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DJ-1 has a role in antioxidative stress to prevent cell death

Takahiro Taira[1,3], Yoshiro Saito[2†], Takeshi Niki[3], Sanae M.M. Iguchi-Ariga[3,4], Kazuhiko Takahashi[2] & Hiroyoshi Ariga[1,3+]

[1]Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan, [2]Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan, [3]CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama, Japan, and [4]College of Medical Technology, Hokkaido University, Sapporo, Japan

Deletion and point (L166P) mutations of DJ-1 have recently been shown to be responsible for the onset of familial Parkinson's disease (PD, PARK7). The aim of this study was to determine the role of DJ-1 in PD. We first found that DJ-1 eliminated hydrogen peroxide *in vitro* by oxidizing itself. We then found that DJ-1 knockdown by short interfering RNA rendered SH-SY5Y neuroblastoma cells susceptible to hydrogen peroxide-, MPP+- or 6-hydroxydopamine-induced cell death and that cells harbouring mutant forms of DJ-1, including L166P, became susceptible to death in parallel with the loss of oxidized forms of DJ-1. These results clearly showed that DJ-1 has a role in the antioxidative stress reaction and that mutations of DJ-1 lead to cell death, which is observed in PD.

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INTRODUCTION

Parkinson's disease (PD) involves an irreversible degeneration of the dopaminergic nigrostriatal pathway. Genes responsible for rare familial early-onset PD, including α-synuclein (Polymeropoulos *et al*, 1997), Parkin (Kitada *et al*, 1998) and UCH-L1 (Leroy *et al*, 1998), have been identified, and they are thought to have a role in ubiquitin–proteasome dysfunction in PD. Various lines of evidence also suggest that oxidative stresses contribute to the cascade leading to dopaminergic cell degeneration in PD, but the mechanisms responsible for nigral dopaminergic cell death are not clear (Nicklas *et al*, 1987; Heikkila & Cohen, 1971; Lotharius & O'Malley, 2000; for a recent review, see Jenner, 2003). DJ-1 was identified by us as an oncogene product (Nagakubo *et al*, 1997). It was later found to be a positive regulator of the androgen receptor (Takahashi *et al*, 2001; Niki *et al*, 2003) and to be related to infertility (Wagenfeld *et al*, 1998, 2000; Welch *et al*, 1998; Klinefelter *et al*, 2002; Okada *et al*, 2002). Deletion and point (L166P) mutations of DJ-1 have recently been shown to be responsible for the onset of familial PD, PARK7 (Bonifati *et al*, 2003), and expression of DJ-1 was induced by oxidative stresses (Mitsumoto & Nakagawa, 2001; Mitsumoto *et al*, 2001; Srisomsap *et al*, 2002). The mechanisms underlying the onset of PD due to DJ-1 mutations, however, have not been clarified. In this study, we analysed the effects of DJ-1 on hydrogen-peroxide-induced cell death. We found that DJ-1 has a role in the antioxidative stress reaction and that mutations of DJ-1 lead to cell death.

[1]Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812, Japan
[2]Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812, Japan
[†]Present address: Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology, Osaka, Japan
[3]CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan
[4]College of Medical Technology, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812, Japan
[+]Corresponding author. Tel: +81 11 706 3745; Fax: +81 11 706 4988; E-mail: hiro@pharm.hokudai.ac.jp

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RESULTS AND DISCUSSION

Elimination of hydrogen peroxide by DJ-1

Expression of DJ-1 was induced in human ECV304 endothelial cells, mouse primary macrophages and human RD4 fibroblast cells that had been subjected to treatment with paraquat, endotoxin and iron, respectively, which yielded reactive oxygen species (Mitsumoto & Nakagawa, 2001; Mitsumoto *et al*, 2001; Srisomsap *et al*, 2002). To examine statistically the expression of DJ-1 in neuroblastoma cells, human neuroblastoma SH-SY5Y cells were treated with various concentrations of hydrogen peroxide for 12 h, and proteins prepared from cells were separated on SDS-containing polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing gel over a pH range of 5–8 (Fig 1A). After blotting with an anti-DJ-1 antibody, the amounts of actin were not changed but DJ-1 was found to be induced by hydrogen peroxide at doses up to 25 μM in a dose-dependent manner, and the pI of DJ-1 was shifted to a more acidic point, from 6.7 to 5.7 (Fig 1A, SDS–PAGE and IEF, respectively). These phenomena were also observed in other cell lines, including human HeLa, monkey Cos1 and mouse NIH3T3 cells (data not shown, see later). Recombinant DJ-1 was expressed in and purified from *Escherichia coli* to near-homogeneity (Fig 1B, left panel). Recombinant DJ-1 was then

