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Crystallization and preliminary crystallographic analysis of DJ-1, a protein associated with male fertility and parkinsonism

Kazuya Honbou,[a] Nobuo N. Suzuki,[a,b] Masataka Horiuchi,[a] Takahiro Taira,[b,c] Takeshi Niki,[b] Hiroyoshi Ariga[b,c] and Fuyuhiko Inagaki[a,b]*

[a]Department of Structural Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan, [b]CREST, Japan Science and Technology Corporation, Japan, and [c]Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan

Correspondence e-mail: finagaki@pharm.hokudai.ac.jp

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DJ-1 was identified as a novel oncogene product that transformed mouse NIH3T3 cells in cooperation with activated Ras. DJ-1 was also correlated with male infertility and parkinsonism. DJ-1 was crystallized using sodium citrate and HEPES at pH 7.5. The crystal belongs to space group $P3_1$ or $P3_2$, with unit-cell parameters a = 75.04, c = 74.88 Å and contains two molecules in an asymmetric unit. An intensity data set was collected to 2.00 Å resolution.

1. Introduction

DJ-1 is a novel oncogene product that transforms mouse NIH3T3 cells in cooperation with activated Ras. DJ-1 consists of 189 amino-acid residues and is expressed in various human organs, especially in testis (Nagakubo *et al.*, 1997). DJ-1 was found to promote androgen receptor (AR) dependent transcription. This function arises from binding of DJ-1 to PIASxα, which inhibits AR-dependent transcriptional activity (Takahashi *et al.*, 2001). CAP1/SP22, a rat homologue of human DJ-1, decreased by exposure of rats to sperm toxicants such as ornidazole, which caused male infertility (Klinefelter *et al.*, 1997; Wagenfeld *et al.*, 1998). In addition, treatment of sperm with a DJ-1 antibody significantly inhibited fertility of sperm *in vivo* and *in vitro* (Klinefelter *et al.*, 2002). The above evidence taken together suggests that DJ-1 plays an important role in male fertility. Recently, DJ-1 mutation was found to be associated with PARK7, a monogenic form of human parkinsonism. Parkinsonism is the second most common neurodegenerative disorder (Lang & Lozano, 1998) and PARK7 is one of autosomal recessive early onset parkinsonism. In these patients, the highly conserved leucine at position 166 was mutated to proline (Bonifati *et al.*, 2003).

Here, we report the preliminary crystallographic studies of human DJ-1. The three-dimensional structure of DJ-1 will be helpful to understand the functional role of DJ-1 in fertility and parkinsonism.

2. Materials and methods

2.1. Molecular clonimg, expression and purification of DJ-1

Full-length human DJ-1 (189 amino acids, 20 kDa) was cloned into pGEX6P-1 as a fusion protein with glutathione *S*-transferase (GST) and expressed in *Escherichia coli* BL21 (DE3). The bacterial culture was grown in 2×YT with 0.1 g l^{-1} ampicillin at 310 K until absorbance at 600 nm reached 0.5. Protein expression was induced by addition of isopropyl-1-thio-β-D-galactoside to 0.1 m*M*. Cells were cultured at 298 K and harvested after 7 h. The cells were collected and disrupted by sonication at 277 K in PBS(−) containing 1 m*M* EDTA, 5 m*M* NaN_3. The supernatant was incubated with glutathione Sepharose 4B (Pharmacia Biotech) and GST-fusion protein was eluted with a solution containing 25 m*M* glutathione (reduced form) pH 8.0. The eluate was dialyzed in cleavage buffer (150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, 50 m*M* Tris–HCl pH 7.0) and the GST tag was cleaved by digestion with 10 units ml^{-1} PreScission Protease (Pharmacia Biotech) for 4 h at 277 K. After digestion, protein solution was again incubated with glutathione Sepharose 4B and both GST and uncleaved GST-fusion protein were removed. Further purification was performed using HiTrap Q (Pharmacia Biotech) followed by HiLoad Superdex 75pg 26/60 (Pharmacia Biotech).

Purified protein contains an additional 15 amino acids at the N-terminus derived from the linker connecting DJ-1 and GST. In order to remove these N-terminal residues, trypsin digestion was performed by incubating 0.5 mg ml^{-1} protein with 10 pmol ml^{-1} trypsin (sequencing grade, Roche Molecular Biochemicals) with a solution containing 0.1 *M* ammonium bicarbonate for 1.5 h at 310 K. After digestion, protein was purified by two passes of HiLoad Superdex 75 pg 26/60 (Pharmacia Biotech). MALDI–TOF MS and N-terminal sequence analyses were performed, which revealed that 15 amino acids were removed from the N-terminus. Thus, intact DJ-1 (1–189) was obtained. A MALDI–TOF MS spectrum of purified DJ-1 is shown in Fig. 1.

2.2. Crystallization

The crystallization conditions for DJ-1 were established by sparse-matrix screening

(Jancarik & Kim, 1991) using Crystal Screen I (Hampton Research) and sitting-drop vapor diffusion. In each trial, a sitting drop of 2 µl of purified protein solution (10 mg ml^{-1} in 5 m*M* NaN_3 solution) was mixed with 0.5 µl of reservoir solution. A single-crystal was grown at 293 K in precipitant solution containing 1.4 *M* sodium citrate, 0.1 *M* HEPES pH 7.5 in 1 d. The crystals reached maximal dimensions of 0.3 × 0.3 × 0.1 mm.

2.3. Data collection and processing

All diffraction data were collected from crystals cooled at 100 K in a cold nitrogen stream using an R-AXIS IV imaging-plate detector on an FR-C X-ray generator (Rigaku). The data collection was performed with 2.0° steps over a total oscillation range of 142°, with an exposure time of 60 min for each frame. The camera distance was 130 mm. The crystal was found to diffract to 2.00 Å resolution and belongs to space group $P3_1$ or $P3_2$, with unit-cell parameters a = 75.04, c = 74.88 Å. All data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystallographic parameters and data-collection statistics are shown in Table 1. The total number of observed reflections was 124 947, which gave 29 240 unique reflections. The resulting data gave an R_{merge} of 9.76 (29.4% for the outer shell, 2.07–2.00 Å) with a completeness of 97.6% (92.6% for the outer shell). The crystal mosaicity was estimated to be 0.446°. The present crystals contain two molecules in the asymmetric unit, with a V_M value (Matthews, 1968) of 3.07 Å^3 Da^{-1}, corresponding to a solvent content of 59%. The crystal data were used for calculating a self-rotation function using *POLARRFN* (Collaborative Computational Project, Number 4, 1994), as shown in Fig. 2. In the κ = 180° section, the self-rotation map shows strong peaks at (ω, φ) = (90, 0), (90, 60), (90, 120), (90, 180), (90, 240) and (90, 300), indicating that there are three local twofold axes related by the crystallographic threefold symmetry. This result fits well with the estimated value of two DJ-1 molecules in the asymmetric unit.

Sequence alignment using *FASTA* (Pearson & Lipman, 1988) has shown that DJ-1 has no significant homology with other proteins of known tertiary structure.

Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.07–2.00 Å).

Space group	$P3_1$ or $P3_2$
Unit-cell parameters (Å)	$a = b$ = 75.04, c = 74.88
Mathews coefficient (Å^3 Da^{-1})	3.07 (2 mols per AU)
Solvent content (%)	59
Resolution range (Å)	50–2.0
Observed reflections	124947
Unique reflections	29240
R_{merge}† (%)	9.76 (29.4)
Completeness (%)	97.6 (92.6)
Mosaicity (°)	0.446

† $R_{merge} = \sum|I_i - \langle I\rangle|/\sum I_i$, where I_i is the observed intensity and $\langle I\rangle$ is the averaged intensity obtained from multiple observation of symmetry-related reflections.

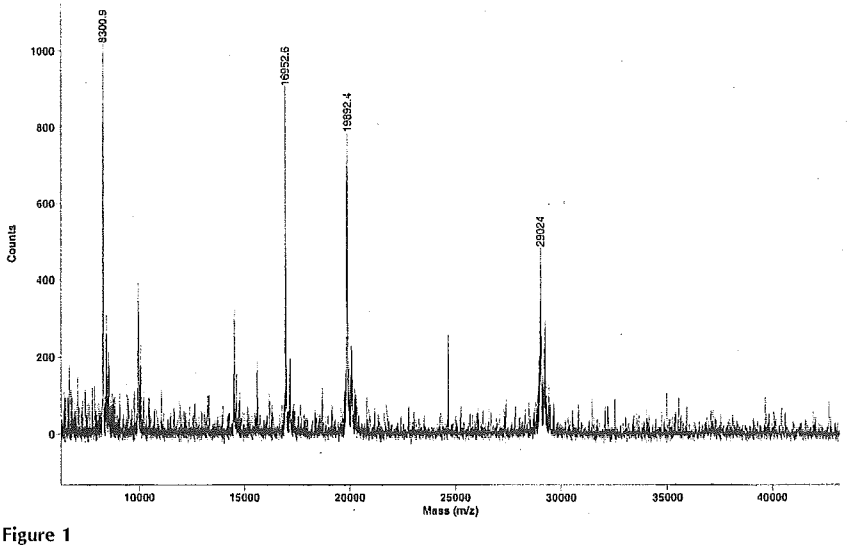

Figure 1
MALDI-TOF/MS spectra of purified DJ-1 using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. Myoglobin (Sigma, M-0630) and carbonic anhydrase (Sigma, C-7025) were used as the internal standard. The observed peak species of (m/z) = 29 024, 169 52.6 and 8300.9 correspond to carbonic anhydrase, myoglobin and doubly charged species of myoglobin, respectively. After calibration, the DJ-1 (m/z) peak was estimated as 19 892.4, in good agreement with full-length DJ-1 (calculated mass 19 891.2).

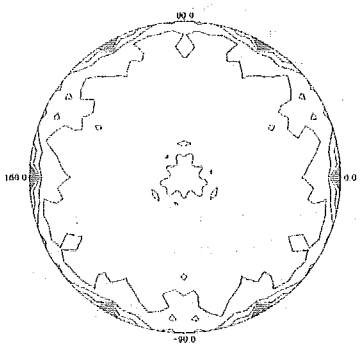

Figure 2
A stereographic projection of the self-rotation function from the DJ-1 data set at κ = 180°. The self-rotation function was calculated using a 29 Å radius of integration and data in the resolution range 7–4 Å. The obvious peaks corresponding to twofold non-crystallographic symmetry can be seen on this section.

Therefore, a heavy-atom multiple isomorphous replacement method for the structure analysis was applied. An extensive search for heavy-atom derivatives is currently under way.

