The DJ-1\textsuperscript{L166P} mutant protein associated with early onset Parkinson’s disease is unstable and forms higher-order protein complexes

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Parkinson’s disease (PD) is a common neurodegenerative disorder that involves the selective degeneration of midbrain dopaminergic neurons. Recently DJ-1 mutations have been linked to autosomal-recessive early-onset Parkinsonism in two European families. By using gel filtration assays under physiological conditions we demonstrate that DJ-1 protein forms a dimeric structure. Conversely, the DJ-1\textsuperscript{L166P} mutant protein shows a different elution profile as compared with DJ-1\textsuperscript{WT} both in overexpression cellular systems or in lymphoblasts cells, suggesting that it might form higher order protein structures. Furthermore we observed that the level of DJ-1\textsuperscript{L166P} mutant protein in the patient’s lymphoblasts was very low as compared with the wild-type protein. We excluded a potential transcriptional impairment by performing quantitative RT–PCR on the patient’s material. Pulse-chase experiments in transfected COS-1 cells and cycloheximide treatment in control and patient lymphoblasts indicated that the mutant protein was rapidly degraded. This rapid turnover and the structural changes of DJ-1\textsuperscript{L166P} mutant protein might be crucial in the disease pathogenesis.

INTRODUCTION

Parkinson’s disease (PD) is a progressive age-related movement disorder with a prevalence of ~2% in the population aged over 65 (1). Clinically, it is characterized by bradykinesia, muscular rigidity, resting tremor and postural instability, which are due to the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta. Insoluble protein aggregates known as Lewy bodies (LBs) are seen in the cytoplasm of dopaminergic neurons and other subcortical and cortical structures. LBs are fibrillar inclusions that mainly consist of α-synuclein and ubiquitin (2).

Although the etiology of the majority of PD cases is still unknown it is likely to be a combination of genetic and environmental factors. The genetic loci and genes linked with familial PD, which account for less than 10% of all cases of PD (3), have provided tremendous insight into the pathogenesis of this disorder by elucidating some of the molecular pathogenic mechanisms (4–6). Recently we reported cloning of the DJ-1 gene, for early-onset recessive PD (7). Other genes identified for familial PD are α-synuclein (8), parkin (9) ubiquitin C-terminal hydrolase L (UCH-L1) (10), and NR4A2 (11).

Despite the remarkable progress in recent years, the etiopathogenesis of PD remains elusive. Several hypotheses have been put forward. Findings in post-mortem PD brains and biochemical studies provide support for two main theories related to oxidative and/or proteolytic stress (12,13). Particular interest in the concept of proteolytic stress has arisen because of the E3 ligase activity of the parkin protein. Parkin mutations associated with familial PD potentially interfere with normal protein degradation through the ubiquitin–proteasome system. Decreasing the E3 activity of parkin results in the accumulation of target proteins such as CDC-cel1 (14), PaelR1 (15), or glycosylated α-synuclein (16). Furthermore, there is growing evidence that the normal metabolism of dopamine in itself might be responsible for high basal levels of oxidative stress in...
the substantia nigra, due to auto-oxidation of dopamine, the subsequent semiquinone formation and polymerization through which radical species are produced. The hydroxyl radical produced can damage proteins, nucleic acids and membrane phospholipids, potentially leading to cellular degeneration.

By studying two families from genetically isolated communities in The Netherlands and Italy, two mutations in the DJ-1 gene were found to be associated with autosomal recessive early-onset PD (7). In the Dutch family, a large homozygous genomic deletion removes approximately 14 kb of genomic DNA, including the first five exons of DJ-1 and approximately 4 kb of promoter sequence. The result of this mutation is that the expression of DJ-1 is completely abolished. In the Italian family, a homozygous point mutation was identified, resulting in the substitution of a highly conserved leucine residue at position 166 of the DJ-1 protein by a proline.