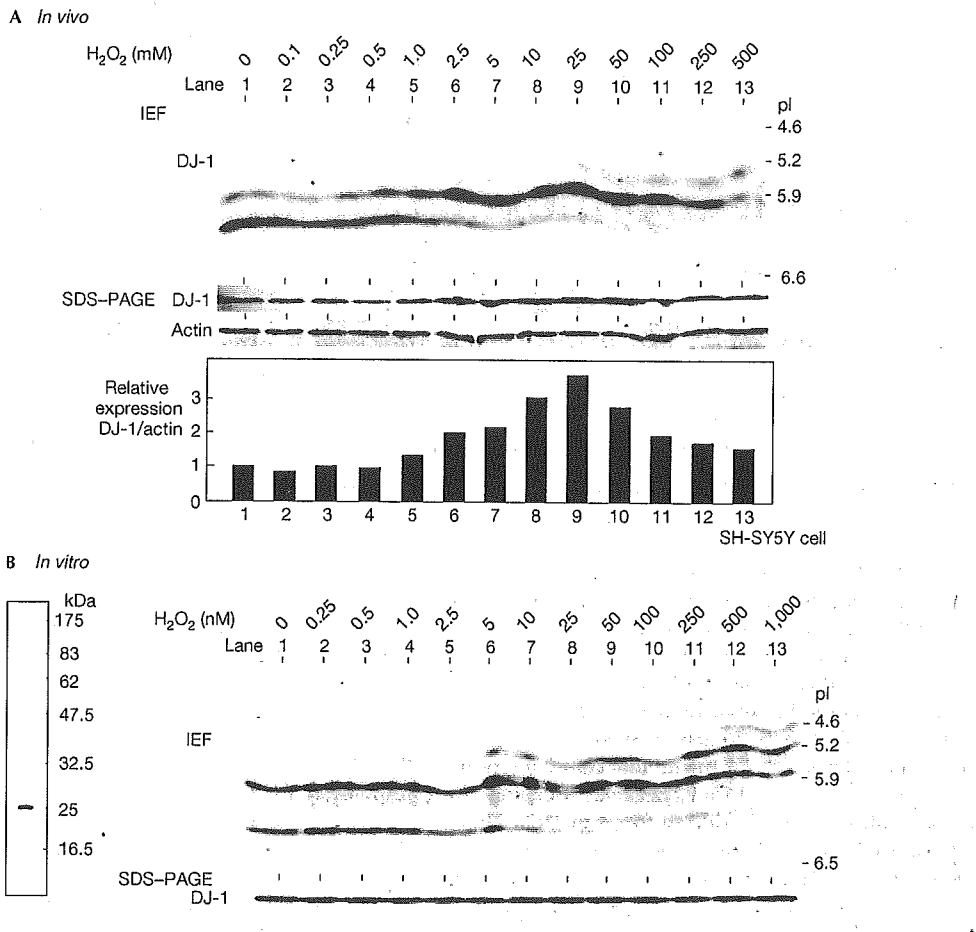

Fig 1 | pI shift of DJ-1 after its treatment with hydrogen peroxide. (A) SH-SY5Y cells were treated with various concentrations of hydrogen peroxide for 12 h, and proteins in the extracts were then analysed by isoelectric focusing phoresis gel (IEF, upper panel) or PAGE containing SDS (SDS–PAGE in the lower panel) as described in Methods. Intensities of the bands of DJ-1 and actin were measured and are shown as 'relative expression'. (B) In all, 1 μg of recombinant DJ-1 was reacted with various concentrations of hydrogen peroxide for 30 min at room temperature and subjected to isoelectric focusing Diet phoresis and SDS–PAGE electrophoresis as described in A (right panel). The recombinant DJ-1 used in this experiment was separated on PAGE containing SDS and stained with Coomassie brilliant blue R-250 (left panel).

reacted with various concentrations of hydrogen peroxide for 30 min, and its pI changes were examined (Fig 1B). Although the total amount of DJ-1 was not affected by hydrogen peroxide, a pI shift in DJ-1 to a more acidic point was observed. However, lower concentrations of hydrogen peroxide were found to be needed in *in vitro* reactions than in *in vivo* culture cells, suggesting that DJ-1 is directly affected by hydrogen peroxide.

To examine the effect of DJ-1 on hydrogen peroxide, recombinant DJ-1 was reacted with hydrogen peroxide at concentrations five times greater than those of DJ-1 for 1 h at 30 °C, and the concentrations of hydrogen peroxide were measured by the scopoletin method (Fig 2A). Although the control bovine serum albumin (BSA) was hardly affected, DJ-1 eliminated approximately 80% of hydrogen peroxide. Elimination of hydrogen peroxide by DJ-1 occurred in a time-dependent manner for up to 30 min (data not shown). We and others have analysed the structure of DJ-1 by X-ray crystallography and found that DJ-1 is present as a dimer (Honbou *et al*, 2003; Huai *et al*, 2003; Lee *et al*, 2003; Tao & Tong, 2003; Wilson *et al*, 2003). Based on this finding, two point-mutants of DJ-1, V51A and C53A, that disrupt the dimer formation of DJ-1 were constructed and tested for their ability to eliminate hydrogen peroxide *in vitro* (Fig 2B). Whereas wild-type DJ-1 eliminated hydrogen peroxide in a dose-dependent manner, the activity levels of the two mutants were only about 20% that of wild-type DJ-1, suggesting that the active form of DJ-1 is a dimer. Members of the family of peroxiredoxin proteins, such as thioredoxin peroxidase, eliminate hydrogen peroxide by the use of electrons provided by thioredoxin, and the resultant oxidized thioredoxin peroxidase is then reduced back to the active form (Jacquot *et al*, 2002; Wood *et al*, 2003). To determine whether DJ-1 is a peroxiredoxin protein, oxidized DJ-1 was reacted with thioredoxin or glutathione and its pI shift was measured. The results showed, however, that the pI of DJ-1 was not changed by these chemicals (data not shown), suggesting that DJ-1 is a new