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Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition

Takanori Yokota,[a,*] Kanako Sugawara,[a] Kaoru Ito,[a] Ryosuke Takahashi,[b] Hiroyoshi Ariga,[c,d] and Hidehiro Mizusawa[a]

[a] *Department of Neurology and Neurological Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[b] *Laboratory for Motor System Neurodegeneration, RIKEN Brain Science Institute (BSI), Saitama 351-0198, Japan*
[c] *Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*
[d] *CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan*

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Abstract

Mutations in DJ-1 gene have been linked to autosomal recessive early onset parkinsonism (AR-EOP). Although the mechanism of neuronal cell death due to DJ-1 mutation has not been fully elucidated, loss of DJ-1 function was considered to cause the phenotype. Here, we demonstrated that the down regulation of endogenous DJ-1 of the neuronal cell line by siRNA enhanced the cell death which was induced by oxidative stress, ER stress, and proteasome inhibition, but not by pro-apoptotic stimulus. The cell death with hydrogen peroxide was dramatically rescued by over-expression of wild-type DJ-1, but not by that of L166P mutant DJ-1. Furthermore, DJ-1 rescued the cell death caused by over-expression of Pael receptor, which was a substrate of Parkin, another gene product for autosomal recessive juvenile parkinsonism. These results suggest that loss of protective activity of DJ-1 from neuro-toxicity induced by these stresses contributes to neuronal cell death in AR-EOP with mutant DJ-1.

Keywords: DJ-1; Park 7; Autosomal recessive early onset parkinsonism; Parkinson's disease; Oxidative stress; Hydrogen peroxide; ER stress; Proteasome inhibition

Although most patients with Parkinson's disease (PD) are sporadic, some of the juvenile PD patients with autosomal recessive inheritance (AR-JP) have mutations in Parkin gene (PARK2) [1]. Recently, DJ-1 has been reported as the second causative gene for autosomal recessive early-onset Parkinsonism (AR-EOP) (PARK7) [2]. Since the inheritance is autosomal recessive and the mutations in the DJ-1 gene include a large deletion, the mutations cause Parkinsonism, probably through a loss of DJ-1 protein or function [2]. DJ-1 first identified as an oncogene [3] and later was also found to be a hydrogen peroxide-responsive protein, suggesting that it may function as an antioxidant [4]. Furthermore, DJ-1 was sumoylated through binding to the SUMO-1 ligase PIAS that modulates the activity of transcription factors [5]. Here, we examined the effect of down regulation or over-expression of DJ-1 on the cell death after the oxidative-, ER-stress, apoptotic stimulation, and proteasome inhibition.

Methods

Plasmid constructs, cell culture, transfection, Western blotting. The constructions of expression plasmids of human DJ-1 were previously reported [5]. The coding region of human DJ-1 cDNA was subcloned into pEGFP-N1 (Clontech) (pEGFP-DJ-1). To make L166P mutant DJ-1 expression vector, thymin was changed to cytosine at position 497 from ORF start in of pEGFP-DJ-1 using the QuickChange site-directed mutagenesis system (Stratagene). siRNA-expressing vector was constructed by a previously reported method [6]. For targeting mouse DJ-1 (GTGATTCC TGTGGATGTCATG), or human DJ-1 (GGTCATTACACCTAC TCTGAGAATCGT), the loop sequence (TTCAAGAGA) flanked

* Corresponding author. Fax: +81-3-5803-0134.
E-mail address: tak-yokota.nuro@tmd.ac.jp (T. Yokota).

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by the sense and antisense siRNA sequence was inserted immediately downstream of U6 promoter in pUC19. As negative controls, siRNA-expressing vectors expressing shuffled siRNA sequence were used. A mouse Neuro2a cell and a human embryonic kidney cell line 293T cells (293T) were maintained in Dulbecco's supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. These siRNA-expression vectors were transfected to these cells with Lipofectamine Plus (Invitrogen). Forty-eight hours after transfection, cells were harvested by TNG buffer (50 mM Tris–HCl, 150 mM NaCl, and 1% Triton X-100) with protease inhibitor cocktail (Roche), separated on 15% SDS–polyacrylamide gels, and transferred onto polyvinylidene difluoride membrane (Bio-Rad). The membrane was probed with anti-human DJ-1 polyclonal antibodies [3], or anti-mouse DJ-1 monoclonal [7], and visualized by using enhanced chemiluminescence detection kit (ECL; Amersham–Pharmacia Biotech).

Assessment of cell death. In order to assess the effect of down regulation or over-expression of DJ-1 by siRNA on the cell death induced by various stresses, Neuro2a or 293T cells in 24-well culture plates were transfected with DJ-1 siRNA vector (1 μg/well), or pEGFP-DJ-1 (0.5 μg/well) with Lipofectamine Plus. At 24 h after transfection of pEGFP-DJ-1, and 48 h after transfection of DJ-1 siRNA vector, various stresses were given to the cells. After 24 h after the stresses cell death was assessed with trypan blue exclusion method, and measurement of cytoplasmic lactate dehydrogenase (LDH) activity with the Cytotox 96 nonradioactive cytotoxicity assay (Promega). In addition, cells with nuclear condensation were counted under a fluorescence microscope in 15–30 min after application of 1.0 mM Hoechst dye (33258).

Results

Both siRNA vectors reduced expression level of endogenous mouse DJ-1 in Neuro2a cells or endogenous human DJ-1 in 293T cells by more than 90% on band intensity of Western blotting at 48 h after the transfection (Fig. 1A). Without stresses, down regulation of DJ-1 or over-expression of wild-type and mutant DJ-1 alone was confirmed to have no effect on cell death by LDH assay (data not shown).

After down regulation of endogenous DJ-1, Neuro2a cells were much more susceptible to the oxidative stress with 0.2 mM H_2O_2 (Fig. 1B). This cell death is apoptotic, because it showed nuclear fragmentation and condensation by Hoechst dye staining, which was also increased after down regulation of DJ-1 (Fig. 1C). In contrast, the cell death induced by proapoptotic stimulus, 0.1 mM staurosporin, was not influenced by down regulation of DJ-1 (Fig. 1D).

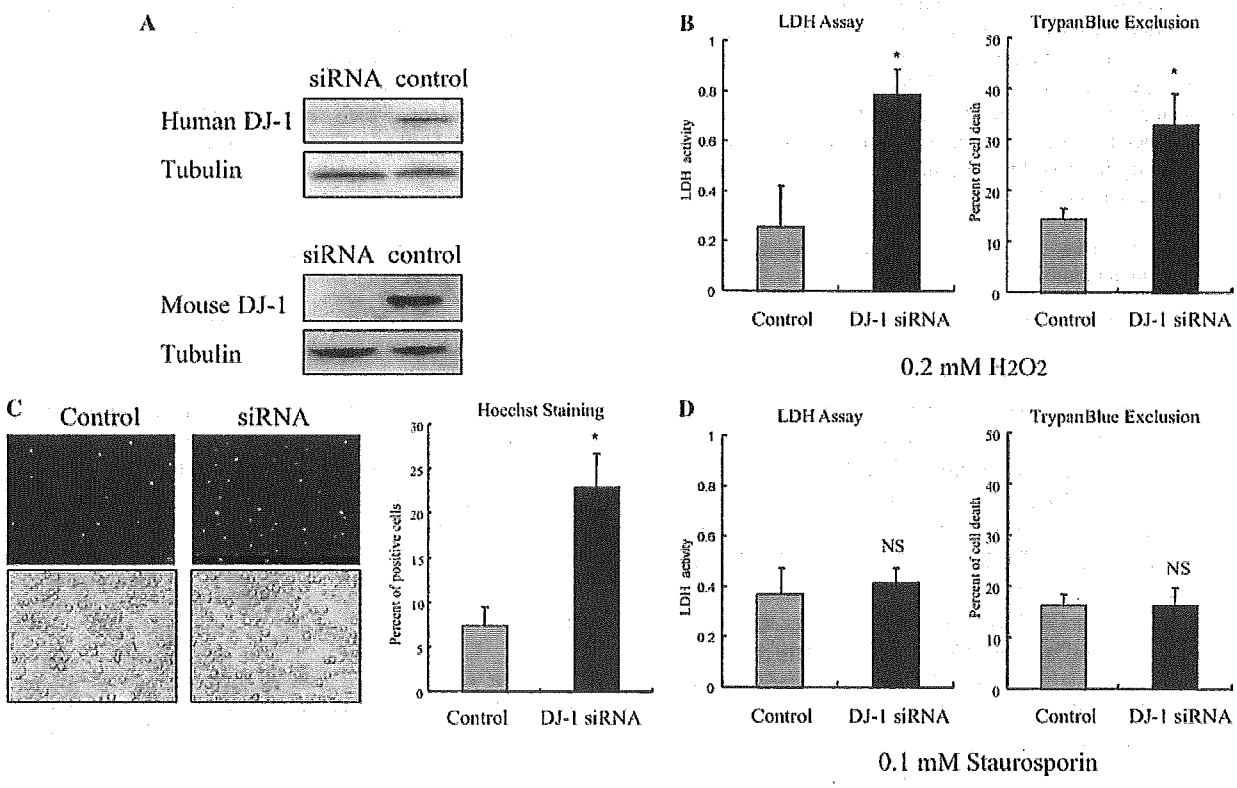

Fig. 1. Effect of down regulation of endogenous DJ-1 on cell death induced by oxidative stress with hydrogen peroxide (H_2O_2). (A) Down regulation of endogenous DJ-1 with siRNA-expressing vector on Western blotting after 48 h after transfection. One microgram of the siRNA-expressing vector for human or mouse was transfected to 293T or Neuro2a cells in 24 well, respectively. (B) Down regulation of endogenous DJ-1 increased cell death of Neuro2a induced with 0.1 mM H_2O_2. $p < 0.01$. (C) Nuclear condensation of Neuro2a cells by Hoechst dye staining was increased by down regulation of endogenous DJ-1 after exposure to H_2O_2. The lower panels were pictures under light field. $p < 0.01$. (D) Down regulation of endogenous DJ-1 increased cell death of Neuro2a did not influence the apoptosis induced by 0.1 mM staurosporin. NS, not significant.

Furthermore, the cell death of Neuro2a cells induced by oxidative stress with 0.4 mM H_2O_2 was dramatically rescued by over-expression of wild-type DJ-1, but not by that of L166P mutant DJ-1 (Figs. 2A and B). The expressions of wild type and mutant DJ-1 proteins were confirmed by GFP-fluorescence (data not shown). In contrast, over-expression of DJ-1 did not influence the cell death caused by 0.2 mM staurosporin (Fig. 2C).

ER stress was given to the cells with tunicamycin and thapsigargin. ER stress-induced cell deaths of Neuro2a cells were also enhanced by down regulation of endogenous DJ-1 expression by the siRNA (Fig. 3A). At higher concentration of tunicamycin (5.0 μg/ml) and thapsigargin (5.0 μM), the induced cell death was decreased by over-expression of wild-type DJ-1, but not by that of L166P mutant DJ-1 (Fig. 3B, left). This rescue effect of DJ-1 overexpression on ER stress-induced cell death, however, is much less than that on oxidative stress-induced cell death. Unexpectedly, at lower concentration of tunicamycin (1.0 μg/ml) and thapsigargin (2.5 μM), over-expression of L166P DJ-1 seems to have a toxic effect (Fig. 3B, right).

