoluble fractions from cells, the 1% Triton X-100-insoluble pellet fraction was solubilized by sonication and boiling in 100 μl 2× SDS sample buffer containing β-mercaptoethanol. Equivalent detergent-insoluble fractions were analyzed by western blotting.

Human brain tissue

Human brain tissue was obtained through the brain donation program of the Morris K. Udall Parkinson's Disease Research Center at Johns Hopkins Medical Institutions (JHMI) according to HIPAA regulations. This research proposal involves anonymous autopsy material that lacks identifiers of gender, race, or ethnicity. The JHMI Joint Committee on Clinical Investigations decided that the studies in this proposal are exempt from Human Subjects Approval because of Federal Register 46.101 exemption number 4. Tissue from six control brains, two AD brains and five PD/DLB brains were utilized for western blot analysis. Table 1 shows clinical details of subjects used in this study. Frontal cortex tissue from four AR-JP brains was collected at the Department of Neurology and Department of Pathology, Juntendo University School of Medicine, Japan (Table 1). AR-JP cases 1 and 3 each carry a homozygous deletion of parkin exon 4, whereas cases 2 and 4 each carry a homozygous deletion of parkin exon 3, as described previously (7,59,67).

Fractionation experiments

Detergent-soluble and -insoluble fractions were prepared from human brain tissue by homogenization of samples in TNE buffer (10 mM Tris–HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA) containing Complete protease inhibitor cocktail (Roche) and detergent (0.5% NP-40). The homogenate was centrifuged (20 min at 100 000g), and the resulting pellet (P1) and supernatant (S1, detergent-soluble) fractions were collected. The P1 fraction was washed once in TNE buffer containing detergent, and the resulting pellet (P2, detergent-insoluble) was homogenized and further solubilized by sonication and boiling in TNE buffer containing 1% SDS

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and 0.5% sodium deoxycholate. Fractions were quantitated using the BCA kit (Pierce) with BSA standards, and analyzed by western blotting.