DJ-1 is ubiquitously expressed in human tissues (7,17) and is present in both the nucleus and cytoplasm of mammalian cells. Although its exact biological function is still largely unknown, previous studies suggest several putative roles for DJ-1. In particular there is evidence that DJ-1 might play an important role in the post-transcriptional control of gene expression. Hod et al. (18) identified DJ-1 as a regulatory subunit (RS) of an unknown 400 kDa complex (RBP) that contains an RNA-binding activity, and demonstrated that the purified recombinant RS has the capacity to bind the complex and inhibit the RNA-binding activity. DJ-1 further acts as a positive transcriptional co-regulator of the androgen receptor (AR) by preventing PIASx/ARIP3 (androgen receptor interacting protein 3) and DJBP (DJ-1 binding protein) from binding to AR (17,19). PIASx/ARIP3 is a member of the PIAS (protein inhibitor of activated STAT) protein family that not only interacts with SUMO-1 but also functions as an E3 type SUMO protein ligase (20). PIASx was first characterized as an AR binding protein that specifically down-regulates the transcriptional activity of the AR. DJ-1 directly binds to the AR binding region of PIASx, thereby absorbing PIASx from the AR–PIASx complex. DJBP directly binds to both DJ-1 and AR in a testosterone-dependent manner and it negatively modulates the AR transcription activity by recruiting histone–deacetylase complexes. DJ-1 antagonizes this inhibition by abrogation of the complexes, thereby restoring AR activity.

Other studies suggest that DJ-1 is a protein responsive to oxidative stress resulting from exposure to H2O2 or the pesticide paraquat (21,22), that might function as an indicator of oxidative stress state in vivo since it is converted into a variant with a lower pl (pI 5.8) on two-dimensional gels in response to small amounts of reactive oxygen species (ROS) produced during aerobic metabolism in murine cells and tissues (22).

We have proposed a ‘loss of function’ mechanism to explain the pathogenicity of both DJ-1 mutations in the Dutch and Italian families (7); however the molecular mechanism underly the DJ-1L166P mutation needs to be clarified. Based on the structure of the PH1704 protease from *Pyrococcus horikoshii* (23), we predicted a molecular model of DJ-1 and assumed that DJ-1 adopts the same α/β sandwich structure as PH1704. The latter was found to form a hexameric ring structure, which can be regarded as a trimer of dimers with the active sites in the dimerization regions. According to our model, L166P is placed right in the middle of a C-terminal helix, providing a straightforward structural explanation for the mutant’s dysfunction: proline is a strong helix breaker and its presence in the mutant is therefore likely to destabilize the terminal helix, which forms part of the trimerization region, and provides a scaffold for the loop formed by residues S155–P158, which is part of the active site cleft. The general pattern of salt bridges and hydrophobic packing in the trimerization region of PH1704 is conserved in the DJ-1 model, which makes it likely that DJ-1 also forms higher aggregates (7). Therefore, here we investigated this hypothesis and demonstrated that, while DJ-1WT protein is likely to form dimers, in contrast DJ-1L166P forms higher-order structure. The suggested structural changes and the resulting aberrant complex formation might have serious consequences for the normal biological function of DJ-1.

In addition we report that the expression of DJ-1L166P in patient lymphoblasts cells is extremely low compared with DJ-1WT in control samples, suggesting that an abnormal turnover of the mutant protein might play an important role in the pathogenic mechanism as well.

**RESULTS**

**Intracellular localization of DJ-1L166P**

DJ-1WT shows diffuse cytoplasmic and nuclear immunoreactivity in several mammalian cells including COS, HeLa and PC12 cells (7,17,18,24). DJ-1L166P, in transfected COS and PC12 cells, has a similar uniform nuclear staining, whereas the cytoplasmic staining appeared to mostly co-localize with mitochondria (7). Since in the DJ-1 molecular model, L166 is placed right in the middle of a carboxyl-terminal helix, it was necessary to rule out the possibility that the mitochondrial localization was a direct consequence of using a C-terminal tagged construct. We therefore transfected COS-1 cells with DJ-1WT and DJ-1L166P expression constructs without added tags. DJ-1 proteins were visualized by indirect immunofluorescence using our recently generated rabbit polyclonal antibody (SN1132) (25) or a mouse monoclonal anti-DJ-1 antibody, and FITC or TRITC-conjugated secondary antibodies. Both antibodies did not stain endogenous DJ-1 in untransfected COS-1 cells. However on transfected cells the results confirmed our prior studies, showing mitochondrial localization in about 50% of the cells transfected with DJ-1WT and DJ-1L166P (Fig. 1). The remaining half showed diffuse cytoplasmic and nuclear staining as seen for DJ-1WT protein. These results were not dependent on the transfection time or cell density.