Proteasome inhibition was made with lactacystin. The cell death induced by lactacystin was enhanced after down regulation of endogenous DJ-1 of Neuro2a cells by siRNA (Fig. 4A). At higher concentration of lactacystin (50 μM) the induced cell death was decreased by over-expression of wild-type DJ-1, but not by that of L166P mutant DJ-1. In contrast, at higher concentration of lactacystin (20 μM) the over-expression of L166P DJ-1 seems to be mildly toxic to the cells (Fig. 4B).

Putative G protein-coupled transmembrane polypeptide receptor (Pael R) was identified as an interacting protein of Parkin, another gene product of AR-JP [16]. Cell death of Neuro2a cells induced by over-expression of Pael R was rescued by co-expressed DJ-1 (Fig. 5).

All above results were confirmed by three independent experiments. The similar results were also obtained with 293T cells except for experiments with lower concentration of tunicamycin, thapsigargin, and lactacystin (data not shown).

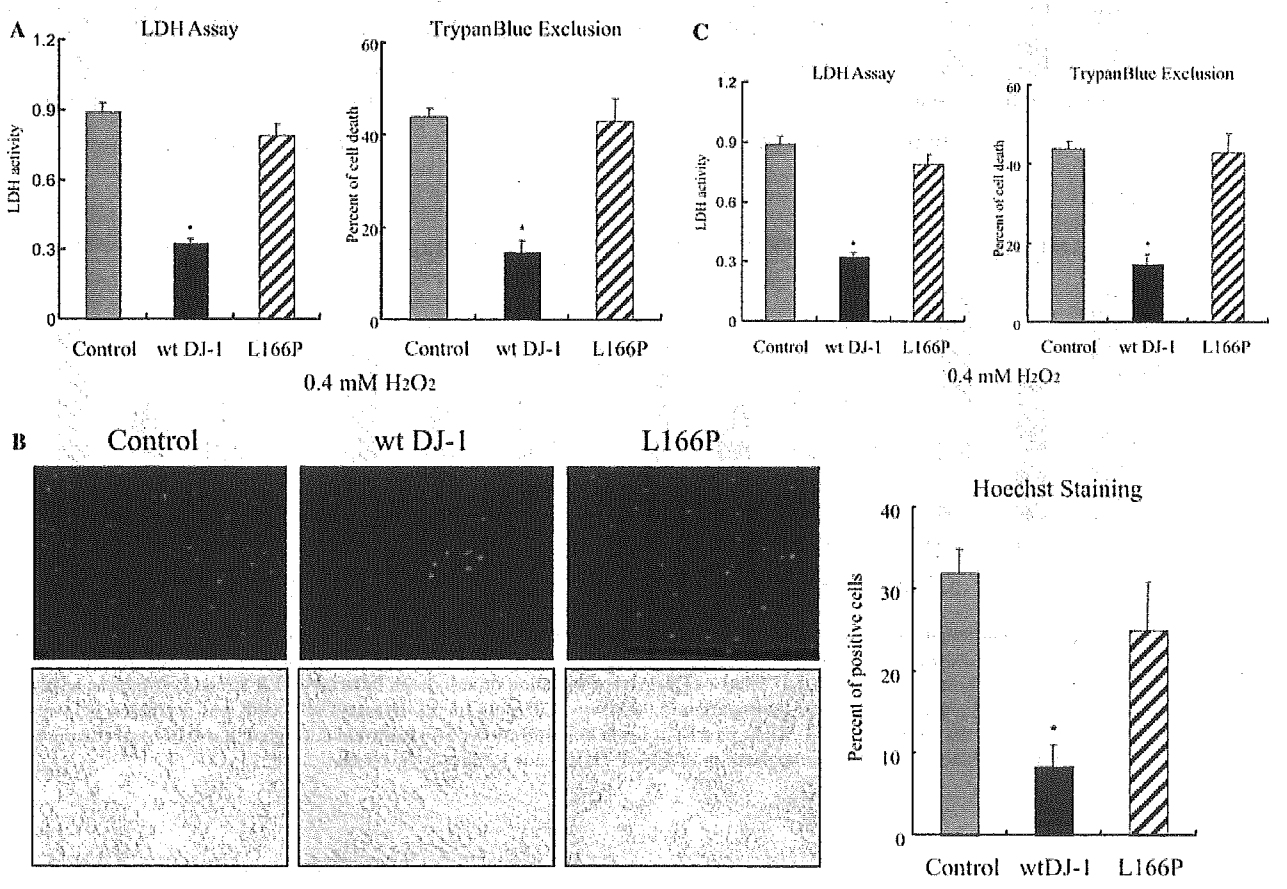

Fig. 2. Effect of DJ-1 over-expression on cell death induced by oxidative stress with H_2O_2. (A) The cell death of Neuro2a induced by 0.4 mM H_2O_2 was rescued by over-expression of wild-type DJ-1, not by that of L166P mutant DJ-1. $p < 0.001$. (B) Nuclear condensation of Neuro2a cells by Hoechst dye staining was much reduced by over-expression of wild-type DJ-1, not by that of L166P mutant DJ-1. The lower panels were pictures under light field. $p < 0.001$. (C) Over-expression of wild-type DJ-1 did not influence the apoptosis of Neuro2a induced by 0.2 mM staurosporin. NS, not significant.

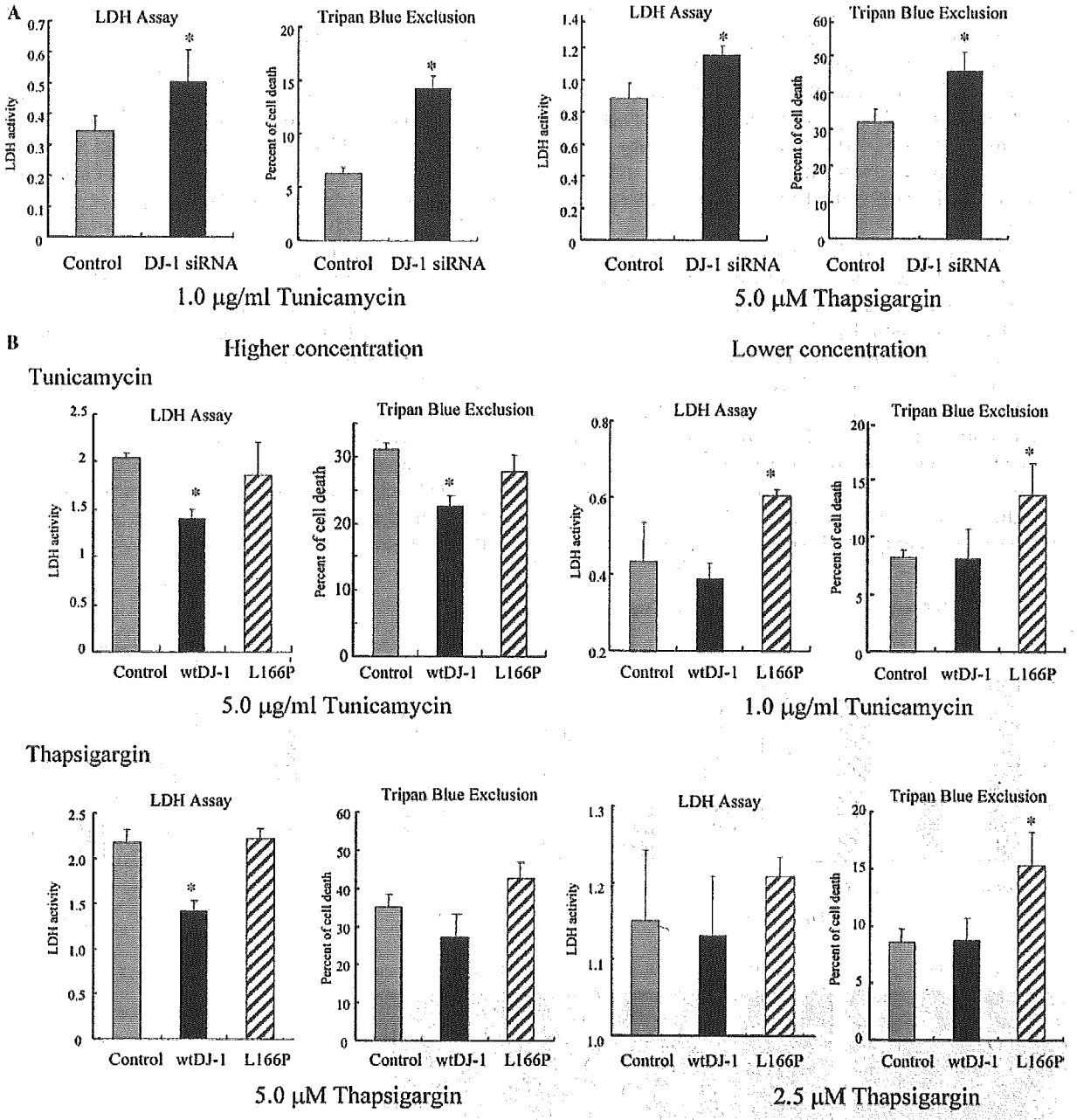

Fig. 3. Effect of DJ-1 down regulation or over-expression on cell death induced by ER stress. (A) Effect of down regulation of endogenous DJ-1 on cell death induced by ER stress. Knockdown of endogenous DJ-1 by siRNA increased cell death of Neuro2a induced by 1.0 μg/ml tunicamycin ($p < 0.01$) and that by 5.0 μM thapsigargin ($p < 0.05$). (B) Effect of DJ-1 over-expression on cell death induced by ER stress. Left panels: at higher concentration of tunicamycin (5.0 μg/ml, $p < 0.01$) and thapsigargin (5.0 μM, $p < 0.05$ only for LDH assay) cell death was decreased by over-expression of wild-type DJ-1, not by that of L166P DJ-1. Right panels: at lower concentration of tunicamycin (1.0 μg/ml, $p < 0.01$) and thapsigargin (2.5 μM, $p < 0.05$ only for trypan blue exclusion), over-expression of L166P DJ-1 tends to have toxic effect.