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REFERENCES

1. Lang, A.E. and Lozano, A.M. (1998) Parkinson's disease. First of two parts. *N. Engl. J. Med.*, **339**, 1044–1053.
2. Lang, A.E. and Lozano, A.M. (1998) Parkinson's disease. Second of two parts. *N. Engl. J. Med.*, **339**, 1130–1143.
3. Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M. and Goedert, M. (1998) α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc. Natl Acad. Sci. USA*, **95**, 6469–6473.
4. Gasser, T. (2003) Overview of the genetics of parkinsonism. *Adv. Neurol.*, **91**, 143–152.
5. Dawson, T.M. and Dawson, V.L. (2003) Rare genetic mutations shed light on the pathogenesis of Parkinson disease. *J. Clin. Invest.*, **111**, 145–151.
6. Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R. *et al.* (1997) Mutation in the α-synuclein gene identified in families with Parkinson's disease. *Science*, **276**, 2045–2047.
7. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, **392**, 605–608.
8. Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., Joosse, M. *et al.* (2003) Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism. *Science*, **299**, 256–259.
9. Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G. *et al.* (2004) Hereditary early-onset Parkinson's disease caused by mutations in *PINK1*. *Science*, **304**, 1158–1160.
10. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L. and Riess, O. (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. *Nat. Genet.*, **18**, 106–108.
11. Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B. *et al.* (2004) The new mutation, E46K, of α-synuclein causes Parkinson and Lewy body dementia. *Ann. Neurol.*, **55**, 164–173.
12. Farrer, M., Kachergus, J., Forno, L., Lincoln, S., Wang, D.S., Hulihan, M., Maraganore, D., Gwinn-Hardy, K., Wszolek, Z., Dickson, D. *et al.* (2004) Comparison of kindreds with parkinsonism and α-synuclein genomic multiplications. *Ann. Neurol.*, **55**, 174–179.
13. Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R. *et al.* (2003) α-Synuclein locus triplication causes Parkinson's disease. *Science*, **302**, 841.
14. Bonifati, V., De Michele, G., Lucking, C.B., Durr, A., Fabrizio, E., Ambrosio, G., Vanacore, N., De Mari, M., Marconi, R., Capus, L. *et al.* (2001) The parkin gene and its phenotype. Italian PD Genetics Study Group, French PD Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. *Neurol. Sci.*, **22**, 51–52.
15. Mizuno, Y., Hattori, N., Mori, H., Suzuki, T. and Tanaka, K. (2001) Parkin and Parkinson's disease. *Curr. Opin. Neurol.*, **14**, 477–482.
16. Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L. and Dawson, T.M. (2000) Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl Acad. Sci. USA*, **97**, 13354–13359.
17. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. *et al.* (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.*, **25**, 302–305.
18. Imai, Y., Soda, M. and Takahashi, R. (2000) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J. Biol. Chem.*, **275**, 35661–35664.
19. Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S. and Selkoe, D.J. (2001) Ubiquitination of a new form of α-synuclein by parkin from human brain: implications for Parkinson's disease. *Science*, **293**, 263–269.
20. Chung, K.K., Zhang, Y., Lim, K.L., Tanaka, Y., Huang, H., Gao, J., Ross, C.A., Dawson, V.L. and Dawson, T.M. (2001) Parkin ubiquitinates the α-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat. Med.*, **7**, 1144–1150.
21. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y. and Takahashi, R. (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of parkin. *Cell*, **105**, 891–902.
22. Staropoli, J.F., McDermott, C., Martinat, C., Schulman, B., Demireva, E. and Abeliovich, A. (2003) Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. *Neuron*, **37**, 735–749.
23. Ren, Y., Zhao, J. and Feng, J. (2003) Parkin binds to α/β tubulin and increases their ubiquitination and degradation. *J. Neurosci.*, **23**, 3316–3324.
24. Corti, O., Hampe, C., Koutnikova, H., Darios, F., Jacquier, S., Prigent, A., Robinson, J.C., Pradier, L., Ruberg, M., Mirande, M. *et al.* (2003) The p38 subunit of the aminoacyl-tRNA synthetase complex is a parkin substrate: linking protein biosynthesis and neurodegeneration. *Hum. Mol. Genet.*, **12**, 1427–1437.
25. Tsai, Y.C., Fishman, P.S., Thakor, N.V. and Oyler, G.A. (2003) Parkin facilitates the elimination of expanded polyglutamine proteins and leads to preservation of proteasome function. *J. Biol. Chem.*, **278**, 22044–22055.
26. Huynh, D.P., Scoles, D.R., Nguyen, D. and Pulst, S.M. (2003) The autosomal recessive juvenile Parkinson disease gene product, parkin, interacts with and ubiquitinates synaptotagmin XI. *Hum. Mol. Genet.*, **12**, 2587–2597.
27. Imai, Y., Soda, M., Hatakeyama, S., Akagi, T., Hashikawa, T., Nakayama, K.I. and Takahashi, R. (2002) CHIP is associated with parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol. Cell*, **10**, 55–67.
28. Hague, S., Rogaeva, E., Hernandez, D., Gulick, C., Singleton, A., Hanson, M., Johnson, J., Weiser, R., Gallardo, M., Ravina, B. *et al.* (2003) Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. *Ann. Neurol.*, **54**, 271–274.
29. Abou-Sleiman, P.M., Healy, D.G., Quinn, N., Lees, A.J. and Wood, N.W. (2003) The role of pathogenic DJ-1 mutations in Parkinson's disease. *Ann. Neurol.*, **54**, 283–286.
30. Hedrich, K., Djarmati, A., Schafer, N., Hering, R., Wellenbrock, C., Weiss, P.H., Hilker, R., Vieregge, P., Ozelius, L.J., Heutink, P. *et al.* (2004) DJ-1 (PARK7) mutations are less frequent than Parkin (PARK2) mutations in early-onset Parkinson disease. *Neurology*, **62**, 389–394.
31. Clark, L.N., Afridi, S., Mejia-Santana, H., Harris, J., Louis, E.D., Cote, L.J., Andrews, H., Singleton, A., Wavrant De-Vrieze, F., Hardy, J. *et al.* (2004) Analysis of an early-onset Parkinson's disease cohort for DJ-1 mutations. *Movement Disord.*, **19**, 796–800.
32. Hedrich, K., Schafer, N., Hering, R., Hagenah, J., Lanthaler, A.J., Schwinger, E., Kramer, P.L., Ozelius, L.J., Bressman, S.B., Abbruzzese, G. *et al.* (2004) The R98Q variation in DJ-1 represents a rare polymorphism. *Ann. Neurol.*, **55**, 145.
33. Moore, D.J., Zhang, L., Dawson, T.M. and Dawson, V.L. (2003) A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. *J. Neurochem.*, **87**, 1558–1567.