**DJ-1 forms dimers and L166P mutation affects DJ-1 complex formation**

The predicted DJ-1 molecular model led to the assumption that DJ-1 (19.9 kDa) is likely to form higher order structures, and the predicted structural consequences and the mitochondrial localization of DJ-1L166P could represent higher-order protein complexes. This hypothesis was investigated by performing gel filtration assays. As DJ-1 is highly expressed in most mammalian cells, cytoplasmic lysates from COS, HeLa and lymphoblasts cells were fractionated on a Superdex 200 PC precision column using physiological conditions (150 mM
NaCl). The collected fractions were analyzed for the presence of endogenous DJ-1 by western blotting using our SN1132 antibody. DJ-1 from untransfected COS and HeLa cells was eluted mainly in fraction number 23, corresponding to a molecular weight of ~35 kDa (data not shown), suggesting therefore that DJ-1 might form a dimer, in agreement with the predicted molecular model. The consequences on complex formation of the L166P mutation were then investigated. COS-1 cells were transfected with a DJ-1WT/V5 or DJ-1L166P/V5 construct. The eluted fractions were visualized by using an anti-V5 antibody. The molecular weight of DJ-1WT/V5 is ~25 kDa and was mainly present in fractions number 22 and 23, corresponding to a molecular weight of ~35–45 kDa (Fig. 2A). This is consistent with DJ-1WT being a dimer. In contrast, DJ-1L166P is present in fractions 19 and 20 (Fig. 2A), corresponding to the elution profile of the protein marker BSA (68 kDa). These results would suggest that DJ-1L166P forms higher-order structures.

Since these experiments were performed using an over-expression system, we further characterized endogenous DJ-1 in lymphoblast cells (Fig. 2B) originating from the patient carrying the homozygous L166P point mutation and from a healthy control individual. Once again DJ-1L166P was eluted one fraction earlier than DJ-1WT, strengthening the suggestion that DJ-1 in normal conditions might assume a dimer conformation and that the L166P mutation affects the structure in such a way that DJ-1L166P forms larger protein complexes.

In order to study the strength of the potential DJ-1/DJ-1 interaction, the experiments were repeated using high salt lysis buffers. Two different salt concentrations were tested: 500 mM NaCl and 1 M NaCl (Fig. 2C), respectively. DJ-1 wild-type and mutant physiological profiles were not affected by the 500 mM NaCl treatment, suggesting that DJ-1 interactions are strong enough to hold even in the presence of high salinity levels. However, in the presence of 1 M NaCl both wild-type and mutant DJ-1 ran as a 68 kDa protein complex. It appears that very high non-physiological salt concentrations induced the wild-type protein to behave as the mutant protein, forming higher order structures.