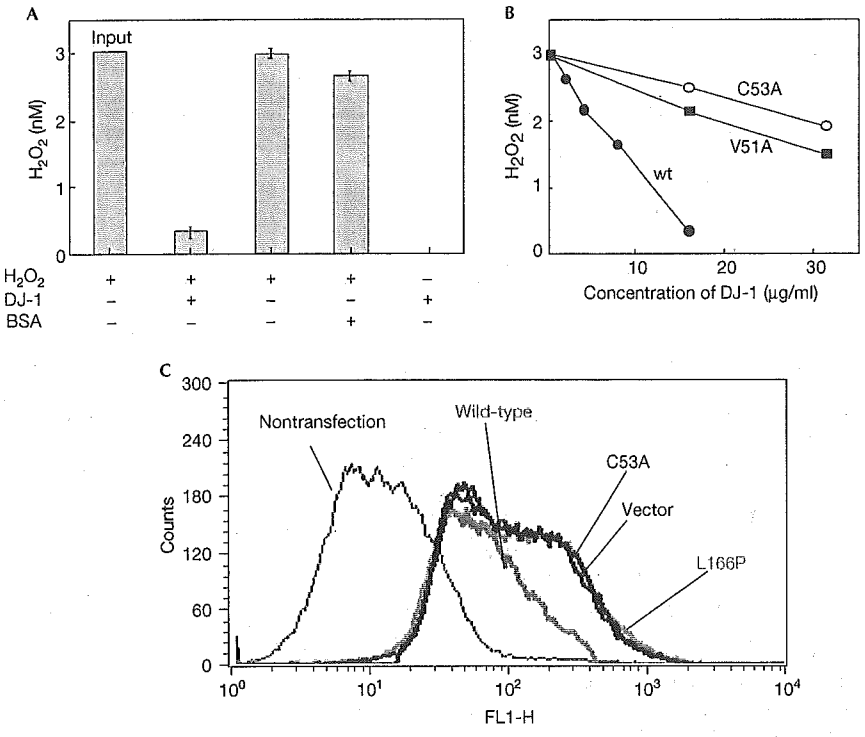

Fig 2 | Elimination of hydrogen peroxide by DJ-1. (A) In all, 3 nM of hydrogen peroxide was reacted with 0.5 nM of DJ-1 for 1 h at 30 °C, and the concentration of hydrogen peroxide was measured as described in Methods. (B) In all, 3 nM of hydrogen peroxide was reacted with various amounts of wild-type, V51A and C53A DJ-1 for 1 h at 30 °C, and the concentrations of hydrogen peroxide were measured as described in Methods. (C) SHSY-5Y cells in 10-cm dishes were transfected with 5 μg each of plasmids used for the establishment of cell lines by the lipofectamine method. At 36 h after transfection, cells were treated with 10 μM hydrogen peroxide for 60 min and then with 5 μM DCFH-DA for 30 min, and analysed by flow cytometry. BSA, bovine serum albumin.

type of scavenger protein acting on hydrogen peroxide. To examine the elimination of hydrogen peroxide by DJ-1 *in vivo*, SH-SY5Y cells were transfected with wild-type and mutant forms of DJ-1. At 36 h after transfection, 10 μM hydrogen peroxide was added to the cells over a period of 60 min, and the cells were then reacted with 2′,7′-dichlorodihydrofluoroscein diacetate (DCFH-DA) for 30 min and analysed by flow cytometry (Fig 2C). Cells containing hydrogen peroxide have high fluorescence intensity and the peak is shifted to the left (compare cells that were not treated with cells transfected with a vector in Fig 2C). Whereas fluorescence-intensities of cells transfected with the two point-mutants V51A and C53A and another mutant of DJ-1, L166P, a mutant with a leucine-to-proline substitution found in a PARK7 patient (Bonifati *et al*, 2003), were shifted to the position of cells transfected with a control vector, that of cells transfected with wild-type DJ-1 was shifted back (Fig 2C), indicating that elimination of hydrogen peroxide by DJ-1 occurs in cultured cells.

Abrogation of hydrogen-peroxide-induced cell death by DJ-1

Reactive oxygen species such as hydrogen peroxide induce cell death, and these effects are related to the onset of PD (Heikkila & Cohen, 1971; Nicklas *et al*, 1987; Lotharius & O'Malley, 2000; for a recent review, see Jenner, 2003). To determine the roles of DJ-1 in cells treated with hydrogen peroxide, siRNA against DJ-1 was transfected into SH-SY5Y cells to knock down the expression of endogenous DJ-1, and viabilities of cells in the presence of various doses of hydrogen peroxide were measured 36 h after transfection (Fig 3). The endogenous levels of DJ-1 and actin in SH-SY5Y cells transfected with wild-type or mutated siRNAs against DJ-1 or against GAPDH, a negative control, were first measured by western blotting with anti-DJ-1 and anti-actin antibodies, and the level of DJ-1 was normalized to that of actin (Fig 3A). Although the mutated siRNAs against DJ-1 or siRNA against GAPDH did not affect the amount of DJ-1, the amount of DJ-1 was reduced to 30% of that without siRNA. As the efficiency of transfection of siRNA into SHSY-5Y cells was about 80%, the expression of DJ-1 in cells containing siRNA against DJ-1 was almost knocked down. Under these conditions, the death of SH-SY5Y cells transfected with siRNA against DJ-1 was accelerated compared to that of nontransfected SH-SY5Y cells and that of cells transfected with mutated siRNAs against DJ-1 or siRNA against GAPDH; concentrations of hydrogen peroxide that gave ID_{50} in cells transfected with siRNA against DJ-1 and with mutated siRNA against DJ-1 were 70 ± 10 and 230 ± 8 μM, respectively (Fig 3B). The same activity of DJ-1 against cell death was also observed in mouse NIH3T3 cells (data not shown). The effect of DJ-1 on the viability of cells in the presence of dopaminergic neurotoxins, 1-methyl-4-phenylpyridium (MPP+) and 6-hydroxydopamine (6-OHDA),