Discussion

Homozygous deletion, missense mutations (L166P, M26I), and compound heterozygous mutation in DJ-1 gene have been reported to be directly pathogenic for the phenotype of AR-EOP [2,8,9]. A homozygous 14 kb large deletion removing exons 1–5 [2], and a compound heterozygous mutation leading to the frameshift in the exon 1 and the splice error in exon 7 [8], both are predicted to result in a loss of functional protein. Recently, L166P DJ-1 protein proved to be unstable and rapidly degraded when expressed in

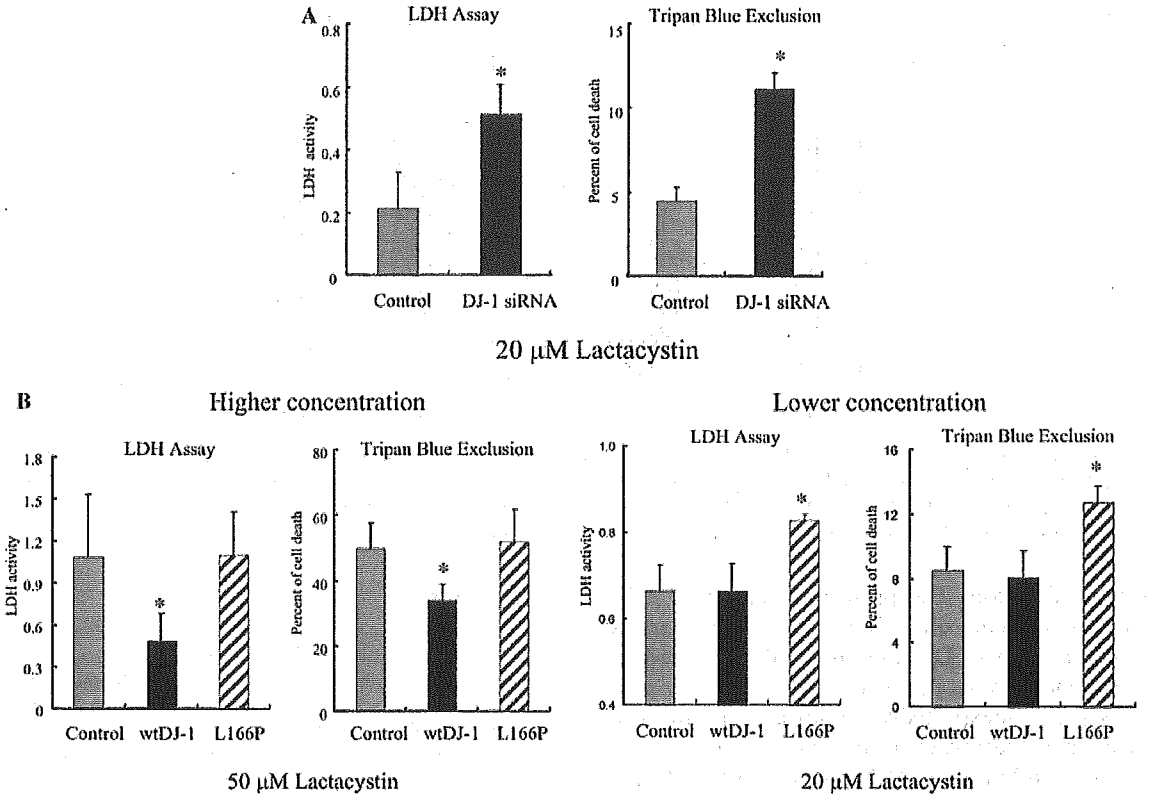

Fig. 4. Effect of DJ-1 down regulation or over-expression on cell death induced by proteasome inhibition with lactacystin. (A) Knockdown of endogenous DJ-1 by siRNA increased cell death of Neuro2a induced with 20 μM lactacystin ($p < 0.01$). (B) Effect of DJ-1 over-expression on cell death induced by lactacystin. Left: at higher concentration of lactacystin (50 μM), cell death was decreased by over-expression of wild-type DJ-1 ($p < 0.01$), not by that of L166P DJ-1. Right: at lower concentration of lactacystin (20 μM), over-expression of L166P DJ-1 tends to have a toxic effect. $p < 0.05$.

culture cells, and the level of L166P DJ-1 protein in patient's lymphoblasts, actually, was very low as compared to the wild type protein [10,11]. Therefore, in at least AR-EOP with mutant DJ-1, loss or reduction of DJ-1 protein function has been considered to cause the phenotype.

The pathophysiology of PD has not been well known, but oxidative stress is suggested to participate in the process of dopaminergic neuronal cell death that undergoes selective degeneration in PD (see recent review, [12]). Ferrous iron (Fe^{2+}) level is elevated and glutathione level is decreased in the substantia nigra, the dopamine-containing region of the brain, in patients with the disorder [13,14]. Accessible iron can react with H_2O_2 produced during oxidative deamination of dopamine to generate hydroxyl radicals (·OH) that can damage proteins, nucleic acids, and membrane phospholipids, leading to cellular degeneration [15]. We showed that neuronal cell death induced by H_2O_2 was enhanced by down regulation of endogenous DJ-1 and was dramatically rescued by over-expression of wild-type DJ-1 but not by that of L166P mutant DJ-1. These results suggest that DJ-1 works as a powerful antioxidant and that loss of its activity is related to dopaminergic neuronal cell death in patients with DJ-1 mutation.

There are several pieces of evidences suggesting that cell death in PD is related to ER-stress and proteasome inhibition. ER-stress maker protein, BiP, was up-regulated in AR-JP (PARK2) brain tissue compared with controls [16]. Parkin protein is E2-dependent E3 ubiquitin–protein ligase and its mutant lost the E3 activity [17]. Over-expression of mutant alpha-synuclein, causative mutation for autosomal dominant PD, produced a proteasome inhibition and sensitized the culture cells to toxicity induced by lactacystin [18,19]. Antisense knockdown of Parkin, another causative gene for autosomal recessive Parkinsonism, increased sensitivity to proteasome inhibitors [20]. Over-expression of Parkin, but not its mutant, specifically suppressed ER-stress-induced cell death [17].

Similar to the results with Parkin, in this study, the cell deaths of Neuro2a induced by ER-stress and proteasome inhibition were enhanced by down regulation of endogenous DJ-1 and slightly suppressed by DJ-1 over-expression. These results suggest that loss of DJ-1 protein activity makes neurons to be vulnerable to ER-stress or

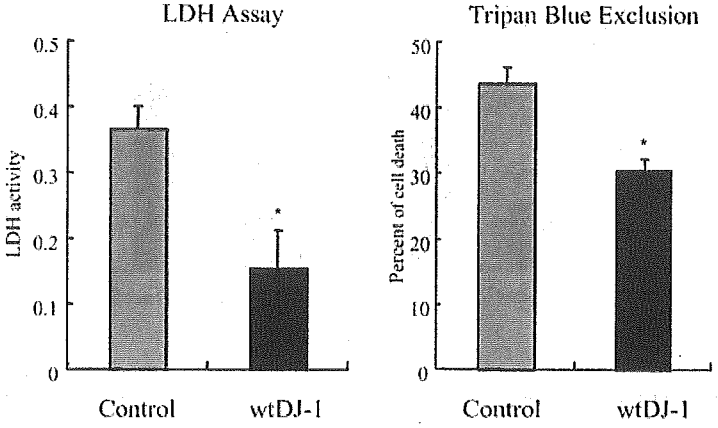

Fig. 5. Effect of DJ-1 over-expression on cell death induced by Pael R over-expression. Cell death was analyzed after co-transfection of 0.3 μg of Pael-R-expressing vector and 0.6 μg wild-type DJ-1 expressing vector (Neuro2a cells in 24 well). Over-expression of DJ-1 rescued cell death induced by Pael R expression ($p < 0.01$). In mock transfection with 0.3 μg pcDNA3.1 (Invitrogen) and 0.6 μg pEGFP-N1, the background cell death showed less than 0.06 in LDH activity and less than 4% in trypan blue exclusion method (data not shown).

proteasome inhibition. The protective activity of DJ-1 from ER stress might be attributed, at least in part, to anti-oxidant activity of DJ-1, because ER stress leads to accumulate endogenous peroxides and promotes oxidative stress [21]. Unexpectedly, at lower concentration of tunicamycin, thapsigargin, and lactacystin, over-expression of L166P mutant DJ-1 not only lost protective activity to these stresses, but also seemed to have a toxic effect on the cells. This mechanism is not known, but an aberrant DJ-1 protein might act in a dominant manner, because heterozygous missense mutations were found in sporadic Parkinson's disease [8,9]. However, further studies are needed to make clear the pathological mechanism of missense mutant DJ-1 under ER-stress and proteasome inhibition.

Pael R is a substrate of Parkin, E3 ubiquitin–protein ligase. Over-expression of parkin, but not mutant, reduced accumulation of insoluble Pael R and suppressed Pael-R-induced cell death. Furthermore, Pael R in insoluble fraction was actually increased in AR-JP brains compared with normal controls. The accumulation of unfolded Pael R is therefore suspected to be causative in AR-JP [16]. Here, DJ-1 over-expression rescues Pael-R-induced cell death. This result also supports that DJ-1 is related to neuronal cell death in patients with Parkinsonism.

In conclusion, our results suggest DJ-1 functions in protecting neuron from oxidative stress and ER stress, and that loss of these activities in mutant DJ-1 contributes to the pathophysiology in AR-EOP.

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The expression of DJ-1 (*PARK7*) in normal human CNS and idiopathic Parkinson's disease

Rina Bandopadhyay,[1,*] Ann E. Kingsbury,[1,*] Mark R. Cookson,[4] Andrew R. Reid,[1] Ian M. Evans,[3] Andrew D. Hope,[1] Alan M. Pittman,[1] Tammaryn Lashley,[2] Rosa Canet-Aviles,[4] David W. Miller,[4] Chris McLendon,[4] Catherine Strand,[2] Andrew J. Leonard,[3] Patrick M. Abou-Sleiman,[2] Daniel G. Healy,[2] Hiroyashi Ariga,[5] Nicholas W. Wood,[2] Rohan de Silva,[1] Tamas Revesz,[2] John A. Hardy[4] and Andrew J. Lees[1]

[1]*Reta Lila Weston Institute of Neurological Studies, Royal Free and UCL Medical School,* [2]*Department of Molecular Neuroscience, Institute of Neurology, Queen Square and* [3]*Department of Molecular Endocrinology, University College London, London, UK,* [4]*Laboratory of Neurogenetics, National Institute of Ageing, NIH, Bethesda, Maryland, USA and* [5]*Hokkaido University, Graduate School of Pharmaceutical Sciences, Sapporo and CREST, Japan Science and Technology Corporation, Kawaguchi, Japan*

Correspondence to: Professor Andrew J. Lees MD, FRCP, Reta Lila Weston Institute of Neurological Studies, Royal Free and UCL Medical School, 46 Cleveland Street, London W1T 4JF, UK.
E-mail: a.lees@ion.ucl.ac.uk

These two authors contributed equally to the work

Summary

Two mutations in the DJ-1 gene on chromosome1p36 have been identified recently to cause early-onset, autosomal recessive Parkinson's disease. As no information is available regarding the distribution of DJ-1 protein in the human brain, in this study we used a monoclonal antibody for DJ-1 to map its distribution in frontal cortex and substantia nigra, regions invariably involved in Parkinson's disease. Western blotting of human frontal cortex showed DJ-1 to be an abundant protein in control, idiopathic Parkinson's disease, cases with clinical and pathological phenotypes of Parkinson's disease with R98Q polymorphism for DJ-1, and in progressive supranuclear palsy (PSP) brains. We also showed that DJ-1 immunoreactivity (IR) was particularly prominent in astrocytes and astrocytic processes in both control and Parkinson's disease frontal cortex, whereas neurons showed light or no DJ-1 IR. Only occasional Lewy bodies (LBs), the pathological hallmarks of Parkinson's disease, showed faint DJ-1 IR, localized to the outer halo. In preclinical studies we showed that DJ-1 is expressed in primary hippocampal and astrocyte cultures of mouse brain. By 2D gel analysis we also showed multiple pI isoforms for DJ-1 ranging between 5.5–6.6 in both control and Parkinson's disease brains, whilst exposure of M17 cells to the oxidizing agent paraquat was manifested as a shift in pI of endogenous DJ-1 towards more acidic isoforms. We conclude that DJ-1 is not an essential component of LBs and Lewy neurites, is expressed mainly by astrocytes in human brain tissue and is sensitive to oxidative stress conditions. These results are consistent with the hypothesis that neuronal–glial interactions are important in the pathophysiology of Parkinson's disease.