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34. Miller, D.W., Ahmad, R., Hague, S., Baptista, M.J., Canet-Aviles, R., McLendon, C., Carter, D.M., Zhu, P.P., Stadler, J., Chandran, J. *et al.* (2003) L166P mutant DJ-1, causative for recessive Parkinson's disease, is degraded through the ubiquitin-proteasome system. *J. Biol. Chem.*, **278**, 36588–36595.
35. Macedo, M.G., Anar, B., Bronner, I.F., Cannella, M., Squitieri, F., Bonifati, V., Hoogeveen, A., Heutink, P. and Rizzu, P. (2003) The DJ-1^{L166P} mutant protein associated with early onset Parkinson's disease is unstable and forms higher-order protein complexes. *Hum. Mol. Genet.*, **12**, 2807–2816.
36. Gorner, K., Holtorf, E., Odoy, S., Nuscher, B., Yamamoto, A., Regula, J.T., Beyer, K., Haass, C. and Kahle, P.J. (2004) Differential effects of Parkinson's disease-associated mutations on stability and folding of DJ-1. *J. Biol. Chem.*, **279**, 6943–6951.
37. Olzmann, J.A., Brown, K., Wilkinson, K.D., Rees, H.D., Huai, Q., Ke, H., Levey, A.I., Li, L. and Chin, L.S. (2004) Familial Parkinson's disease-associated L166P mutation disrupts DJ-1 protein folding and function. *J. Biol. Chem.*, **279**, 8506–8515.
38. Tao, X. and Tong, L. (2003) Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. *J. Biol. Chem.*, **278**, 31372–31379.
39. Honbou, K., Suzuki, N.N., Horiuchi, M., Niki, T., Taira, T., Ariga, H. and Inagaki, F. (2003) The crystal structure of DJ-1, a protein related to male fertility and Parkinson's disease. *J. Biol. Chem.*, **278**, 31380–31384.
40. Wilson, M.A., Collins, J.L., Hod, Y., Ringe, D. and Petsko, G.A. (2003) The 1.1-A resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease. *Proc. Natl Acad. Sci. USA*, **100**, 9256–9261.
41. Huai, Q., Sun, Y., Wang, H., Chin, L.S., Li, L., Robinson, H. and Ke, H. (2003) Crystal structure of DJ-1/RS and implication on familial Parkinson's disease. *FEBS Lett.*, **549**, 171–175.
42. Lee, S.J., Kim, S.J., Kim, I.K., Ko, J., Jeong, C.S., Kim, G.H., Park, C., Kang, S.O., Suh, P.G., Lee, H.S. *et al.* (2003) Crystal structures of human DJ-1 and *Escherichia coli* Hsp31, which share an evolutionarily conserved domain. *J. Biol. Chem.*, **278**, 44552–44559.
43. Wilson, M.A., St Amour, C.V., Collins, J.L., Ringe, D. and Petsko, G.A. (2004) The 1.8-A resolution crystal structure of YDR533Cp from *Saccharomyces cerevisiae*: a member of the DJ-1/ThiJ/PfpI superfamily. *Proc. Natl Acad. Sci. USA*, **101**, 1531–1536.
44. Kinumi, T., Kimata, J., Taira, T., Ariga, H. and Niki, E. (2004) Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide-mediated oxidation *in vivo* in human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.*, **317**, 722–728.
45. Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S.M., Takahashi, K. and Ariga, H. (2004) DJ-1 has a role in antioxidative stress to prevent cell death. *EMBO Rep.*, **5**, 213–218.
46. Mitsumoto, A. and Nakagawa, Y. (2001) DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. *Free Radic. Res.*, **35**, 885–893.
47. Mitsumoto, A., Nakagawa, Y., Takeuchi, A., Okawa, K., Iwamatsu, A. and Takanezawa, Y. (2001) Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. *Free Radic. Res.*, **35**, 301–310.
48. Yokota, T., Sugawara, K., Ito, K., Takahashi, R., Ariga, H. and Mizusawa, H. (2003) Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. *Biochem. Biophys. Res. Commun.*, **312**, 1342–1348.
49. Zhang, Y., Dawson, V.L. and Dawson, T.M. (2000) Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol. Dis.*, **7**, 240–250.
50. Dawson, T.M. and Dawson, V.L. (2003) Molecular pathways of neurodegeneration in Parkinson's disease. *Science*, **302**, 819–822.
51. Sherer, T.B., Kim, J.H., Betarbet, R. and Greenamyre, J.T. (2003) Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and α-synuclein aggregation. *Exp. Neurol.*, **179**, 9–16.
52. Sherer, T.B., Betarbet, R., Stout, A.K., Lund, S., Baptista, M., Panov, A.V., Cookson, M.R. and Greenamyre, J.T. (2002) An *in vitro* model of Parkinson's disease: linking mitochondrial impairment to altered α-synuclein metabolism and oxidative damage. *J. Neurosci.*, **22**, 7006–7015.
53. Manning-Bog, A.B., McCormack, A.L., Li, J., Uversky, V.N., Fink, A.L. and Di Monte, D.A. (2002) The herbicide paraquat causes up-regulation and aggregation of α-synuclein in mice: paraquat and alpha-synuclein. *J. Biol. Chem.*, **277**, 1641–1644.
54. Lee, S.J. (2003) α-synuclein aggregation: a link between mitochondrial defects and Parkinson's disease? *Antioxid. Redox Signal*, **5**, 337–348.
55. Betarbet, R., Sherer, T.B., Di Monte, D.A. and Greenamyre, J.T. (2002) Mechanistic approaches to Parkinson's disease pathogenesis. *Brain Pathol.*, **12**, 499–510.
56. Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V. and Greenamyre, J.T. (2000) Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat. Neurosci.*, **3**, 1301–1306.
57. Chung, K.K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, V.L. and Dawson, T.M. (2004) S-Nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science*, **304**, 1328–1331.
58. Canet-Aviles, R.M., Wilson, M.A., Miller, D.W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M.J., Ringe, D., Petsko, G.A. and Cookson, M.R. (2004) The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proc. Natl Acad. Sci. USA*, **101**, 9103–9108.
59. Shimura, H., Hattori, N., Kubo, S., Yoshikawa, M., Kitada, T., Matsumine, H., Asakawa, S., Minoshima, S., Yamamura, Y., Shimizu, N. *et al.* (1999) Immunohistochemical and subcellular localization of Parkin protein: absence of protein in autosomal recessive juvenile parkinsonism patients. *Ann. Neurol.*, **45**, 668–672.
60. Hering, R., Strauss, K.M., Tao, X., Bauer, A., Woitalla, D., Mietz, E.M., Petrovic, S., Bauer, P., Schaible, W., Muller, T. *et al.* (2004) Novel homozygous p.E64D mutation in DJ1 in early onset Parkinson disease (PARK7). *Hum. Mutat.*, **24**, 321–329.
61. Bonifati, V., Oostra, B.A. and Heutink, P. (2004) Linking DJ-1 to neurodegeneration offers novel insights for understanding the pathogenesis of Parkinson's disease. *J. Mol. Med.*, **82**, 163–174.
62. Tofaris, G.K., Layfield, R. and Spillantini, M.G. (2001) α-Synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. *FEBS Lett.*, **509**, 22–26.
63. David, D.C., Layfield, R., Serpell, L., Narain, Y., Goedert, M. and Spillantini, M.G. (2002) Proteasomal degradation of tau protein. *J. Neurochem.*, **83**, 176–185.
64. Pawlyk, A.C., Giasson, B.I., Sampathu, D.M., Perez, F.A., Lim, K.L., Dawson, V.L., Dawson, T.M., Palmiter, R.D., Trojanowski, J.Q. and Lee, V.M. (2003) Novel monoclonal antibodies demonstrate biochemical variation of brain parkin with age. *J. Biol. Chem.*, **278**, 48120–48128.
65. Rizzu, P., Hinkle, D.A., Zhukareva, V., Bonifati, V., Severijnen, L.A., Martinez, D., Ravid, R., Kamphorst, W., Eberwine, J.H., Lee, V.M. *et al.* (2004) DJ-1 colocalizes with tau inclusions: a link between parkinsonism and dementia. *Ann. Neurol.*, **55**, 113–118.
66. Neumann, M., Muller, V., Gorner, K., Kretzschmar, H.A., Haass, C. and Kahle, P.J. (2004) Pathological properties of the Parkinson's disease-associated protein DJ-1 in α-synucleinopathies and tauopathies: relevance for multiple system atrophy and Pick's disease. *Acta Neuropathol. (Berl.)*, **107**, 489–496.
67. Hattori, N., Kitada, T., Matsumine, H., Asakawa, S., Yamamura, Y., Yoshino, H., Kobayashi, T., Yokochi, M., Wang, M., Yoritaka, A. *et al.* (1998) Molecular genetic analysis of a novel Parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the Parkin gene in affected individuals. *Ann. Neurol.*, **44**, 935–941.