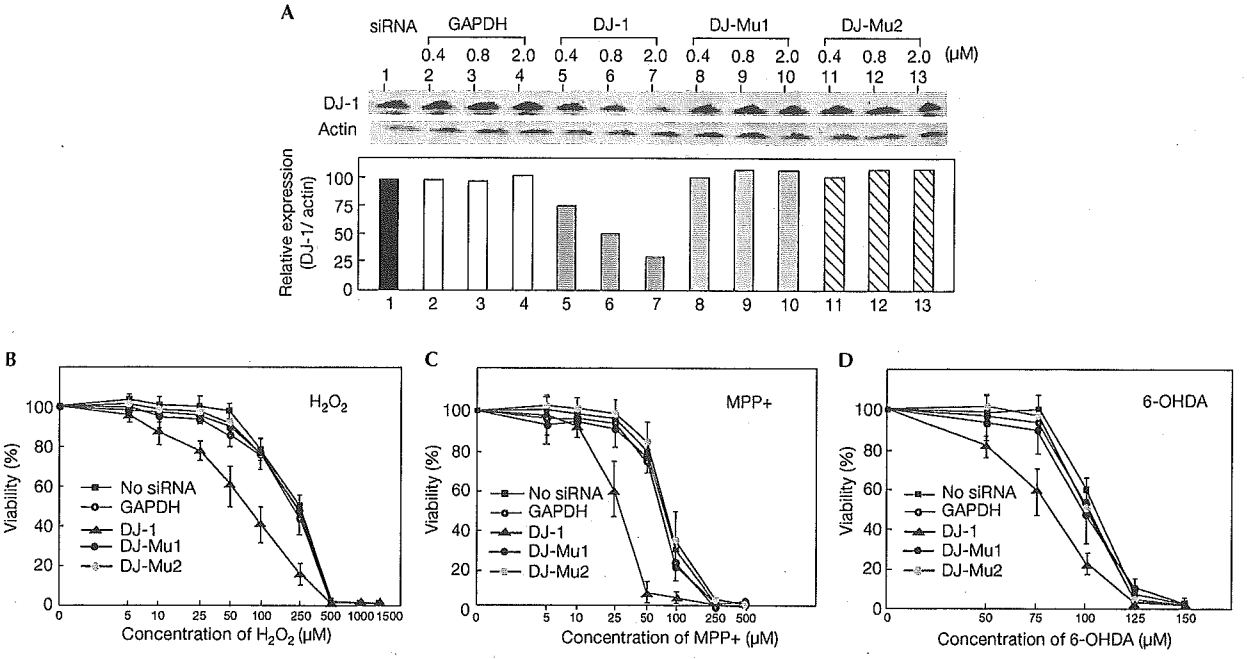

Fig 3 | Acceleration of hydrogen-peroxide-induced cell death in DJ-1-knockdown cells. (A) SH-SY5Y cells were transfected with siRNAs targeting GAPDH and DJ-1. At 36 h after transfection, the proteins in the extracts were blotted with an anti-DJ-1 antibody or anti-actin antibody as described in Methods. The intensities of the bands in western blotting shown in A (upper panel) were measured by an Odyssey system, and the intensity of DJ-1 was normalized to that of actin (lower panel). SH-SY5Y cells were transfected with 2 μM of siRNAs targeting GAPDH and DJ-1. At 36 h after transfection, cells were reacted with various concentrations of hydrogen peroxide (B), MPP+ (C) or 6-OHDA (D) for 12 h, and their viabilities were measured by an MTT assay.

both of which induce oxidative stress in cells (Heikkila & Cohen, 1971; Nicklas *et al*, 1987; Lotharius & O'Malley, 2000), was also examined, and results similar to those for cells treated with hydrogen peroxide were obtained (Fig 3C,D, respectively). These results clearly indicate that DJ-1 has a role in oxidative-stress-induced cell death.

The role of DJ-1 in hydrogen-peroxide-induced cell death was further confirmed by experiments using established NIH3T3 cell lines harbouring exogenously added Flag-tagged wild type or various mutants of DJ-1. The Flp-In system (InVitrogen) was used to establish these cell lines: CMV promoter-based expression vectors surrounded by Flp sequences at both the 5′ and 3′ ends were inserted into a site containing the FRT sequence of a chromosome in mouse NIH3T3 cells by Flp recombinase that had been supplied by co-transfection of its expression vector. All the wild-type DJ-1 and mutant DJ-1 proteins were thereby expressed on the same backgrounds in cells, and these were confirmed by western blotting with anti-Flag and anti-actin antibodies (Fig 4A). Two other mutants of DJ-1 were used in addition to the C53A and L166P mutants: C106S, a mutant with a cysteine-to-serine substitution corresponding to a catalytic site for protease activity, as the X-ray crystal structure suggests that DJ-1 has protease activity (Honbou *et al*, 2003); and K130R, a mutant with a lysine-to-arginine substitution corresponding to a major sumoylation site. When pI shifts of wild-type DJ-1 and mutants of DJ-1 were measured after the addition of two doses of hydrogen peroxide to these cell lines, only small changes to acidic points were observed in all the mutants of DJ-1 compared with that of wild-type DJ-1 (Fig 4B). Viabilities of these cell lines in the presence of hydrogen peroxide were then determined (Fig 4C). Contrary to the case of DJ-1-knockdown cells, cells harbouring exogenously added wild-type DJ-1 were much more resistant to hydrogen peroxide than parental nontransfected cells. Conversely, of the cell lines harbouring mutants, the cell line harbouring L166P was found to be most sensitive to hydrogen peroxide, and the C53A, C106S and K130R lines followed in terms of sensitivity to hydrogen peroxide. To examine the effect of DJ-1 statistically on the viability of cells in the presence of 400 μM hydrogen peroxide, a time-course experiment was carried out using these cell lines (Fig 4D,E). Although pI shifts of wild-type DJ-1 began at 0.5 h after the addition of hydrogen peroxide and continued up to 12 h, only small changes to acidic points were observed in all the mutants of DJ-1 at this concentration of hydrogen peroxide (Fig 4D). Furthermore, all the cell lines, except for the cell line harbouring wild-type DJ-1, began to die 30 min after hydrogen peroxide treatment and continued to die up to 6 h after (Fig 4E). The cell line harbouring L166P was again most sensitive to hydrogen peroxide, and the cell line harbouring wild-type DJ-1 was resistant to hydrogen-peroxide-induced cell death at a concentration of 400 μM. About 50% of parental cells had died at 6 h after hydrogen peroxide treatment. As the half-life of DJ-1 was estimated to be about 6 h (Macedo *et al*, 2003; Miller *et al*, 2003), a reduced amount of DJ-1 in cells may affect cell susceptibility to hydrogen-peroxide-induced cell death. The possibility that different susceptibilities of cell lines to hydrogen-peroxide-induced cell death were due to different activities of