Keywords: DJ-1; Parkinson's disease; immunohistochemistry; 2D gel electrophoresis; paraquat

Abbreviations: DA = dopaminergic; 2DGE = two-dimensional gel electrophoresis; GFAP = glial fibrillary acidic protein; IR = immunoreactivity, immunoreactive; LB = Lewy body; LN =Lewy neurite; PSP = progressive supranuclear palsy; SN = substantia nigra

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Introduction

Parkinson's disease is an incurable, inexorably progressive neurodegenerative disorder affecting around 2% of the population over the age of 65 (de Rijk *et al.*, 1997). The cardinal presenting clinical features comprise bradykinesia, rigidity and resting tremor, with a therapeutic response to L-dopa (Yahr *et al.*, 1969). Selective and severe loss of dopaminergic (DA) neurons projecting from the substantia nigra (SN) to the striatum is responsible for the major motor handicaps. The pathological hallmark is the accumulation of eosinophilic, proteinaceous, intracytoplasmic inclusions known as Lewy bodies (LBs) in the substantia nigra, locus coeruleus, dorsal nucleus of the vagus, parahippocampal gyrus and other brainstem and cortical regions (Forno, 1996). Currently, three Parkinson's disease genes, two other candidate genes and a further six distinct loci responsible for rare Mendelian forms of Parkinson's disease have been identified (Dawson and Dawson, 2003; Hardy *et al.*, 2003). However, the molecular mechanisms leading to neurodegeneration in Parkinson's disease remain poorly understood.

Two point mutations (A30P, A53T) in the α-synuclein (*PARK1*) gene cause autosomal dominant, early-onset Parkinson's disease in some families (Polymeropoulos *et al.*, 1997), and α-synuclein is a major component of LBs (Spillantini *et al.*, 1997), thus implicating its involvement in the aetiopathology of sporadic Parkinson's disease. The production of α-synuclein in transgenic mice and transgenic fruit flies may induce disturbances of motor behaviour (Masliah *et al.*, 2000) and LB-like inclusions (Feany and Bender, 2000). A variety of mutations (homozygous deletions, multiplications of exons, point-mutations and insertions) in the parkin gene (*PARK2*) cause autosomal recessive juvenile Parkinson's disease (Kitada *et al.*, 1998). Parkin functions as an E3 ubiquitin protein ligase (Shimura *et al.*, 2000) and its levels are low or absent in patients with autosomal recessive juvenile Parkinson's disease (Shimura *et al.*, 1999). Neuropathological examination shows that neuronal death occurs preferentially in the SN and locus coeruleus and LBs are absent in the brains of most parkin-mutated cases (Mori *et al.*, 1998). Recently parkin has been reported to be a component of LBs and Lewy neurites (LNs) (Schlossmacher *et al.*, 2002), suggesting its possible involvement in the pathological processes mediating idiopathic Parkinson's disease. A point mutation in the ubiquitin carboxy terminal hydrolase L1 gene (*UCH-L1*) (Leroy *et al.*, 1998) has been reported to cause autosomal dominant Parkinson's disease in two affected siblings in a German family. Brain pathology is not yet available from these patients but UCH-LI has been shown to be a component of LBs (Lowe *et al.*, 1990).

Recently, two homozygous mutations in the DJ-1 gene (*PARK7*) have been shown to cause early-onset autosomal recessive Parkinson's disease in families in the Netherlands and Italy (Bonifati *et al.*, 2003). In the Dutch family isolate there was a deletion in exons 1–5 that included the promoter start site of the DJ-1 gene. In the Italian family, a Leu-Pro substitution at position 166 segregated with Parkinson's disease. It is thought that the Dutch patients are unlikely to produce any DJ-1, whereas the point mutation in the Italian family could lead to an impairment of its normal function (Bonifati *et al.*, 2003). The neuropathological changes occurring in individuals affected by these mutations are not known. Other DJ-1 mutations, with homozygous, compound heterozygous and heterozygous genotypes, may confer disease susceptibility in young-onset Parkinson's disease, as shown by further recent genetic studies (Abou-Sleiman *et al.*, 2003; Hague *et al.*, 2003). Some of these mutations may be population-specific (Abou-Sleiman *et al.*, 2003).

DJ-1 is an 189 amino acid protein with multiple functions. It was first identified and cloned as a c-*myc* protein interactor by yeast-two hybrid screening from a HeLa cDNA library (Nagakubo *et al.*, 1997). In association with *ras*, it transformed NIH3T3 cells, suggesting it has oncogenic potential and may be involved in *ras*-mediated signalling pathways (Nagakubo *et al.*, 1997). The rat DJ-1 homologue, CAP1/sP22, was cloned from rat sperm and is important in fertilization (Wagenfeld *et al.*, 1998). An RNA-binding regulatory subunit, RS, purified from rat hepatoma cells (Hod *et al.*, 1999), is almost identical to DJ-1 and has the capacity to bind and inhibit RNA-binding activity. The structure of DJ-1 has some homology to the bacterial proteins ThiJ and Pfp1, which are involved in thiamine synthesis and protease activity respectively (Lee *et al.*, 2003).

DJ-1 may be involved in the regulation of transcription (Takahashi *et al.*, 2001). By binding with protein inhibitor of activated STAT (signal transducers and activators of transcription) (PIAS), a family of (SUMO-1) ligases, DJ-1 can positively regulate transcription of androgen-responsive genes (Takahashi *et al.*, 2001). It can be post-translationally modified by SUMO, a small ubiquitin-like modifier at amino acid position 130 (Takahashi *et al.*, 2001). One DJ-1 molecule interacts with another as seen in yeast-two hybrid system, and L166P mutation down-regulates this interaction (Miller *et al.*, 2003). DJ-1 appears to be sensitive to oxidative stress conditions and undergoes an alteration in its pI (from 6.2 to 5.8) when cultured endothelial cells are treated with paraquat (Mitsumoto *et al.*, 2001). The crystal structure of DJ-1 has been reported, and it suggests that DJ-1 proteins may only function as dimers and that the L166P mutation may disrupt the dimerization of the protein (Honbou *et al.*, 2003; Tao and Tong, 2003).

DJ-1 is expressed in many tissues, including the brain (Nagakubo *et al.*, 1997). DJ-1 mRNA expression is greater in subcortical regions (Bonifati *et al.*, 2003), raising the possibility that it may be important in basal ganglia function. DJ-1 is also expressed by a number of cell lines, is associated with microtubules and localizes to both the nucleus and the cytoplasm (Hod *et al.*, 1999).

Table 1 *Clinical and post-mortem details of cases included in the study*

Case	Sex	Age (years)	Post-mortem delay (h)*	Cause of death
Controls				
C1	F	88	49	COAD
C2	F	88	unknown	Embolism, ischaemia
C3	F	73	28	Bronchopneumonia, bronchial cancer
C4	M	81	40	COAD
C5	F	92	45	MI, IHD
C6	M	85	43	Oesophageal cancer
C7	M	79	56	Prostate cancer
Parkinson's disease				
P1	F	88	37	Parkinson's disease
P2	M	70	46	Bronchopneumonia
P3	M	73	25	Parkinson's disease, chest infection
P4	M	78	72	Bronchopneumonia
P5	F	77	48	Parkinson's disease, chest infection
P6	M	82	27	Bronchopneumonia
P7	M	75	45	Bronchopneumonia
P8	M	88	104	Cerebrovascular accident
P9	M	81	76	Parkinson's disease
P10	M	55	8	Parkinson's disease
P11	F	88	115	Lung cancer
P12	M	80	16	Parkinson's disease
P13	M	66	20	Cerebrovascular accident
P14	M	79	71	Bronchopneumonia
Parkinson's disease with DJ-1 R98Q polymorphism				
P15	M	75	5	Parkinson's disease
P16	F	72	20	MI
P17	F	82	7.3	MI
PSP				
PSP1	M	76	47	PSP
PSP2	M	77	45	PSP
PSP3	F	78	50	Cancer of the colon, parkinsonism

*From death to fixation of brain. Cause of death was as stated on death certificate or hospital post-mortem report. COAD = chronic obstructive airway disease; MI = myocardial infarction; IHD = ischaemic heart disease, PSP = progressive supranuclear palsy.

The function and detailed distribution of DJ-1 in the brain is unknown. It is also not known whether it is associated with LBs and LNs. The aim of this study was to delineate the distribution of endogenous DJ-1 protein in human brain and cultured cells. We also examined the distribution of the protein in normal and pathological states and examined the pI shift of this protein in response to oxidative stress and in Parkinson's disease.

Material and methods

Cases

Brain tissue was obtained from the Queen Square Brain Bank for Neurological Disorders. Human tissue was collected with the informed consent of next of kin and with the permission of the National Hospital for Neurology and Neurosurgery and the Institute of Neurology Joint Research Ethics Committee. All brains were neuropathologically evaluated to confirm the clinical diagnosis and to exclude undiagnosed pathology in the neurologically normal controls. Details of all cases are outlined in Table 1. Three of our cases harbour a heterozygous G to A substitution at position 98 in exon 5, and had the classical clinical and pathological phenotype of Parkinson's disease. This polymorphism results in the substitution of an alanine for a glycine residue (Abou-Sleiman *et al.*, 2003).

Western blotting

Human brain tissue from the frontal cortex of control, Parkinson's disease, Parkinson's disease with DJ-1 heterozygous mutations and progressive supranuclear palsy (PSP) were homogenized in isotonic sucrose (10% homogenates) buffered with HEPES (Sigma, Poole, UK) and spun at 12 000 *g* for 10 min to remove cellular debris. Protein from the supernatants was measured by the bicinchoninic acid method (Biorad, Hemel Hempstead, UK) using BSA as standard. Ten micrograms of protein from each supernatant was

solubilized in NuPAGE (Invitrogen, Paisley, UK) sample buffer and loaded onto 10% Bis-Tris gels and run with the MES (morpholinoethane sulphonic acid) buffer system (Invitrogen). Protein bands were subsequently electroblotted onto Hybond P (Pharmacia Biotech, UK) membrane. Duplicate membranes were blocked with 5% milk (Marvel) in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 (PBS-T). Western blot analysis was carried out using DJ-1 antibody (clone 3E8; Stressgen, San Diego, CA, USA) at 1 : 5000 dilution or β-tubulin (clone SAP.4G5; Sigma) also diluted 1 : 5000, followed by incubation with horseradish peroxidase-conjugated mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1 : 10 000 dilution. Three 5 min washes with PBS-T were performed between each antibody treatment. The blots were developed by the enhanced chemiluminescence (ECL) method using standard procedures and captured onto Kodak Biomax (Amersham, UK) autoradiography film.