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Genetic Brief

Mutation of the Parkin Gene in a Persian Family: Clinical Progression over a 40-Year Period

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Abstract: We report on an Israeli family originating from Iran in which 4 of 7 brothers born from a consanguineous marriage had juvenile Parkinsonism. Linkage analysis of markers covering the autosomal recessive juvenile Parkinsonism (AR-JP, PARK2, *Parkin* gene, OMIM #602544) gene resulted in a maximal logarithm of odds score of 2.18. A homozygous deletion that expanded from exon 4 to exon 6 was identified in all the patients. Significant clinical heterogeneity was present between siblings. © 2005 Movement Disorder Society

Key words: Parkinson's disease; genetics; Parkin; PARK2; clinical heterogeneity

Parkinson's disease, with prevalence close to 2% after age 65, is one of the most frequent neurodegenerative disorders.[1] The classic symptoms include bradykinesia, rigidity, resting tremor, and postural instability. In 1998, Kitada and coworkers[2] identified a novel gene, designated parkin, in which homozygous mutations cause autosomal recessive juvenile Parkinsonism (AR-JP), which typically has an onset before age 40. Since then, many different parkin mutations, ranging from exon rearrangements (deletions and duplications), point mutations, and small deletions have been described in different families from several populations.[3–5] The phenotype of patients with disease caused by parkin mutations is broad but includes dystonia at onset, diurnal fluctuations, slow disease progression, and a good response to levodopa at low doses.[5] We describe an Israeli family with AR-JP due to a parkin mutation.

PATIENTS AND METHODS

Patients

We analyzed a Persian Jewish family who emigrated from Iran to Israel in the early 1950s. There were 4 brothers of 7 siblings born to fist-degree consanguineous parents who had juvenile Parkinsonism (see Fig. 1). Neither parent has Parkinsonism. The affected cases fulfilled the UK Parkinson's Disease Society Brain Bank criteria for disease.[6]

Genetic Analysis

After informed consent was obtained, blood samples were collected from all 4 patients and 3 unaffected brothers, and DNA extraction was performed using standard methods. Genotyping of the microsatellite markers D6S308, D6S441, D6S1581, D6S264, and D6S446 was conducted by the polymerase chain reaction (PCR) using fluorochrome-labeled primers. PCR products were run simultaneously in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

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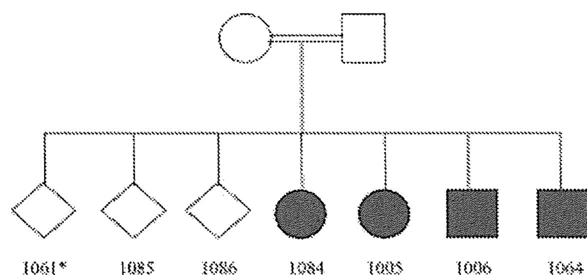

FIG. 1. Pedigree of the Israeli family. Squares represent men, circles represent women. Gender of healthy family members has been masked for confidentiality reasons. Black symbols indicate individuals manifesting autosomal recessive juvenile Parkinsonism. The healthy sibling carrying a heterozygous deletion within the *Parkin* gene is depicted by an asterisk. All the patients carried a homozygous deletion of the exons 4 through 6.