L166P does not affect DJ-1 interaction with PIASxα/ARIP3 protein

Since there is an effect of the L166P mutation on cellular localization and complex formation of DJ-1, we then tested whether known protein interactions would be disrupted by the mutation. Previous studies have shown an interaction between DJ-1 and PIASxα/ARIP3 (24). To determine whether DJ-1L166P retained this capacity, we transiently co-transfected COS-1 cells with either DJ-1WT/V5 or DJ-1L166P/V5 and FLAG-PIASxα/ARIP3 or FLAG-PIASxα/ARIP3D347-418 constructs. FLAG-PIASxα/ARIP3D347-418 lacks the putative zinc-binding structure containing the conserved cysteines and histidine necessary for its interaction with the E2 SUMO-1 conjugase (26). From total cell lysates, proteins were immunoprecipitated with an anti-V5 antibody and successively visualized with an anti-FLAG antibody. As shown in Figure 3, DJ-1L166P interacted efficiently with both PIASxα/ARIP3 wild-type and PIASxα/ARIP3D347-418, suggesting therefore that the L166P mutation does not affect the DJ-1 capacity to bind to PIASxα/ARIP3 proteins.
To investigate whether the L166P mutation might have an effect on the nuclear co-localization of PIASx/ARIP3 and DJ-1 in mammalian cells (24), transfected COS-1 cells were stained with a mouse monoclonal anti-V5 and a rabbit polyclonal anti-FLAG antibodies, DJ-1 and PIASx/ARIP3 proteins were subsequently detected with FITC and TRITC-conjugated secondary antibodies, respectively. No difference was observed in the nuclear co-localization of DJ-1 L166P/V5 with PIASx/ARIP3 as compared with DJ-1 wild-type (data not shown).

DJ-1 does not directly interact with tau and alpha-synuclein

Tau protein aggregates such as neurofibrillary tangles and Pick bodies are the pathological hallmark of many neurodegenerative disorders. We recently observed co-localization of DJ-1 in a subset of such tau aggregates in the brains of several tauopathies including Pick's disease and Alzheimer's disease (25), demonstrating a possible role of DJ-1 in the pathogenesis of both dementia and Parkinsonism. Here we performed co-transfection experiments in COS-1 cells with both DJ-1 wild-type/V5 and DJ-1 L166P/V5 and tau constructs to determine if the observed co-localization and/or interaction between DJ-1 and tau might also occur in non-pathological conditions. After 24 or 48 h of transfection, cytoplasmic lysates were immunoprecipitated with a phosphorylation-independent anti-tau antibody and analyzed by immunoblotting with anti-V5 and anti-tau antibodies. No co-immunoprecipitation of DJ-1 proteins was observed (data not shown) suggesting that the co-localization seen in tau aggregates might be dependent on the hyperphosphorylation state of tau protein in brain aggregates or is mediated by a third as yet unknown protein.

Figure 2. Distribution of DJ-1 after gel filtration. (A) and (C) elution fractions from COS-1 cells transfected with DJ-1 WT/V5 or DJ-1 L166P/V5 lysated by using 150 mM (A) and 1 M NaCl (C), respectively. (B) Elution fractions from lymphoblast cells from a healthy control (2.5 × 10⁶) and from the Italian patient carrying the DJ-1 L166P mutation (5 × 10⁶) lysated in lysis buffer containing 150 mM NaCl. DJ-1 was detected using SN1132 and V5 antibodies.
To investigate whether DJ-1 is able to interact with α-synuclein, the major component of Lewy bodies, we co-transfected COS-1 cells with either DJ-1 WT/GFP or DJ-1L166P/GFP and α-synuclein/V5 constructs. Cytoplasmic lysates were fractionated by gel filtration using physiological conditions. The collected fractions were visualized with anti-GFP and anti-V5 antibodies to detect DJ-1 and α-synuclein, respectively. As previously shown by Xu et al. (27), α-synuclein forms 54–83 kDa soluble protein complexes and this pattern essentially does not change after DJ-1 co-expression (Fig. 4). Furthermore we were not able to co-immunoprecipitate DJ-1 and α-synuclein in co-transfection experiments on COS-1 cells with either DJ-1 WT or DJ-1 L166P with GFP-tag and alpha-synuclein-V5 constructs (data not shown), suggesting therefore that DJ-1 and α-synuclein do not directly interact.