Production of recombinant DJ-1 and antibody preabsorption

DJ-1 cDNA clone was purchased from HGMP (Cambridge, UK; clone No-1309796, GB.Acc – AA770265) and the plasmid DNA was used as the template for the polymerase chain reaction (PCR). The full-length DJ-1 cDNA was constructed using the forward primer 5′-AAAAGAGCTCTGGTCATCCTG-3′ and the reverse primer 5′-GTCTTTAAGAACAAGTGGACG-3′. The PCR reaction was run on a 60–50°C touchdown programme followed by 20 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 30 s. The correct sequence was verified by sequencing. The PCR product was subcloned into pTrcHis2-topo-cloning/expression vector (Invitrogen) and transformed into one-shot *E.coli* competent cells (Invitrogen). DJ-1 protein was expressed by induction with IPTG (an inducer of β-galactosidase activity in bacteria, Promega, Southampton, UK) (1 mM). The DJ-1 protein was purified using His-select HC nickel affinity gel (Sigma) according to the manufacturer's instructions. The purified fractions were run on SDS–PAGE for analysis, and dialysed against PBS containing 0.1 M PMSF. For preabsorption studies, DJ-1 antibody (1 : 5000, 1 : 3000) was incubated with 50 μg of recombinant DJ-1 fusion protein at 37°C for 1 h. The sample was further incubated sequentially at 4°C overnight. The solution was centrifuged for 10 min at 13 000 r.p.m. and the supernatant was used for blocking specific band on immunoblots and tissue sections.

Immunohistochemistry and immunofluorescence on brain sections

Formalin-fixed sections (6 μm) were first dewaxed in xylene. Endogenous peroxidase was blocked with 0.6% hydrogen peroxide in methanol for 10 min. Following rehydration, antigens were retrieved by autoclaving in citrate buffer (10 mM, pH 6) for 10 min. Sections were blocked with 10% normal goat serum (Sigma) before treating with monoclonal antibody to DJ-1 (Stressgen) and glial fibrillary acidic protein (GFAP; rabbit polyclonal; Dako, UK) at 1 : 3000 dilution overnight at 4°C. Biotinylated secondary anti-mouse antibody was used at 1 : 1000 (Vector Laboratories, Peterborough, UK) for 1 h, followed by avidin-biotin peroxidase complex (ABC) (Vector Laboratories) for 30 min. For visualization, hydrogen peroxide-activated DAB (diamino benzidine) was used. Three 5 min washes in PBS were carried out between each step. Tissue sections were lightly counterstained with haematoxylin, dehydrated through graded alcohols, cleared with xylene and mounted in mounting medium (Merck, UK). For specificity of staining, some sections were stained in the absence of primary antibody or with antibody that had been preabsorbed with recombinant DJ-1.

For double immunolabelling of DJ-1 and GFAP in astrocytes, antigens were retrieved by microwaving for 20 min in citrate buffer (10 mM, pH 6). DJ-1 and GFAP antibodies were used as above. The DJ-1 signal was visualized using the tetramethyl rhodamine and GFAP with the fluorescein signal amplification kit (Perkin Elmer, UK). Sections were washed thoroughly in PBS and mounted in Aquamount (Merck) and scanned using a Leica TCS40 laser confocal microscope.

Immunoelectron microscopy

Small blocks of substantia nigra were obtained from fresh tissue and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.15 M Sorenson's phosphate buffer (pH 7.4) for 3 h. After fixation, the tissue were washed and stored in 0.15 M phosphate buffer (pH 7.4) overnight at 4°C, followed by dehydration in graded ethanols and embedded in London resin white (Agar Scientific, Stansted, UK). Ultrathin sections mounted on nickel grids were floated on a droplet of ammonium chloride (0.5 M) for 1 h, followed by incubating buffer containing 1.0% BSA, 1.0% normal goat serum, 0.1% sodium azide and 0.1% Tween 20 in PBS (pH 8.2) for 30 min. The sections were incubated in anti-DJ-1 (1 μg/μl, Stressgen) overnight at 4°C, washed and treated with goat anti-mouse gold conjugate (20 nm, BB International, Cardiff, UK) for 4 h at room temperature. After incubation with secondary antibody, the sections were washed in series of droplets of distilled water, stained with 0.5% uranyl acetate and examined in a Philips CM10 electron microscope operating at 80 kV. Control sections were prepared by omitting the primary antibody.

Primary cell culture and staining

Primary neurons and astrocytes from postnatal day 2 mouse pups were dissociated as described previously (Petrucelli *et al.*, 2002). Glial cells were cultured in feeder layers alone for 2–4 weeks and stained for GFAP using a monoclonal antibody G-A-5 (Sigma). These glial-enriched cultures were >95% GFAP-positive. Neuron-enriched cultures were prepared by dissecting and dissociating hippocampi, also from P2 mouse pups, which were plated in serum-free medium for 3 days prior to staining. Neurons were identified by staining with monoclonal antibody to microtubule-associated protein 2 (MAP2) (clone AP-20; Sigma) and represented approximately 75% of the cells in these cultures, the rest being contaminating glial cells. For DJ-1 staining, cells were preincubated with 500 nM Mitotracker CMTMRos (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C prior to staining. Cells were fixed in 4% paraformaldehyde in Dulbecco's PBS (DPBS) for 30 min at room temperature, permeabilized with 0.1% Triton X-100 and quenched with 0.1 M glycine. After washing in DPBS, non-specific immunoreactivity was blocked with DPBS containing 10% foetal bovine serum and 0.1% Triton X-100 and incubated with sheep polyclonal anti-DJ-1 (gift of Gary Klinefelter; 1 : 1000) overnight at 4°C. This antibody was used in preference to the monoclonal antibody used for human tissue, which did not recognize DJ-1 in mouse brain extracts (data not shown). Cells were incubated with

AlexaFluor 488-conjugated donkey anti-sheep IgG conjugated prior to mounting under ProLong Antifade medium (Molecular Probes, Eugene, OR, USA). Slides were examined using a Zeiss LSM510 confocal microscope using independent excitation for both channels. Omission of primary antibody was used to evaluate non-specific fluorescence and in all cases gave no signal.

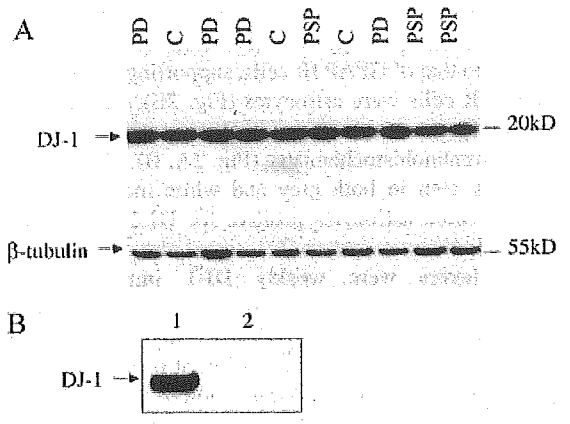

Fig. 1 (**A**) Immunoblot analysis of DJ-1 in human brain. Lysates of brain tissue of control (C), Parkinson's disease and PSP cases were analysed by immunoblot assay with DJ-1 antibody (*upper panel*). Each lane contained 10 μg of protein. Equivalent loadings were confirmed in replicate blots probed with β-tubulin (*lower panel*). Markers on the right of the blot indicate molecular weights in kilodaltons. (**B**) Preabsorption of DJ-1 antibody with recombinant DJ-1. The specific signal for DJ-1 (lane 1) shown is blocked by an excess of recombinant DJ1 (lane 2).

2D gel electrophoresis (2DGE)

M17 human dopaminergic neuroblastoma cells were grown in Optimem (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and either left untreated or exposed to 100 μM paraquat (Sigma) for 20 h. Cytosolic protein extracts were made as described previously (Allen *et al.*, 2003). For 2DGE, 500 μg of protein was separated first on 13 cm immobilized pH gradient (IPG; Amersham Biosciences) strips using 3.0–10.0 linear gradients according to the manufacturer's instructions on the IPGPhor system (Amersham Biosciences). The second dimension was resolved on 10–20% SDS–PAGE gels (Jule, Milford, CT, USA). Gels were blotted to Immobilon polyvinylidene difluoride (PVDF) (Amersham Biosciences) membranes and probed with monoclonal antibody to DJ-1 (Stressgen; 1 : 1000). Blots were developed using peroxidase-labelled secondary antibodies (Jackson Immunochemicals; 1 : 5000) and ECL-plus (Amersham Biosciences). pI was calibrated using creatine phosphokinase carbamylated standards (Amersham Biosciences) and molecular weight using Precision prestained markers (Biorad). A similar protocol was used for 2DGE for human extracts as above with some modifications. Briefly, for the first dimension, human brain homogenates (10 μg) from control and Parkinson's disease subjects, including those with heterozygous changes in the DJ-1 gene, were applied to IPG strips and separated as above. Proteins were blotted onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences) which were probed for DJ-1 as detailed in the western blotting section. Protein spots were visualized with an ECL kit (Amersham Biosciences) according to the manufacturer's instructions.

Results

Western blotting

Immunoblotting of homogenized tissue from frontal cortex of control, idiopathic Parkinson's disease and PSP cases with

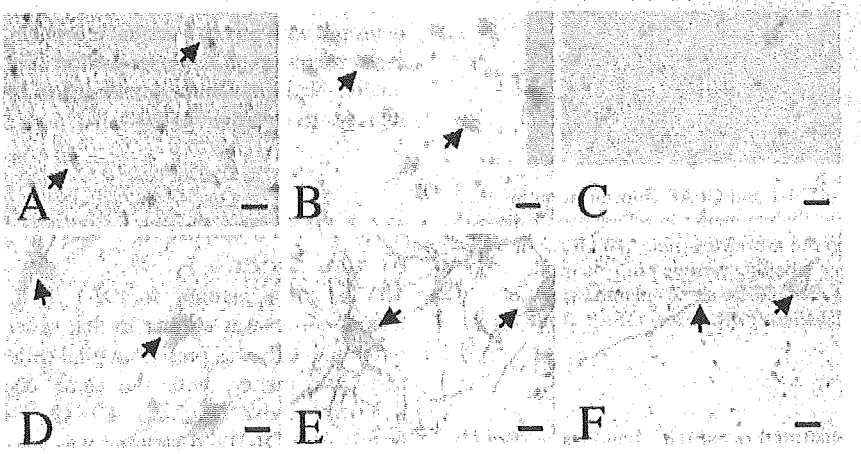

Fig. 2 Immunohistochemistry for DJ-1 in control, and Parkinson's disease tissue. Examples of DJ-1 IR in astrocytes (arrows) in the grey matter from control cortex (**A**) and the white matter in a Parkinson's disease case (**B**). This staining was specific, as a preabsorbed DJ-1 antibody with recombinant DJ-1 shows no positive astrocytic labelling in the white matter from a case of idiopathic Parkinson's disease (**C**). A high-power picture of astrocytes shows both cytoplasmic and nuclear staining for DJ-1 (**D**). Astrocyte morphology was demonstrated with GFAP staining in white matter of a case of idiopathic Parkinson's disease (**E**). In some non-DA neurons from the nigra of a Parkinson's disease case there was cytoplasmic and axonal staining for DJ-1 (**F**). Scale bars = 20 μm for A, B and C, and 10 μm for **D**, **E** and **F**.