We performed two-point linkage analysis using a parametric model with a recessive mode of inheritance, a frequency of the susceptibility allele of 0.01%, and a homozygous penetrance of 100%. Pair-wise linkage calculations were performed using MLINK.

Testing for gene dosage alterations in *Parkin* exons was carried out by quantitative duplex PCR using the ABI PRISM 7900 sequence detection system (Applied Biosystems). The β-globin gene was coamplified with *Parkin* exons 1 through 12 and served as endogenous reference as previously described.[7] The dosage of *Parkin* exons relative to β-globin and normalized to control DNA was determined using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

Molecular Findings

The two-point maximum logarithm of odds score was 2.18 for markers D6S264 and D6S446 at a theta = 0. These markers are located 3 megabases (MB) and 7 MB, respectively, from the *Parkin* gene, thus indicating a PARK2 mutation segregating with the disease.

Real-time quantitative PCR-based gene dosage assays evidenced homozygous deletion of exons 4 to 6 in all the patients. Only 1 of the healthy siblings (individual 1061) carried a heterozygous deletion. The other 2 siblings did not harbor a deletion.

Clinical Description

Clinical characteristics are shown in Table 1.

Patient 1084.

On her first examination (at which time she was 28 years old), the patient had hypomimia, rest tremor in all extremities and in the tongue, mild rigidity, and bradykinesia in all limbs. Treatment with anticholinergic agents was initiated with beneficial response. Nine years later, she started treatment with L-dopa/carbidopa (125 mg/day) with marked response. At the age of 38, she developed oral and limb dyskinesias. She could not tolerate either the combination of L-dopa/carbidopa (125 mg t.i.d.) or a high dose of L-dopa and, until present, is treated with pure L-dopa (100 mg/day). After a disease duration of 35 years, severe freezing is reported with recurrent falls, yet without postural instability. At that time, she was still L-dopa–responsive with wearing off fluctuations. Other medications are selegiline 5mg/day, and trihexyphenidyl 5 mg/day. She could not tolerate dopamine agonists (pergolide and ropinirole). Over the next years, her symptoms slowly progressed. At the time of her most recent examination, she had marked difficulties with walking, severe freezing gait, marked rigidity, and dyskinesias of the left foot.

Patient 1064.

The patient started treatment with trihexyphenidyl with good response. During the first years of the disease, there were no other major complaints besides depression and anxiety. Twelve years after disease onset, he complained of migrating pain in different parts of the body, constipation, depression, and insomnia. He also could not tolerate carbidopa and was treated with low doses of L-dopa (100 mg/day) with moderate response. He remained stable over the course of 10 years and even stopped taking L-dopa, as he felt worsening of his symptoms (pain, tremor, general weakness) under all the

TABLE 1. *Patient clinical characteristics*

	Patient 1084	Patient 1005	Patient 1006	Patient 1064
Age at onset (yr)	18	21	19	39
Age at last examination (yr)	67	62	65	67
Initial symptom	Legs rest tremor	Legs rest tremor	Postural instability	Right extremities rest tremor
Dystonia	+	–	+	+
Psychiatric symptoms	–	–	–	+
Diurnal fluctuations	+	–	+	–
Slow progression	+	+	+	+

L-dopa preparations. He was treated only with trihexyphenidyl and selegiline 5 mg/day. The most prominent symptoms were psychiatric, with psychomotor restlessness, marked anxiety, akathisia, and unexplained fears.

Patient 1006.

At age 32, he was first examined at our clinic. His main complaints were fatigue and excessive sweating. On examination, head and limb tremor was noticed, more on the left side of the body, mild rigidity, and marked retropulsion. At that time, L-dopa treatment was initiated with amelioration of the tremor and improvement of postural instability. Three years later motor fluctuations, dyskinesias, and dystonia of the right foot emerged. The patient presented intolerance to carbidopa and responded to very low doses of L-dopa (200 mg/day). The parkinsonian symptoms progressed slowly, but 31 years after disease onset, he became very disabled due to freezing gait with recurrent falls. On his recent examination, he was still taking very low doses of pure L-dopa (50 mg q.i.d), selegiline 5 mg/day, trihexyphenidyl 5 mg/day, and amantadine 200 mg/day. On examination while *on*, the patient had hypomimia, dysarthria, no tremor, mild rigidity in all extremities, was able to arise from chair with assistance, and gait was impaired with freezing episodes and postural instability. While *off*, he had marked hypomimia, rigidity, and bradykinesia of all four limbs, and he was almost akinetic, with severe and frequent freezing episodes.

Patient 1005.