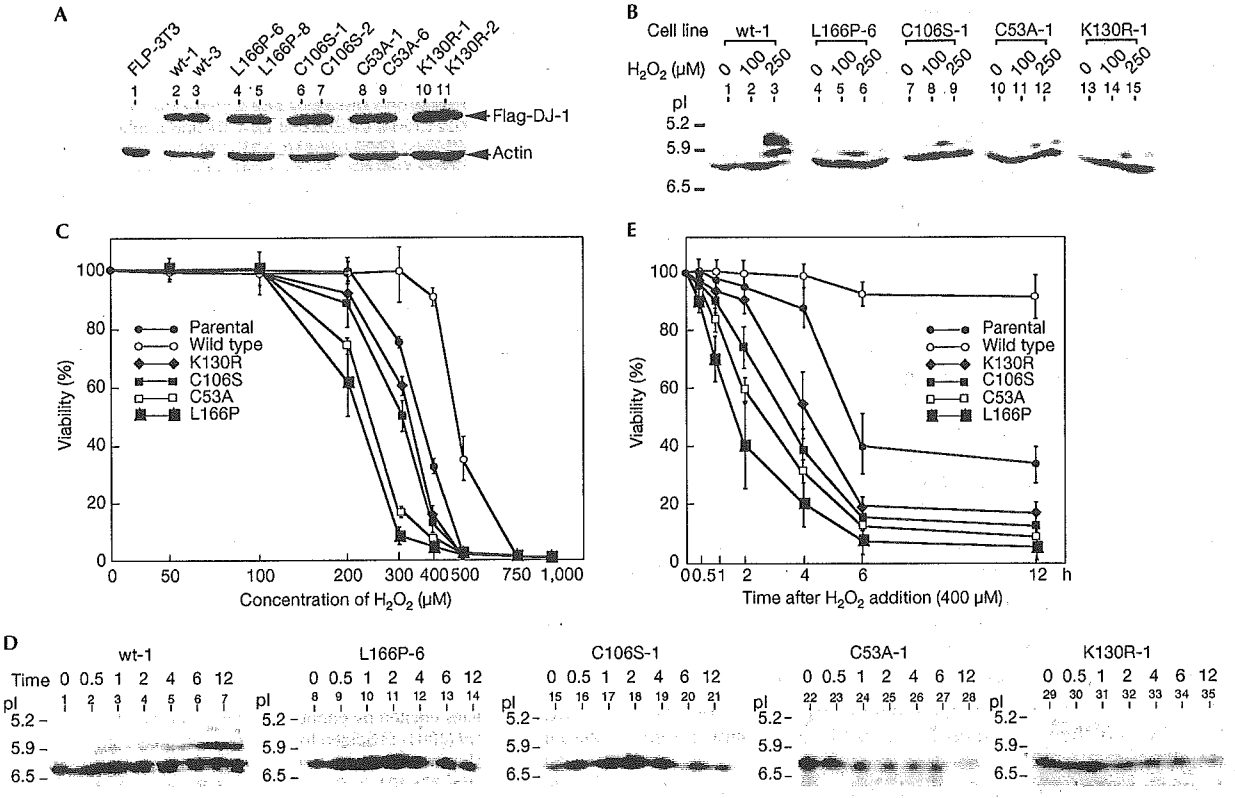

Fig 4 | Acceleration of hydrogen-peroxide-induced cell death in cells harbouring various mutants of DJ-1, including L166P, which is observed in PARK7 patients. (A) Expression levels of Flag-DJ-1 and actin in NIH3T3 cell lines harbouring Flag-tagged wild-type DJ-1 and various mutants of DJ-1 were examined by western blotting. Two different cell lines were used for each construct. (B) NIH3T3 cell lines were reacted with 100 and 250 μM of hydrogen peroxide for 12 h. Proteins in cells were then subjected to isoelectric focusing phoresis and blotted with an anti-DJ-1 antibody. (C) NIH3T3 cell lines were reacted with various concentrations of hydrogen peroxide for 24 h and their viabilities were measured by an MTT assay. (D) NIH3T3 cell lines were reacted with 400 μM of hydrogen peroxide. Proteins in cells at various times after treatment with hydrogen peroxide were then subjected to isoelectric focusing phoresis and blotted with an anti-DJ-1 antibody. (E) NIH3T3 cell lines were reacted with 400 μM hydrogen peroxide, and cell viabilities were measured by an MTT assay at various times after treatment with hydrogen peroxide.

antioxidant enzymes, and not to DJ-1, was ruled out by measuring the enzyme activities of superoxide dismutase, glutathione peroxidase and catalase in the cell lines that had been treated with hydrogen peroxide (see supplementary information online). The results showed that the enzyme activities of all the cell lines were similarly induced by treatment with hydrogen peroxide. These results clearly indicate that DJ-1 has a role in oxidative stress and that mutations of DJ-1 lead to oxidative-stress-induced cell death.

DJ-1 is involved in transcriptional regulation, tumorigenesis, fertilization and early onset of PD (PARK7), and its expression is induced by oxidative stresses. The mechanisms underlying these phenomena, however, have not been clarified. In this study, we found that DJ-1 eliminated hydrogen-peroxide by oxidizing itself and that this activity was a prerequisite for protection of cells against hydrogen-peroxide-induced cell death, as was demonstrated by an experiment using DJ-1-knockdown cells. We also found that mutations of DJ-1 abrogated this activity, leading to hypersusceptibility to cell death. The X-ray crystal structure revealed that DJ-1 functions as a dimer and possesses protease activity. The finding that cell lines containing mutants of DJ-1 became hypersusceptible to cell death is explained as follows. There are heterodimers formed by endogenously present wild-type DJ-1 in NIH3T3 cells and mutant DJ-1 and there are homodimers formed by mutants such as L166P and K130R, which still possess the β-helix that is necessary for dimerization. The antioxidative stress activities of these hetero- and homodimers are lost or weakened. Proper localization in cells is also thought to be necessary for DJ-1 to exert its functions, because L166P is localized in the mitochondria (Bonifati *et al*, 2003), whereas wild-type DJ-1 is localized in the cytoplasm and nucleus. Localization of proteins is determined by their interaction with other proteins, modification of proteins such as sumoylation or proper conformation of proteins, and these situations may also be true for DJ-1.

METHODS

Isoelectric focusing and western blotting. SH-SY5Y and NIH3T3 cells, as well as NIH3T3 cells harbouring wild-type or mutants of DJ-1, were cultured in Dulbecco's modified Eagle's medium

supplemented with 10% calf serum. The cells were then treated with various concentrations of hydrogen peroxide for 12 or 24 h, and cell extracts were prepared in a mixture containing 2% NP-40 and phosphate-buffered saline (PBS). Proteins in the extracts were then separated in pH 5–8 ranges of isoelectric focusing phoresis gel or 12.5% PAGE containing SDS, transferred onto nitrocellulose membranes, and blotted with an anti-DJ-1 polyclonal antibody or anti-actin antibody (MAB1501R, Chemicon). The proteins that reacted with primary antibodies were visualized with IRDye800-conjugated or Alexa Fluor680-conjugated secondary antibodies using an infrared imaging system (Odyssey, LI-COR). When recombinant DJ-1 was used in the experiment, GST-free DJ-1 was prepared as described previously.