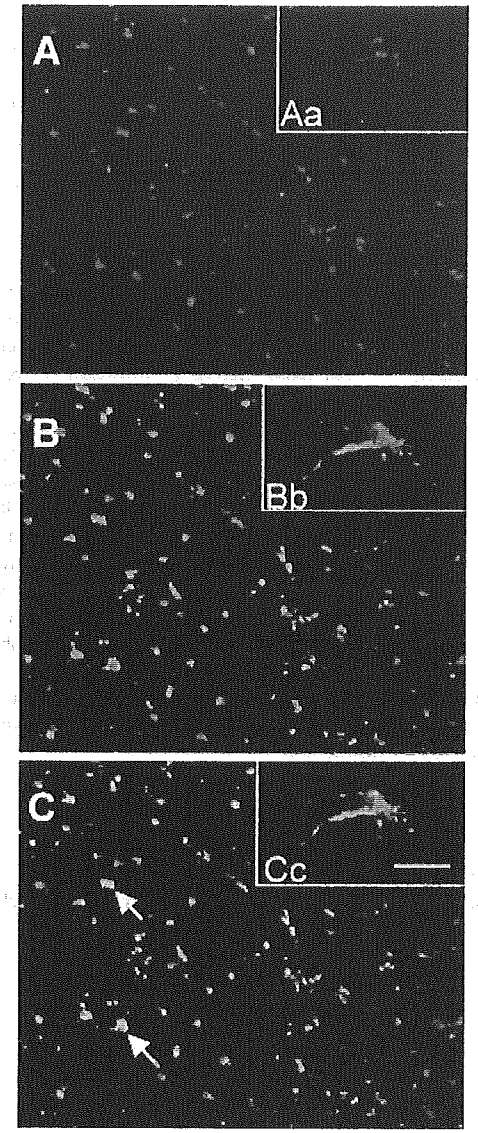

Fig. 3 Confocal images of DJ-1 and GFAP colocalization in astrocytes in frontal cortical white matter in a Parkinson's disease case. (**A**) DJ-1 localization in astrocytes (red). (**B**) GFAP in astrocytes (green). Yellow labelling (arrows) in **C** is true colocalization. Insets **Aa**, **Bb** and **Cc** show enlarged views of a single astrocyte double-labelled for DJ-1 and GFAP. Scale bar = 15 μm.

DJ-1 antibody demonstrated a single band at ~20 kDa (Fig. 1A), which was not detected when the antibody was preabsorbed with excess (50 μg) recombinant DJ-1 (Fig. 1B). Omission of primary antibody also did not give any band for DJ-1 (data not shown). There was no obvious difference in expression levels of DJ-1 in controls, Parkinson's disease or PSP cases. Our Parkinson's disease cases with DJ-1 R98Q polymorphism also gave a similar intensity band (data not shown).

DJ-1 IR in control brain tissue

Frontal cortex

Strong DJ-1 IR was observed in a proportion of glial cells with morphological attributes of astrocytes and DJ-1 IR glia were present in both grey (Fig. 2A) and white matter. In the grey matter, staining for DJ-1 was diffuse throughout the brain parenchyma with some individual glial profiles that were most common in the deep layers, along the white matter border. They were also seen at the cortical surface. DJ-1 IR in glia was present throughout the cell, in cytosol and glial processes. The morphology and distribution of DJ-1 IR cells was similar to that of GFAP IR cells, supporting the view that most DJ-1 IR cells were astrocytes (Fig. 2E), but the dense network of fibres revealed by GFAP staining was not seen with DJ-1 immunohistochemistry (Fig. 2A, B). Glial nuclear staining was seen in both grey and white matter (Fig. 2). There were more astrocytes positive for DJ-1 in the white matter than in the grey matter. Occasional cortical neurons of the deep layers were weakly DJ-1 immunoreactive. Specificity of glial staining by DJ-1 was demonstrated by incubation following pre-absorption of the antibody with recombinant DJ-1 (Fig. 2C) or omission of primary antibody when no staining was seen. Colocalization of DJ-1 and GFAP in astrocytes was also evident by double-labelling immunofluorescence.

Midbrain

Immunopositive cells with astrocyte morphology were observed in the neuropil surrounding DA nigral neurons. Very few were seen among the nigral cell groups. In some cases there was diffuse staining for DJ-1 throughout the nigral neuropil, with no distinct glial morphology; however, in these cases we observed a fair proportion of positively stained glial nuclei. Neuromelanin-containing neurons of the substantia nigra were negative for DJ-1 IR.

DJ-1 IR in Parkinson's disease

Frontal cortex

The pattern of staining for DJ-1 IR in Parkinson's disease frontal cortex was similar to that observed in control brain tissue. DJ-1 IR was present in glial cells in both white matter and grey matter, with the same distribution pattern as described above (Fig. 2B, D). Glial cell processes were positive for DJ-1 and staining was also seen in their nuclei and cytoplasm. DJ-1 and GFAP were found to colocalize in a number of astrocytes, as seen by double- immunofluorescence labelling (Fig. 3A–C). The majority of neuronal perikarya remained negative for DJ-1 IR but occasional neurons in the deep layers were DJ-1-positive. We did not observe any DJ-1-positive cortical LBs or LNs. A similar pattern of staining was seen in brains of Parkinson's disease subjects with *DJ-1* R98Q polymorphisms.

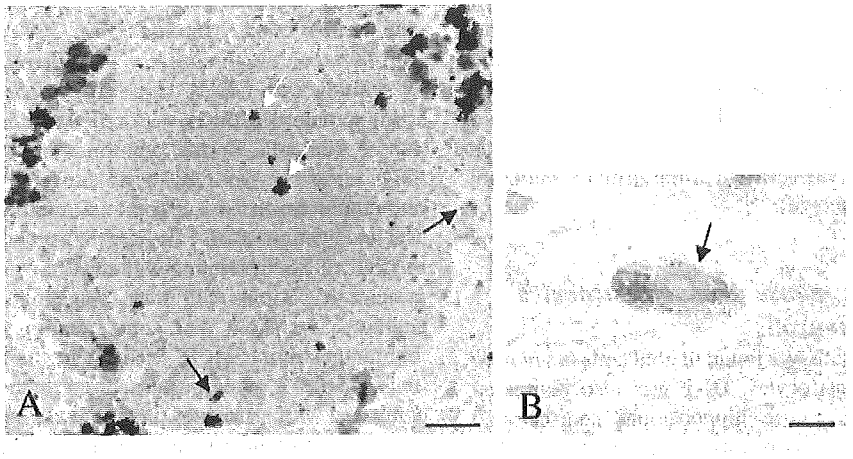

Fig. 4 Electron microscopic (**A**) and immunohistochemical (**B**) localization of DJ-1 in LBs from a case of idiopathic Parkinson's disease. Note that in (**A**), immunogold particles (20 nm) are sparsely scattered throughout the LBs. Some moderate to heavy clustering of gold particles is seen within circular structures (white arrows) in the core of the LB. Some gold conjugates are localized to structures resembling mitochondria at the periphery of the LB (black arrows). Scale bar = 2 nm. (**B**) Using immunohistochemistry, rare LB showed only light immunostaining for DJ-1 at the periphery (arrow) Scale bar = 10 μm.

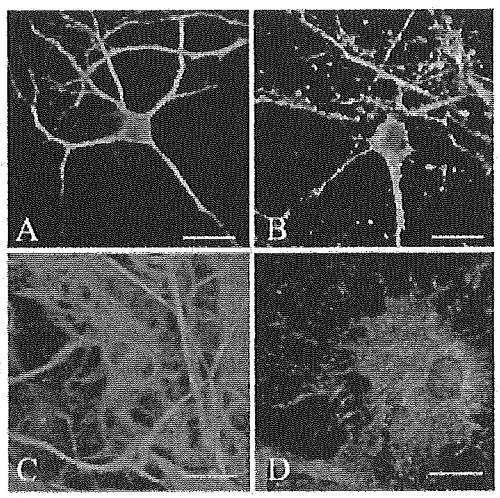

Fig. 5 Expression of DJ-1 in mouse primary neurons and astrocytes *in vitro* and comparison with mitochondrial staining. Cultures enriched in neurons (**A**, **B**) and glia (**C**, **D**) were stained for MAP2 (**A**) or GFAP (**C**) to demonstrate the distinct neuronal and glial morphologies respectively. DJ-1 was expressed in both cell types (green staining in **B** and **D**) but showed minimal overlap with mitotracker (red). Scale bar = 20 μm.

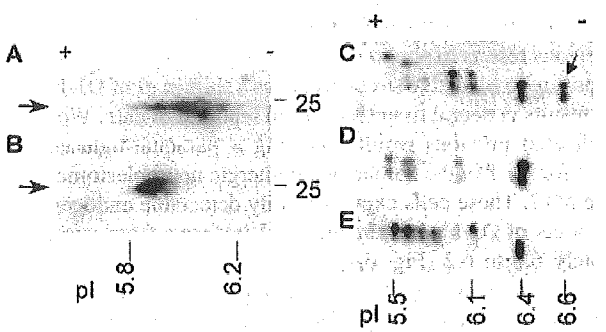

Fig. 6 Multiple isoforms of DJ-1 in neural tissue. (**A**, **B**) pI shift of endogenous DJ-1 in a human cell line under conditions of oxidative stress. Blots of 2D gels from (**A**) untreated or (**B**) paraquat-treated M17 cells using monoclonal antibody to human DJ-1. The arrow indicates endogenously expressed human DJ-1 (~20 kDa) isoforms, with more acidic isoforms towards the left of the blot (+). Molecular weight markers on the right of each blot are in kDa. The markers below the blot indicate approximate pI values of the different species of DJ-1. (**C**–**E**). 2D gel electrophoresis of human frontal cortex extracts. Representative blots of 2DGE from control (**C**, $n = 3$), Parkinson's disease (**D**, $n = 3$) and Parkinson's disease tissue with DJ-1 R98Q polymorphisms (**E**, $n = 2$). Multiple pI isoforms were detected for DJ-1 in all the samples. The arrow indicates the 6.6 isoform, which appears to be absent or diminished in Parkinson's disease and Parkinson's disease brains with DJ-1 R98Q polymorphisms (**D**, **E**) All the spots are ~20 kDa, corresponding to native DJ-1.