Eleven years from disease onset, low doses of L-dopa were initiated with very good response. During the course of the disease there were diurnal variations without sleep benefit, as she felt worse in the morning hours. Her symptoms included fatigue, weight loss, excessive sweating, and pain on the right hand. On her last examination, the most prominent symptom was rest and postural tremor of all extremities and increased tonus more on the right side of the body. Hand and leg movements were normal. She had no difficulties rising from a chair, and there was no gait abnormality.

DISCUSSION

We report on an AR-JP family with disease duration of more that 40 years. Gene dosage analysis revealed an exon 4 to 6 homozygous deletion in *Parkin*. We did not sequence the entire gene and, therefore, cannot rule out any further point mutations in this family.

This mutation has been described also by other authors[8–10]and, as far as we know, this is the third deletion in the *Parkin* gene described in an Israeli family suffering from AR-JP. The same deletion extent was described in two Israeli brothers with first-degree cousin parents.[10] Although both siblings had good response to L-dopa and presented dystonia during the course of the disease, it is noteworthy that none of them exhibited resting tremor. Another Jewish Yemenite kindred also has been described to harbor an exon 3 homozygous deletion.[11] Finally, a single base pair deletion at nucleotide 202 in exon 2 of the *Parkin* gene also has been identified in another Arabic Muslim Israeli family.[12] These studies demonstrate the existence of allelic and phenotypic heterogeneity of *Parkin* mutations in the Israeli population.

The patients described in the present study had beneficial response to L-dopa, slow progression of the disease, and diurnal fluctuations, common among patients with *Parkin* mutations. Our cases share similar clinical features to other parkin cases, including age at onset, symptoms at disease onset, beneficial response to L-dopa, and the occurrence of nonmotor symptoms. However, some features are unique to this kindred: all members have hypersensitivity to L-dopa, responding dramatically to very low doses of L-dopa (at this time, they are treated with strikingly low doses of L-dopa, between 50–100 mg b.i.d. or q.i.d.), and attempts to increase the dose resulted in general complaints; a wide interval of age at onset, ranging from 18 to 39 years, is also presented; psychiatric symptoms seem to be present in only 1 of the 4 patients; and an asymmetrical motor symptom at onset is also reported only by Patient 1064. Dystonia, a common sign in AR-PD patients, was not present in Patient 1005, whereas the other siblings presented different dystonic signs. This phenotypic heterogeneity could be due to the interaction of *Parkin* with other genes (epistasis) and/or other environmental effects.

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REFERENCES

1. de Rijk MC, Tzourio C, Breteler MM, et al. Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON Collaborative Study. European Community Concerted Action on the Epidemiology of Parkinson's disease. J Neurol Neurosurg Psychiatry 1997;62:10–15.
2. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392:605–608.
3. West AB, Maidment NT. Genetics of parkin-linked disease. Hum Genet 2004;114:327–336.
4. Abbas N, Lucking CB, Ricard S, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 1999;8:567–574.
5. Ujike H, Yamamoto M, Kanzaki A, Okumura K, Takaki M, Kuroda S. Prevalence of homozygous deletions of the parkin gene

in a cohort of patients with sporadic and familial Parkinson's disease. Mov Disord 2001;16:111–113.
6. Hughes AJ, Daniel SE, Kilford L, Lees AJ. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 1992;55:181–184.
7. Dogu O, Johnson J, Hernandez D, et al. A consanguineous Turkish family with early-onset Parkinson's disease and an exon 4 parkin deletion. Mov Disord 2004;19:812–816.
8. Nichols WC, Pankratz N, Uniacke SK, et al. Linkage stratification and mutation analysis at the Parkin locus identifies mutation positive Parkinson's disease families. J Med Genet 2002;39:489–492.
9. Foroud T, Uniacke SK, Liu L, et al. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. Neurology 2003;60:796–801.
10. Portman AT, Giladi N, Leenders KL, et al. The nigrostriatal dopaminergic system in familial early onset parkinsonism with parkin mutations. Neurology 2001;56:1759–1762.
11. Nisipeanu P, Inzelberg R, Blumen SC, et al. Autosomal-recessive juvenile parkinsonism in a Jewish Yemenite kindred: mutation of Parkin gene. Neurology 1999;53:1602–1604.
12. Nisipeanu P, Inzelberg R, Abo Mouch S, et al. Parkin gene causing benign autosomal recessive juvenile parkinsonism. Neurology 2001;56:1573–1575.

Preserved Myocardial [^{123}I]Metaiodobenzylguanidine Uptake in Autosomal Recessive Juvenile Parkinsonism: First Case Report

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Abstract: A decrease in myocardial uptake of iodine-123–labeled metaiodobenzylguanidine (^{123}I-MIBG) has been reported in idiopathic Parkinson's disease (PD) using ^{123}I-MIBG myocardial scintigraphy. However, the patient with autosomal recessive juvenile parkinsonism (AR-JP), caused by the *parkin* gene, presented here showed normal ^{123}I-MIBG myocardial uptake, suggesting that AR-JP is a distinct disease entity from PD. Although the clinical features of AR-JP are sometimes quite similar to those of late-onset idiopathic PD, ^{123}I-MIBG myocardial scintigraphy may be a powerful tool to differentiate PD from other parkinsonian syndromes, including AR-JP. © 2005 Movement Disorder Society