Establishment of cell lines harbouring wild-type DJ-1 or mutants of DJ-1. *Kpn*I–*Xho*I fragments containing the CMV promoter and Flag-tagged wild-type or mutant DJ-1s were inserted into *Kpn*I–*Xho*I sites of pcDNA5/FRT. These plasmids were cotransfected with pOG44, an expression vector for Flp recombinase, into Flp-In™3T3 cells (InVitrogen) by the calcium phosphate precipitation method, and the cells were cultured in the medium in the presence of 100 μg/ml zeocin and 100 μg/ml hygromycin for 14 days. The cells that were resistant to both drugs were then selected, and expression of Flag-DJ-1 was examined by western blotting with an anti-Flag antibody (M2, Sigma).

Cell viability assay. Cells were cultured in a 96-well plate and treated with various amounts of hydrogen peroxide for 12 or 24 h. Cell viability was then measured by an MTT assay using a cell counting kit 8 (DOJINDO).

Knockdown of DJ-1 in cells. The nucleotide sequences for wild-type and mutated siRNA (DJ-1, DJ-Mu1 and DJ-Mu2) targeting DJ-1 were as follows: DJ-1: 5′-UGGAGACGGUCAUCCCUGUdTdT-3′ (upper strand) and 3′-dTdTACCUCUGCCAGUAGGGACA-5′ (lower strand); DJ-Mu1: 5′-UCCAGACGGUCAUCCCUGUdTdT-3′ (upper strand) and 3′-dTdTAGGUCUGCCAGUAGGGACA-5′ (lower strand); DJ-M2: 5′-UGGAGACGGAGAUCCCUGUdTdT-3′ (upper strand) and 3′-dTdTACCUCUGCCUCUAGGGACA-5′ (lower strand). The siRNA targeting GAPDH was purchased from Greiner (Japan). Various amounts of siRNA were transfected into SH-SY5Y cells using Oligofectamine reagent (InVitrogen) according to the supplier's manual.

Measurement of hydrogen peroxide. Various amounts of hydrogen peroxide were reacted in 200 μl mixtures with recombinant DJ-1 for 1 h at 30 °C, and 1 ml of PBS, 400 μl of 10 μM scopoletin and 400 μl of 32.5 U/ml horseradish peroxidase were then added to the mixture. After 5 min at 25 °C, fluorescence at Ex 366 nm and Em 460 nm was measured.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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Co-localization with DJ-1 Is Essential for the Androgen Receptor to Exert Its Transcription Activity that Has Been Impaired by Androgen Antagonists

Takahiro TAIRA,[a,c] Sanae Marta Margarita IGUCHI-ARIGA,[b,c] and Hiroyoshi ARIGA*[,a,c]

[a] *Graduate School of Pharmaceutical Sciences, Hokkaido University;* [b] *College of Medical Technology, Hokkaido University; Kita-ku, Sapporo 060–0812, Japan: and* [c] *CREST, Japan Science and Technology Corporation; 4–1–8 Honcho, Kawaguchi, Saitama 332–0012, Japan.* Received December 24, 2003; accepted January 12, 2004

DJ-1 was first identified as a novel candidate of an oncogene product in cooperation with an activated *ras*, and DJ-1 was later found to be a positive regulator of the androgen receptor (AR) transcription activity that was repressed by PIASxα. DJ-1 was also found to be an infertility-related protein that was reduced in rat sperm treated with sperm toxicants that cause infertility in rats. To determine the roles of DJ-1 in the AR function, the effects of several androgen antagonists, some of which had been identified as endocrine-disrupting chemicals, on AR transcription activity and localization of AR and DJ-1 in Cos7 cells were examined. Co-localization of DJ-1 with the AR as dot-like spots in the nucleus was first found in cells that had not been treated with chemicals. Although all of the chemicals tested inhibited AR transcription activity to an average of 25% of that without chemicals, there were two classes affecting the localization of the two proteins; one changes the AR from dot-like spots to diffuse spaces in the nucleus and the other still keeps the AR in the dot-like spots. The localization of DJ-1, on the other hand, was found to be dramatically changed by all of the chemicals, resulting in loss of co-localization with the AR. These results indicate that DJ-1 is an essential factor for the AR to exert its full activity.

Key words DJ-1; androgen receptor; endocrine disrupter

DJ-1 was identified as an oncogene product that transforms mouse NIH3T3 cells in cooperation with activated *ras*.[1] The human DJ-1 gene is mapped to 1p36.2—36.3, where many chromosome aberrations in cancer have been reported.[2] DJ-1 was found to be more strongly expressed in the testis than in other tissues and also in sperm, suggesting that DJ-1 has at least two functions, one function in somatic cells, especially in germ cells, and one function in sperm.

With regard to the function of DJ-1 in somatic cells, we have identified PIAS (Protein Inhibitor of Activated Stat)xα/ARIP3 (androgen receptor-interacting protein 3) and DJBP as DJ-1-binding proteins.[3,4] PIASxα, a member of the PIAS family of proteins, was characterized at first as a testis-specific androgen receptor coregulator.[5,6] We have shown that PIASxα inhibits the transcription activity of the androgen receptor (AR) by binding to the DNA-binding domain of AR and that DJ-1 antagonizes this inhibition by sequestering PIASXα from the AR in CV-1, Cos1 and TM4 sertoli cells, indicating that DJ-1 is a positive regulator of the AR.[3] DJBP, a novel DJ-1-binding protein, recruited histone deacetylase complex to the AR to repress its transcription activity and DJ-1 restored its repressed activity by absorbing DJBP.[4]

The AR is a member of the nuclear receptor superfamily and plays a role as a ligand-dependent transcription factor. After a ligand binds to the AR, the AR is translocated into the nucleus and binds to the androgen-responsive element, ARE, on the androgen-activating gene that affects development, growth and regulation of male reproductive functions.[7—10] Transcriptional activity of the AR is known to be regulated by various coregulators that bind to the respective domain of AR. More than 30 proteins of such coregulators have been reported,[11] (see homepage at http://ww2.mcgill.ca/androgendb). The AR was located in the nucleus as a dot-like spot, in which coactivators, including TIF2/GRIP, SRC1 and CBP, were colocalized,[12,13] and that this localization of the AR was abrogated to diffuse spaces in the nucleus by chemicals, including OHF, vinclozolin, p,p′-DDE and nitrofen, thereby leading to the repression of the AR transcription activity.[14] These results suggest that the distinct localization of the AR with proper coactivators is essential for exertion of its full transcription activity.