Low level of DJ-1L166P protein compared to DJ-1 wild-type

Throughout our experiments, DJ-1L166P appeared to be poorly expressed. To investigate whether this was a biologically relevant finding, COS-1 cells were co-transfected with either DJ-1 WT/V5 or DJ-1L166P/V5 and α-synuclein/V5 constructs. Cytoplasmic lysates were fractionated by gel filtration using physiological conditions. The collected fractions were visualized with anti-GFP and anti-V5 antibodies to detect DJ-1 and α-synuclein, respectively. As previously shown by Xu et al. (27), α-synuclein forms 54–83 kDa soluble protein complexes and this pattern essentially does not change after DJ-1 co-expression (Fig. 4). Furthermore we were not able to co-immunoprecipitate DJ-1 and α-synuclein in co-transfection experiments on COS-1 cells with either DJ-1 WT or DJ-1L166P with GFP-tag and alpha-synuclein-V5 constructs (data not shown), suggesting therefore that DJ-1 and α-synuclein do not directly interact.

Quantitative RT-PCR experiments were therefore performed on total RNA isolated from lymphoblast cells of the L166P patient and control samples using DJ-1 primers that span the exons 3 and 5 and exons 5 and 6. Primers specific for the HPRT gene were also included in the assay to provide a normalization control for each sample. We found no significant difference in DJ-1 transcription levels between the patient and control samples (data not shown).

To study possible differences in the stability of the normal and mutated protein, we performed pulse chase-experiments and determined the half-life of DJ-1 WT/V5 and its mutant L166P counterpart in transiently transfected COS-1 cells. After 3 h labeling with [35S]trans-labeled methionine, newly synthesized DJ-1 protein was collected at different time points (0, 3, 6 and 24 h), and immunoprecipitated with anti V5 antibody. While
**DISCUSSION**

The results of the analyses of DJ-1\textsuperscript{WT} by gel fractionating assays on either lymphoblast cells and overexpression cellular systems strongly suggest that DJ-1\textsuperscript{WT} forms dimers, as was predicted from the molecular model (7). It was also demonstrated that DJ-1\textsuperscript{L166P} is present in a higher order protein complex and that its expression levels in transfection studies and patients’ lymphoblasts are very low compared with the wild-type protein.

The L166 residue is highly conserved in evolution among the DJ-1 proteins and, based on the sequence homology between DJ-1 and the PH1704 from *Pyrococcus horikoshi*, it was previously suggested that the L166 amino acid is located right in the middle of a carboxyl terminal helix. This carboxyl terminal helix is thought to be involved in the formation of DJ-1 high order structures (7). Therefore the change of the 166 residue from leucine to proline might have a destabilizing effect, forcing the dimer to assume a different structure as compared with the wild-type protein. The shift in molecular size observed for the mutant protein in our gel filtration experiments might be a direct consequence of protein misfolding that affects migration of the protein under native conditions. Alternatively, the extended elution pattern might be explained by DJ-1 present as a monomer, forming complexes with other unidentified protein. Such binding would be quite strong, as it was not disrupted under high salt conditions.

Although it might be relevant to determine the exact structure of DJ-1\textsuperscript{L166P}, the structural change might not be the crucial event for the disease pathogenesis as we have demonstrated that the mutation does not alter the known protein interaction with Piasxz/ARIP3.

Recently, two independent studies reported the crystal structure of DJ-1, revealing that the wild-type protein indeed forms dimers and that this dimer formation is probably correlated to DJ-1 biological function (28,29), in agreement with our results. Any attempt to obtain the crystal structure of the mutant protein failed, but light scattering experiments showed that it might exist in solution as monomer (28).

Interestingly, in our earlier study (7) we saw a change in the localization of DJ-1\textsuperscript{L166P} as compared with the wild-type protein. In both COS-1 cells and PC12 cells we observed a co-localization of the mutated protein with mitochondria, while DJ-1\textsuperscript{WT} showed a diffuse localization in cytoplasm and nuclei. We confirmed this result after ruling out the possibility that such co-localization was artificially determined by the presence of a C-terminal tag in the eukaryotic expression constructs used.

