Association of DJ-1 and parkin mediated by pathogenic DJ-1 mutations and oxidative stress
Darren J. Moore1,2, Li Zhang1,2, Juan Troncoso2,3, Michael K. Lee3, Nobutaka Hattori6, Yoshikuni Mizuno6, Ted M. Dawson1,4 and Valina L. Dawson1,2,4,5,*

1Institute for Cell Engineering, 2Department of Neurology, 3Department of Pathology, 4Department of Neuroscience and 6Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, MD, 21205, USA and 6Department of Neurology, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113-0033, Japan

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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/DBL 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

*To whom correspondence should be addressed at: Department of Neurology, Institute for Cell Engineering, Johns Hopkins University School of Medicine, 733 N. Broadway, Suite 731, Baltimore, MD 21205, USA. Tel: +1 4106143359; Fax: +1 4106149568; Email: vdawson@jhmi.edu

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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 M261) 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 homo-dimer 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 shift in pI which may possess both chaperone activity and weak proteolytic activity (39–42). 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 pI 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 >> 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 weakly 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.
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 co-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
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 autoubiquitination and (ii) DJ-1 mutants can differentially associate

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.
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 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.
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 pI-value (44,45,58). SH-SHYSY 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
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
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.

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).
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 restricts 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 cingulum 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 20S 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 favor 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>
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<tr>
<th>Subjects</th>
<th>Diagnosis</th>
<th>Brain region</th>
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<th>Age (year)</th>
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<td>Control 1</td>
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<td>FRNTL</td>
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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.
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

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.
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 poly-ubiquitination 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 20S 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/DBL 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/DBL 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 DBL brains (65,66), the increased levels of insoluble DJ-1 observed in PD/DBL 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, 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% CO₂ 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 and once with PBS, and submitted to IP, as described earlier.

**Ubiquitination assay**

Human 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 with 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/DBL 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.
REFERENCES