To further characterize the function of DJ-1 in the AR transcription machinery, the transcription activity and localization of the AR and DJ-1 were examined after the cells had been treated with chemicals that rendered AR transcription activity. The results showed that co-localization of DJ-1 with the AR was drastically hampered by the chemicals, irrespective of the proper or improper localization of the AR, suggesting that DJ-1 is a factor determining the AR transcription activity in cells.

MATERIALS AND METHODS

Chemicals Vinclozolin, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (p,p′-DDE) and nitrofen were purchased from Kanto Chemical Industries. Ornidazole, epichlorohydrin (ECH) and dihydrotestosterone (DHT) were purchased from Sigma. Tamoxifen citrate was purchased from Biomol Research Labo. All of the chemicals were handled according to the guidelines of the company and the Ministry of International Trade and Industry. Hydroxyflutamide (OHF) was kindly provided by Nippon Kayaku, Co. Ltd.

Luciferase Assay Monkey Cos7 cells were cultured in Dulbecco's modified Eagle's medium with 10% calf serum. Two micrograms of pARE$_2$-TATA-Luc, a reporter plasmid, 0.5 μg of pcDNA3-F-rAR, and 0.5 μg of pCMV-β-gal, a β-galactosidase expression vector, were transfected into cells approximately 60% confluent in a 6-cm dish in the presence of 10^{-8} M DHT by the calcium phosphate method.[15] Chemicals were added to the medium for 12 h from 36 to 48 h after

* To whom correspondence should be addressed. e-mail: hiro@pharm.hokudai.ac.jp

transfection, and whole cell extracts were prepared by the addition of the Triton X-100-containing solution from a Pica gene kit (Wako Pure Chemicals Co., Ltd.) to the cells. About a one-fifth volume of the extract was used for the β-galactosidase assay to normalize the transfection efficiency as described previously,[3] and the luciferase activity due to the reporter plasmid was determined using a Pica gene kit and a luminometer, Lumat LB 9507 (EG & G Berthold). The same experiments were repeated three to five times.

Indirect Immunofluorescence Monkey Cos7 cells were transfected with DNAs and treated with chemicals by exactly the same protocols as those used in the luciferase assay described above. Forty-eight hours after transfection, the cells were fixed with a solution containing 4% paraformaldehyde and reacted with a mouse anti-FLAG monoclonal antibody (M2, Sigma) and an anti-DJ-1 polyclonal antibody.[1] The cells were then reacted with a rhodamine-conjugated anti-mouse IgG or FITC-conjugated anti-rabbit IgG and observed under a confocal laser fluorescent microscope.

RESULTS AND DISCUSSION

DJ-1 in the epididymis and sperm of rats and mice exposed to male or testis/sperm toxicants, including ornidazole and epichlorohydrin (ECH), were reduced and that treatment of animals with these chemicals lead to infertility.[19—22] The effects of these chemicals on the AR, however, have not been investigated. Nawata and his colleagues, on the other hand, reported that both OHF, an anti-androgen agent, and androgen-antagonists, including OHF, vinclozolin, p,p′-DDE and nitrofen, repressed AR transcription activity in monkey Cos7 cells by abrogation of the proper localization of the AR in the nuclei.[14] The effects of these chemicals on DJ-1, however, have not been investigated. To first examine or confirm the effects of these chemicals on AR transcription activity, Cos7 cells were transfected with pARE2-TATA-Luc as a reporter and with pcDNA3-F-AR in the presence of 10^{-8} M testosterone. The plasmid pCMV-β-gal was also cotransfected into cells to normalize the transfection efficiency. Chemicals were added to cells for 12 h from 36 h to 48 h after transfection, and then cell lysates were prepared and their luciferase activities were measured (Fig. 1). The results showed that ethanol, which had been used to dissolve the chemicals, had no effect but that OHF, an anti-androgen used as a positive control, repressed the AR transcription activity in a dose-dependent manner and that 10^{-6} M OHF repressed the AR activity to 14.9% of that without OHF (Fig. 1A). Tamoxifen, an anti-estrogen and very weak anti-androgen agent, was found to repress, to some extent, the AR activity. The three chemicals vinclozolin, p,p′-DDE and nitrofen, which have been identified as endocrine-disrupting (ED) chemicals, were found to repress the AR transcription activity in a dose-dependent manner as previously described (Fig. 1A).[14] Ornidazole and ECH, on the other hand, were also found to repress AR transcription activity in a dose-dependent manner, and 10^{-6} M ornidazole and ECH repressed AR activities to 25.9% and 28.8%, respectively, of those without chemicals (Fig. 1B). These results indicate that ornidazole and ECH can be classified as androgen antagonists.