Although the biological relevance of this finding is not yet clear, particularly because DJ-1 does not have a canonical mitochondrial localization signal, we cannot exclude DJ-1\textsuperscript{L166P} being shuttled to mitochondria after binding to other proteins. Interestingly, it has been suggested that DJ-1 has antioxidant activity as it is modified by oxidative stress generated by H\textsubscript{2}O\textsubscript{2} and paraquat. Several studies suggested a prominent role for oxidative stress and oxidizing toxins in inducing nigral cell degeneration. In this respect it is tempting to explain the detrimental consequences of the L166P mutation by its inability to maintain a dimer formation and by its sequester in protein complexes co-localized with mitochondria resulting in an absence of functional protein in the cytoplasm. By developing and using specific DJ-1 antibodies on post-mortem brain from patients with several neurodegenerative disorders, including Alzheimer’s disease and Pick’s disease, co-localization of DJ-1 was observed in a subset of tau aggregates that suggests a
potential chaperone activity for DJ-1 (25). These results are in agreement with the finding that the DJ-1 yeast homolog is strongly up-regulated after treatment with azetidin-2-carboxylic acid, a toxic analog of proline that activates heat shock factors via accumulation of thermally misfolded proteins (30).

DJ-1 therefore may perform a chaperone-like role that protects cells against toxicity from abnormally aggregated proteins and it may no longer be able to perform this function when mutated.

Strengthening the arguments for the detrimental effects of the L166P mutation is the observation of the low expression level for DJ-1L166P protein in both an overexpression system and in patient’s lymphoblast cells. By pulse-chase experiments and cycloheximide assays it was demonstrated that the mutant protein is highly unstable as it is rapidly degraded in contrast to the wild-type protein, which is highly stable. If the steady-state level of DJ-1 protein has biological relevance, then it is likely that an aberrant turn over of the mutant protein might be responsible for the loss of function of DJ-1. Studies on cystic fibrosis (31), α1-antitrypsin deficiency (32), mitochondrial acyl-CoA dehydrogenase (33) deficiencies and many other diseases have indeed shown that enhanced proteolytic degradation of mutant proteins is a common molecular mechanism (34–36) and might play a key role here as well. Interestingly, recently the involvement of the ubiquitin–proteosome pathway in the degradation of DJ-1L166P has been reported (37).

In conclusion, our results would suggest that the reduced stability of DJ-1L166P could well explain the loss of function proposed for the L166P mutation. The dramatic reduction observed in the steady-state level of DJ-1 would consequently impair the protein capability to perform its normal biological functions, leading therefore to Parkinsonism.

**Figure 5.** Steady-state level of DJ-1. Lymphoblast cells, 1 × 10⁶ cells (lanes 1, 3 and 5) and 2.5 × 10⁶ cells (lanes 2, 4 and 6) from a healthy control (DJ-1), a patient carrying the deletion of exons 1–5 (DJ-1Δ1–5) and a patient carrying the L166P point mutation (DJ-1L166P) were lysed in sample buffer and resolved by 12% SDS–PAGE. DJ-1 expression was visualized with SN1132 antibody. After stripping the blot was labeled with anti-α-tubulin antibody.

**Figure 6.** DJ-1L166P is a short-lived protein. (A) [35S]methionine pulse chase of COS-1 cells transfected with DJ-1WT/V5 or DJ-1L166P/V5. Four time points were taken at 0, 3, 6 and 24 h. Lysates were immunoprecipitated with anti-V5 antibody, samples were run on 12% SDS–PAGE gel and exposed on phosphoimager. (B) Lymphoblast cells (2.5 × 10⁶ cells) from a control sample and a patient carrying the L166P mutation were treated with cycloheximide (final concentration 50 μg/ml) for the indicated time points. Total lysates were run on 12% and labeled with SN1132 antibody. Different exposure times have been used to detect DJ-1WT and DJ-1L166P, respectively.