Key words: autosomal recessive juvenile parkinsonism; Parkinson's disease; metaiodobenzylguanidine

Myocardial scintigraphy using iodine-123–labeled meta-iodobenzylguanidine (^{123}I-MIBG) is used to differentiate idiopathic Parkinson's disease (PD) from other neurological diseases.[1–4] Low myocardial ^{123}I-MIBG uptake is a consistent finding in PD.[1–3] A recent neuropathological study suggests that low ^{123}I-MIBG uptake indicates the involvement of postganglionic sympathetic function in PD.[5]

Recently, autosomal recessive juvenile parkinsonism (AR-JP) has been found to be caused by the *parkin* gene.[6] Although the clinical features of AR-JP are sometimes quite similar to those of late-onset idiopathic PD, neuropathological studies have revealed no Lewy bodies in the substantia nigra and locus ceruleus of most cases of AR-JP, suggesting that the pathological findings and disease process of AR-JP are different from those of PD.[7,8] In the present study, we investigated cardiac sympathetic function in a patient with AR-JP using ^{123}I-MIBG scintigraphy and evaluated the clinical applicability of this method.

Case Report

The patient was a 62-year-old, Japanese man with a 32-year history of parkinsonism. He was the only son of consanguineous parents from Niigata Prefecture, Japan. At 30 years of age, dystonic posture of the legs and gait disturbance developed. At presentation, the clinical features included diurnal fluctuation and slow progression of the disease. The parkinsonian triad was mild, and the tremor was usually of the fine type. These clinical features markedly improved during sleep. Treatment with levodopa effectively improved the condition, which exhibited a benign clinical course. He was treated with 500 mg of L-dopa/carbidopa and 22.5 mg of bromocriptine per day, which resulted in marked improvement in bradykinesia and rigidity. However, the wearing-off phenomenon was marked, and the response to L-dopa gradually decreased. AR-JP was diagnosed on the basis of the medical history, physiological and neurological examinations, routine laboratory tests, magnetic resonance imaging studies of the brain, and *parkin* gene mutation analysis, including quantitative analysis using real-time polymerase chain reaction (PCR) to detect a compound heterozygote (Fig. 1).[9] Results of quantitative PCR amplification analysis showed that the relative dose value for exons 2 to 4 was approximately 0.5, indicating that this patient was a compound heterozygote for exons 2 and 3 to 4 deletions or exons 2 to 3 and 4 deletions. Alternatively, it is possible that this patient was a heterozygote for deletions of exons 2 to 4. Considering the clinical information, including the age of onset and the mode of

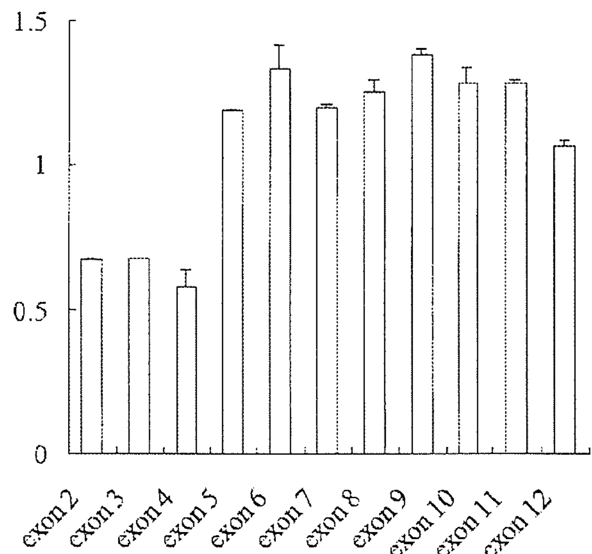

FIG. 1. Real-time polymerase chain reaction shows heterodeletion in exons 2, 3, and 4, indicating compound heterozygosity. The vertical axis represents the ratio of each exonic dose of the *parkin* gene to the dose of the β-actin gene.

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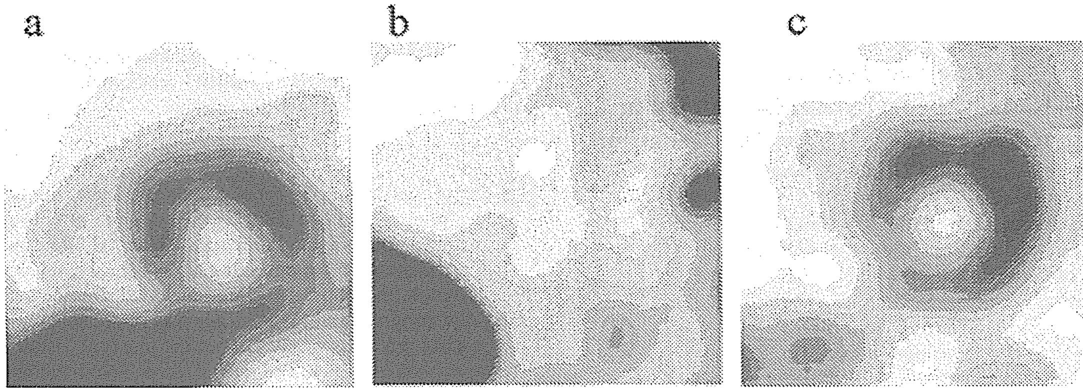

FIG. 2. Short-axis views in the early phase of single-photon emission computed tomography of iodine-123–labeled metaiodobenzylguanidine (^{123}I-MIBG) myocardial scintigraphy in a 62-year-old man with autosomal recessive juvenile parkinsonism (**a**), a 58-year-old man with idiopathic Parkinson's disease (Hoehn and Yahr Stage II, **b**), and a healthy 62-year-old man (**c**).

inheritance, the patient may be a compound heterozygote rather than heterozygote for a deletion of exons 2 to 4.