Midbrain

In Parkinson's disease nigra, in addition to glial staining, DJ-1 IR was observed only in occasional LBs and a single LN. However the majority of LBs and pale bodies were negative. In the few LBs that were stained, DJ-1 was mainly localized to the periphery (Fig. 4B). The cytoplasm of DA neurons in Parkinson's disease brains was lightly labelled and staining was also seen in neurons of the third nerve nucleus. Some non-melanized neurons and their processes were also lightly labelled (Fig. 2F). In immunoelectron microscope studies, some scattered immunogold labelling of DJ-1 was observed within a small number of nigral LBs. In some, immunogold

particles were found to be concentrated around circular structures (Fig. 4A) In addition, some conjugated gold particles were observed on structures resembling mitochondria around the periphery of the LB (Fig. 4A). No immunogold labelling of the fibrillar material *per se* was detected. No labelling was seen when the primary antibody was omitted (data not shown).

DJ-1 expression in mouse primary astrocytes and hippocampal neurons

Given that DJ-1 expression was found in glial cells *in vivo*, we also cultured cortical astrocytes. DJ-1 was also expressed endogenously in both mouse hippocampal neurons and astrocytes (Fig. 5). Previous results (Bonifati *et al.*, 2003) have shown a possible colocalization of DJ-1, especially the L166P mutant, with mitochondria. However, in both these cultured cell types, endogenous DJ-1 signal (green) did not appear to colocalize with the Mitotracker probe (red) (Fig. 5 B, D).

Multiple isoforms of DJ-1 in oxidative stress models and human brain

Previous results in non-neuronal cell lines have indicated that exposure to oxidative stress produces a shift in pI of DJ-1, but no results in neural tissue have been reported to date. We first replicated previous results showing a paraquat-induced pI shift for DJ-1 in the human dopaminergic neuroblastoma cell line M17. These cells express readily detectable endogenous amounts of DJ-1 with a range of pI isoforms from approximately 6.0 to 6.2 (Fig. 6A). Exposure to 100 μM paraquat resulted in the accumulation of acidic isoforms with a pI of 5.8 (Fig. 6B). We next performed 2DGE of human frontal cortex extracts and found at least six different pI isoforms of DJ-1. In control brain, they ranged from 5.5 to 6.6, the 6.4 pI isoform being the most prominent. Compared with controls (Fig. 6C), in Parkinson's disease samples the 6.6 pI isoform appears to be missing or diminished (Fig. 6D). Additionally, a marked difference in the distribution of the more acidic DJ-1 isoforms was seen in the Parkinson's disease subjects with DJ-1 R98Q polymorphism compared with both control subjects and other Parkinson's disease cases (Fig. 6E).

Discussion

This is the first report on the distribution of DJ-1 protein in the human brain. We have used an antibody for DJ-1 that recognizes a single band corresponding to DJ-1 from human frontal cortex homogenates by western blotting, but not rodent DJ-1 (data not shown). DJ-1 is expressed in high amounts in the frontal cortex, a region of predilection for LB deposition, in control, PSP and Parkinson's disease cases (Fig. 1A). Using this highly specific antibody, we have shown that the major cell type expressing DJ-1 IR in human brain is glial rather than neuronal (Fig. 2A, B, D) We have corroborated the findings on DJ-1 immunohistochemistry by staining with GFAP (Fig. 2E), which produces a similar staining pattern with respect to the morphology and distribution of glial cells. Further confirmation of the localization of DJ-1 in astrocytes has been shown by double-labelling immunofluorescence and confocal microscopy (Fig. 3A–C). The localization of DJ-1 IR in both the cytoplasm and nucleus of glial cells is in keeping with the known distribution of this protein in various cell lines (Nagakubo *et al.*, 1997). In addition, we show that some neurons are weakly DJ-1 immunopositive (Fig. 2F). However, the relative concentrations of DJ-1 protein in neurons may be much lower compared with astrocytes. Staining was specific, as recombinant DJ-1 protein was able to abolish immunoreactivity in both blotting and staining techniques.

The presence of significant amounts of DJ-1 in glial cells in the brain is of interest as the two other Parkinson's disease genes, α-synuclein and parkin, are predominantly neuronal. However, it has been recently shown that parkin expression in glial cells is up-regulated during unfolded protein stress (Ledesma *et al.*, 2002). We have shown that DJ-1 is expressed by mouse primary astrocytes and neurons (Fig. 5). A difference between *in vivo* and *in vitro* results is that cultured mouse hippocampal neurons did express detectable DJ-1, suggesting either that there are species differences or that the tissue culture environment promotes the expression of this stress-responsive protein. As DJ-1 is responsive to oxidative stress (see below), it is possible that DJ-1 is up-regulated in the culture environment due to exposure to free radicals.

The brain regions chosen for this study are known to be vulnerable to pathological damage in Parkinson's disease, including LB and LN accumulation. In both the typical Parkinson's disease cases and in the cases of Parkinson's disease with DJ-1 R98Q polymorphism examined here, DJ-1 IR was localized to only a few nigral LBs, indicating that DJ-1 protein is not an essential component of LBs and is unlikely to be important in their formation in Parkinson's disease. This result is in contrast to the localization of α-synuclein and parkin, which are present in most LBs and LNs (Spillantini *et al.*, 1998; Schlossmacher *et al.*, 2002).

Our 2D gel analysis of human brain extracts and cells exposed to oxidative stress, shows the existence of a range of pI isoforms for DJ-1 protein. This may be specific to brain tissue as only two pI isoforms for DJ-1 have been reported in human endothelial cell cultures and in mouse lung tissue (Mitsumoto and Nakagawa, 2001). In addition, we show that DJ-1 protein responds to the oxidative stressor paraquat by exhibiting more acidic pI isoforms in a neuroblastoma cell line (Fig. 6A), in agreement with a previous study showing DJ-1 sensitivity to paraquat (Mitsumoto *et al.*, 2001). Oxidative stress factors are believed to be implicated in the pathogenesis of Parkinson's disease (Jenner, 2003) and it has been suggested that the shift in pI of DJ-1 is a useful indicator of oxidative stress status both *in vivo* and *in vitro* (Mitsumoto and Nakagawa, 2001). Thus the presence of multiple isoforms

of DJ-1 in control and Parkinson's disease brains and the tendency of the most alkaline pI isoform to be absent from Parkinson's disease cases (Fig. 6D,E) could point to the involvement of oxidative stress in (Abbas *et al.*, 1999) Parkinson's disease. Whether these factors are the cause of the differences in the DJ-1 pI isoform distribution in the brain between cases of Parkinson's disease and cases with DJ-1 R98Q polymorphism remains to be determined, but it is clear that there is greater complexity in DJ-1 expression in the human brain compared with other systems. Our Parkinson's disease cases heterozygous for DJ-1 mutation showed a similar expression pattern for DJ-1 protein compared with idiopathic Parkinson's disease cases and phenotypically they were similar to late-onset Parkinson's disease cases. It is possible that in these individuals, the DJ-1 R98Q polymorphism is non-pathogenic or that the DJ-1 variant together with other yet unknown variants are responsible for the disease phenotype (Abou-Sleiman *et al.*, 2003). In this respect, the DJ-1 R98Q polymorphism may be similar to parkin mutation, for which a single heterozygous mutation may confer disease susceptibility (Abbas *et al.*, 1999) indistinguishable from idiopathic Parkinson's disease. The effect of the G to A heterozygous DJ-1 mutation on the biological function of the protein, however, remains to be studied.

There is increasing interest in the possibility that glial cells may be major contributors to oxidative stress in Parkinson's disease (Czlonkowska *et al.*, 2002; Teismann *et al.*, 2003). The post-mortem Parkinson's disease brain exhibits some increase in astroglia, as seen by GFAP staining (Forno *et al.*, 1992; Mirza *et al.*, 2000). Furthermore, there is an inverse correlation between numbers of GFAP-positive astrocytes and DA cell loss, and areas with a sparse astrocytic response show greater cell loss (Damier *et al.*, 1993). In addition, histological examination of familial Parkinson's disease cases with parkin mutations has also shown gliosis in the nigra (Ishikawa and Takahashi, 1998; Hayashi *et al.*, 2000; Hishikawa *et al.*, 2001). Astrocytes, however, are known for their protective effects on neurons through their capacity to secrete glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), and may be protective (Czlonkowska *et al.*, 2002). Addition of astrocytes to neuronal cultures prevents cell death caused by a number of neurotoxic compounds (Hou *et al.*, 1997; Tieu *et al.*, 2001; Mena *et al.*, 2002). Furthermore, astrocytes are rich in glutathione peroxidase, which catalyses the removal of hydrogen peroxide by formation of oxidized glutathione and prevents the formation of hydroxyl radicals by a reaction between hydrogen peroxide and heavy metals. The levels of this important enzyme have been demonstrated to be reduced in homogenates of substantia nigra in Parkinson's disease (Sofic *et al.*, 1992; Sian *et al.*, 1994; Pearce *et al.*, 1997). It is possible that some of this reduction in glutathione peroxidase takes place in nigral glia. Any change in the normal physiological role of astrocytes may therefore contribute to DA cell death by reduced secretion of neurotrophic factors and a reduced capacity of glia to cope with free radical production. The abundance of DJ-1 in astroglia suggests a prominent role for this protein in glial biology and in some neurodegenerative processes, and may also indicate that astrocytes are subjected to oxidative stress in Parkinson's disease.

Present concepts of disease pathogenesis suggest a unifying pathway involving α-synuclein, parkin and UCH-L1, the three genes associated with familial forms of Parkinson's disease, within the ubiquitin–proteasome system (UPS) (Cookson, 2003; Hardy *et al.*, 2003). In this context, reduced proteasome function in Parkinson's disease cases, indicating a defect in the UPS, has been reported (McNaught and Jenner, 2001). How this relates to oxidative stress is unclear, but there is some evidence that the proteasome has a major role in the degradation of oxidatively damaged proteins (Grune *et al.*, 2003; Shringarpure *et al.*, 2003). Furthermore, oxidative stress can inhibit proteasome function (Ding and Keller, 2001) and formation of intracellular protein aggregates is dependent on oxidative events (Demasi and Davies, 2003). Therefore, the pathways involving proteasome function and oxidative stress may intersect, as has been highlighted by others (Chung *et al.*, 2001; Jenner, 2003). It is not yet clear whether DJ-1 will be part of this pathway or whether it will involve new pathways contributing to a common end-point (Cookson, 2003). Interestingly, mutant DJ-1, but not the wild-type protein, is degraded by the UPS in cell culture (Miller *et al.*, 2003). Further work on the cell biology of DJ-1 is needed to establish its precise role in the loss of DA neurons. However, our study provides further data to suggest that glia may be important in the pathogenesis of Parkinson's disease and that interactions between neuronal and glial function should be investigated in greater depth.

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