**MATERIALS AND METHODS**

**Plasmids and their construction**

DJ-1WT/V5 and DJ-1L166P/V5 have been previously described (7). Coding sequence of DJ-1, DJ-1L166P and alpha-synuclein were amplified by PCR and cloned in pcDNA3.1/CT-GFP-TOPO and pcDNA.3.1/V5-His TOPO (Invitrogen), respectively. For the untagged constructs, DJ-1WT and DJ-1L166P cDNAs were amplified by reverse transcriptase-treated total RNA from a control sample and from a patient carrying L166P mutation using the following forward and reverse primers: 5' ggggtgcaggcttgtaaacat 3' and 5' tgacttccatacttccgaa 3'. PCR products were cloned in TOPO TA cloning vector (Invitrogen), and EcoRI fragments were then transferred to pcDNA3.1 expression vector (Invitrogen). Fidelity of the constructs was verified by sequencing using the Big Dye terminator version 3 (Applied Biosystems). The longest tau isoform containing four microtubules-binding repeats (4R tau) cloned into pcDNA3.1 has been described elsewhere (38).
pFLAG-PIASxz/ARIP3 and pFLAG-PIASxz/ARIP3<sup>A347-418</sup> were kindly provided by Jorma J. Palvimo (26).

### Antibodies

The following primaries antibodies were used: mouse monoclonal anti-V5 antibody from Invitrogen; mouse monoclonal anti-FLAG antibody M2 from Sigma; mouse monoclonal against green fluorescent protein from Clontech; mouse monoclonal anti-α-tubulin from Sigma; and rabbit polyclonal anti DJ-1 SN1132 was developed and characterized by us as described elsewhere (25).

*Secondary antibodies.* Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were purchased from Amersham and goat anti-mouse FITC-conjugated, and goat anti-rabbit TRITC-conjugated were from Sigma.

### Cell culture and transfection

COS-1 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen) and were kept at 37°C in 5% CO<sub>2</sub>. Lymphoblast cells from control individuals and the Italian patient carrying the L166P mutation and from the Dutch patient carrying the deletion of exons 1–5 were cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics (Invitrogen). The day before transfection COS-1 cells were seeded on 6-cm-diameter dishes. Cells were transiently transfected with an expression plasmid containing DJ-1<sup>WT</sup> or DJ-1<sup>L166P</sup> either with V5, GFP or untagged, and p-FLAG-PIASxz/ARIP3 or p-FLAG-PIASxz/ARIP3<sup>A347-418</sup> or α-synuclein/V5, or 4R tau using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s recommendations.

### Gel filtration

A Precision column PC 3.2/30 pre-packed with Superdex 200 was used in a SMART system (Pharmacia) to determine by gel filtration the molecular mass of DJ-1. The optimal range for separation of globular proteins in this column is 10–600 kDa, with an exclusion limit of 1300 kDa. In order to calibrate the column and to determine the molecular masses of the eluting fractions, four protein markers were applied in each of the three buffers used for protein fractionation: 150 mM NaCl (physiological buffer), 500 mM and 1 M NaCl (high-salt buffers). The more salt present in the running buffer, the longer was the retention time of the protein in the column. Before running, the column was equilibrated with lysis buffer [50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl and protease inhibitor cocktail (Complete from Roche)]. Lysates were homogenized by sonication and then centrifuged at 13 000g for 10 min to obtain a cytoplasmic supernatant. Cytoplasmic lysate (2/3) was injected into the SMART system and the protein profile was monitored at 280 nm with a column flow rate of 50 μl/min. Fractions (50 μl each) were collected separately.

### Gel electrophoresis and immunoblotting

Proteins present in total lysates and protein fractions were resolved in 2 × sodium dodecyl sulfate (SDS) sample buffer, resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Schleicher and Schuell). Residual protein-binding sites were blocked by incubation of the membrane in PBS supplemented with 5% (w/v) dry milk and 0.1% Tween 20 for 1 h at room temperature. Immunodetections were made using the appropriate primary antibodies: rabbit polyclonal SN1132 (1:1000), mouse monoclonal anti-V5 antibodies (1:5000), mouse monoclonal anti GF (1:1000), and mouse monoclonal anti GFP (1:1000) for 1 h at room temperature. Following washing, the membranes were incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase and the reaction products were visualized by using the enhanced chemiluminescence detection reagents (ECL kit, Amersham Biosciences) according to the manufacturer’s instructions.