Single-photon emission computed tomography (SPECT) ^{123}I-MIBG scintigraphy of the heart was performed with an intravenous injection of 111 MBq of ^{123}I-MIBG (Daiichi Radioisotope Laboratories Co., Tokyo, Japan). Early and delayed SPECT was obtained 30 minutes and 4 hours after injection, respectively. Short-axis reconstruction of ^{123}I-MIBG early SPECT acquisitions showed normal ^{123}I-MIBG uptake (Fig. 2). The mean values of the heart to mediastinum (H/M) ratio in the early and delayed phases were 1.99 and 2.13, respectively. In our hospital, the same ratios in age-matched healthy control subjects ($n = 19$) were early phase, 2.02 ± 0.12 (range, 1.86–2.25), delayed phase, 2.05 ± 0.15 (range, 1.85–2.38).

Discussion

To our knowledge, this is the first report of ^{123}I-MIBG myocardial scintigraphy in a patient with AR-JP. Myocardial uptake of ^{123}I-MIBG was normal in this patient (Fig. 2). The H/M ratios in the early and delayed phases in this patient were well within the range of healthy control subjects, although the disease duration was more than 30 years. These findings might explain the tendency for cardiac sympathetic function to be normal in patients with AR-JP.

In PD, ^{123}I-MIBG uptake of postganglionic cardiac sympathetic neurons is reduced at an early stage of the disease in almost all patients with a clinical severity score of Hoehn and Yahr stage II or higher.[1,3] In addition, ^{123}I-MIBG uptake was reduced even in patients whose symptoms had been present less than 5 years, although the reduction was significant in patients with advanced disease.[1] The pathological background of PD is a systemic distribution of Lewy bodies and Lewy neuritis, spreading to the peripheral autonomic nervous system, including the cardiac plexus.[10,11] However, neuropathological studies in AR-JP have revealed selective degeneration with gliosis of the pigmented neurons of the substantia nigra and locus ceruleus, but generally no Lewy bodies,[7,8,12] suggesting that the pathological findings and disease process of AR-JP differ from those of PD. Considered together, our findings in AR-JP and those of previous studies in PD allow us to speculate that AR-JP might be a disease entity distinct from PD, although the clinical features of both diseases are quite similar.

Although this study consisted of only 1 patient with AR-JP, our findings indicate that cardiac sympathetic nerve denervation occurs in PD, which accounts for the decrease in cardiac uptake of ^{123}I-MIBG, but not in AR-JP. In this regard, quantification of cardiac ^{123}I-MIBG uptake has been considered a valuable tool to identify patients with PD and to distinguish them from patients with other parkinsonian syndromes, including AR-JP.[1–4] Further studies involving ^{123}I-MIBG uptake and neuropathological examination of the sympathetic nervous system should be performed in a larger population of patients with *parkin* mutations to confirm our conclusion.

References

1. Orimo S, Ozawa E, Nakade S, Sugimoto T, Mizusawa H. ^{123}I-metaiodobenzylguanidine myocardial scintigraphy in Parkinson's disease. J Neurol Neurosurg Psychiatry 1999;67:189–194.
2. Braune S, Reinhardt M, Schnitzer R, Riedel A, Lucking CH. Cardiac uptake of [^{123}I]MIBG separates Parkinson's disease from multiple system atrophy. Neurology 1999;53:1020–1025.
3. Druschky A, Hilz MJ, Platsch G, et al. Differentiation of Parkinson's disease and multiple system atrophy in early disease stages by means of I-123-MIBG-SPECT. J Neurol Sci 2000;175:3–12.
4. Taki J, Nakajima K, Hwang EH, et al. Peripheral sympathetic dysfunction in patients with Parkinson's disease without autonomic failure is heart selective and disease specific. Eur J Nucl Med 2000;27:566–573.
5. Orimo S, Oka T, Miura H, et al. Sympathetic cardiac denervation in Parkinson's disease and pure autonomic failure but not in multiple system atrophy. J Neurol Neurosurg Psychiatry 2002;73:776–777.
6. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998; 392:605–608.
7. Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998;51:890–892.
8. Paviour DC, Surtees RA, Lees AJ. Diagnostic considerations in juvenile parkinsonism. Mov Disord 2004;19:123–135.
9. Kobayashi T, Matsumine H, Zhang J, Imamichi Y, Mizuno Y, Hattori N. Pseudo-autosomal dominant inheritance of PARK2: two families with parkin gene mutations. J Neurol Sci 2003;207:11–17.