### Co-immunoprecipitation assays

**DJ-1 and PIAS.** COS-1 cells were co-transfected with DJ-1/ V5 or DJ-1<sup>L166P</sup>/V5 and FLAG-tagged PIASxz/ARIP3 or PIASxz/ARIP3<sup>A347-418</sup> constructs. Twenty-four hours after transfection, cells were collected and lysed in physiological buffer as previously described (24) and cell homogenates were prepared as described above. Five percent of the cell extracts were immunoblotted with the anti-FLAG M2 monoclonal mouse antibody (1:1000), and the rest of the samples were immunoprecipitated with 2 μg of monoclonal mouse anti-V5 antibody, overnight at 4°C followed by addition of protein G Sepharose-beads (Amersham). Bound proteins were detected with anti-FLAG antibody (1:1000).

**DJ-1 and tau.** COS-1 cell were co-transfected with DJ-1<sup>WT</sup>/V5 or DJ-1<sup>L166P</sup>/V5 and 4R tau in pcDNA3. Twenty-four and 48 h after transfection, cells were lysed in physiological buffer containing 1% NP40 and immunoprecipitated with either anti-V5 or anti-tau (H-7) antibodies. Detection was performed by using anti-V5 (1:5000) and anti-tau (1:2000) antibodies.

**DJ-1 and α-synuclein.** COS-1 cells were co-transfected with DJ-1<sup>WT</sup>/GFP or DJ-1<sup>L166P</sup>/GFP and α-synuclein/V5. Twenty-four hours after transfection, cells were lysed in physiological buffer containing 1% NP40 or 0.5% sodium deoxycholate and immunoprecipitated with anti-V5 antibody. Detection was performed by using anti-GFP antibody (1:1000).

**DJ-1 half-life.** For pulse chase experiments COS-1 cells were transfected with DJ-1<sup>WT</sup>/V5 or DJ-1<sup>L166P</sup>/V5. Twenty-four hours after transfection, cells were starved in methionine/cysteine/glutamine-free DMEM medium (ICN) for 1 h, pulsed for 3 h with 20 μCi/ml of [<sup>35</sup>S]methionine/cysteine (ICN), rinsed and chased for the indicated periods of time in DMEM complete plus 10% FCS. Cell lysates were obtained in lysis buffer with 1% NP-40 and were immunoprecipitated with 2 μg anti-V5 antibody and protein G sepharose. Immunoprecipitates were resolved by 12% SDS–PAGE, visualized by
phosphoimaging and quantified with ImageQuant analysis software. DJ-1 half-life was investigated in lymphoblast cells by inhibiting protein synthesis with 50 μg/ml cycloheximide (Sigma) at the time points indicated. Cell pellets were resuspended in sample buffer and after sonication, were analyzed by immunoblot with SN1132 antibody.

Quantitative RT–PCR

For the quantitative assay the following primer combinations were used to amplify DJ-1 and HPRT genes: forward 1 5’agagagacagtatgagcttctttgccg 3’ and reverse 1 5’ctgtctctatatttcccctgc 3’ and forward 2 5’ggagatactgaaggagcagg 3’ and reverse 2 5’gcaagaggtgtgttgaac 3’ spanning exons 3 and 5 and exons 5 and 6, respectively for DJ-1; forward 3 5’ggagtggatttggaaagggtg 3’ and reverse 3 5’tgcgagcacacagagggctaca 3’ for HPRT. PCR reactions were prepared by using qPCR Core Kit for SYBR Green from Eurogentec and the iCycler iQ termocycler from BioRad.

Immunofluorescence

The day before transfection, COS-1 cells were seeded on glass coverslips. Transfections were performed with Lipofectamine Plus reagent (Invitrogen) and 0.4 μg/ml cycloheximide. Twenty-four hours after transfection, cells were rinsed and mounted on a glass slide in Vectashield mounting medium (Vector) containing DAPI. Images were captured using the Power Gene FISH system on a Leica DMRXA microscope at ×1000 amplification.

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