It has been reported that the AR was localized in the nucleus as a dot-like spot and that this localization was disrupted by chemicals used in the experiment for which the results are shown in Fig. 1A.[14] To determine the localization of the AR and DJ-1 in cells exposed to these chemicals, monkey Cos7 cells were transfected and treated with chemicals under exactly the same conditions as those in the experiments on AR transcription activity as shown in Fig. 1. Two days after transfection, the cells were stained with anti-FLAG and anti-DJ-1 antibodies, and the proteins were detected by rhodamine- and FITC-conjugated secondary antibodies, respectively, and then visualized under a confocal laser microscope (Fig. 2). Three independent experiments were carried out and the typical results were shown. Endogenous DJ-1 (green) and the AR (red) were found to be localized in nuclei as dot-like spots and that these two proteins were co-localized as shown by the yellow color in a merged figure (Fig. 2, Merge). Cell nuclei were identified by DAPI staining (data not shown). Although the number of dots in the nuclei of

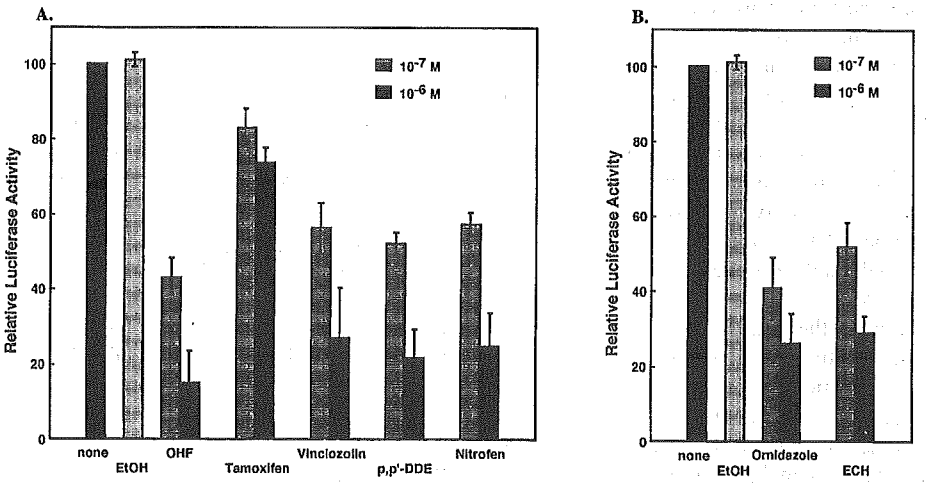

Fig. 1. Effects of Chemicals on Transcriptional Activity of the AR

(A) Cos7 cells were transfected with an expression vector for FLAG-AR together with pARE$_2$-TATA-Luc, a reporter plasmid for AR transcription activity. At 36 to 48 h after transfection, 10^{-7} or 10^{-6} M OHF, tamoxifen, vinclozolin, p,p′-DDE and nitrofen were added to the cells, and then cell extracts were prepared and the luciferase activity was measured as described in Experimental procedures. (B) Cos7 cells were transfected with DNAs and treated with 10^{-7} or 10^{-6} M ornidazole and ECH under exactly the same conditions as those described in (A), and the luciferase activities were measured as in (A).

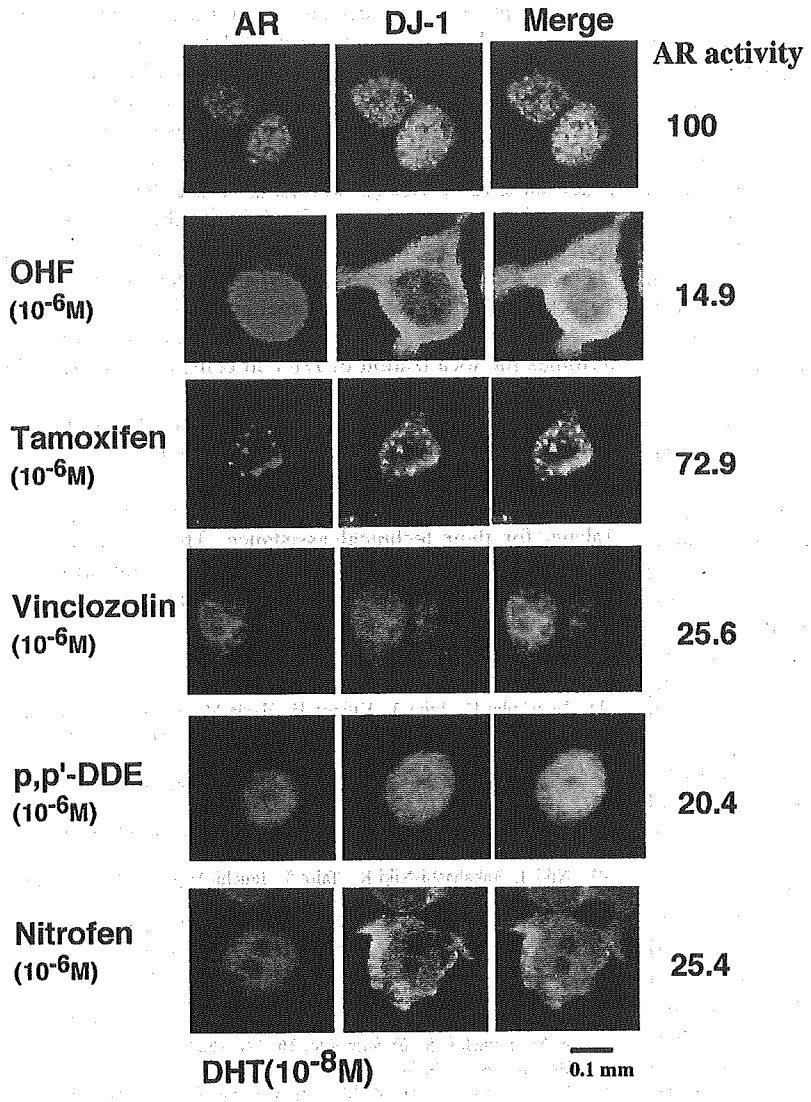

Fig. 2. Abrogation of Co-localization of DJ-1 with the AR by an Anti-androgen Agent or Androgen Antagonists

Cos7 cells were transfected with an expression vector for FLAG-AR together with $pARE_2$-TATA-Luc, a reporter plasmid for the AR transcription activity. At 36 to 48 h after transfection, 10^{-6} M OHF, tamoxifen, vinclozolin, p,p'-DDE and nitrofen were added to the cells. The cells were then fixed, reacted with an anti-FLAG monoclonal antibody (M2, Sigma) and an anti-DJ-1 polyclonal antibody,[1] and visualized with a rhodamine-conjugated anti-mouse IgG and an FITC-conjugated anti-rabbit IgG, respectively. The two figures have been merged. The average AR activities determined by the luciferase assay as described in the legend to Fig. 1 are indicated on the right sides of the figures.

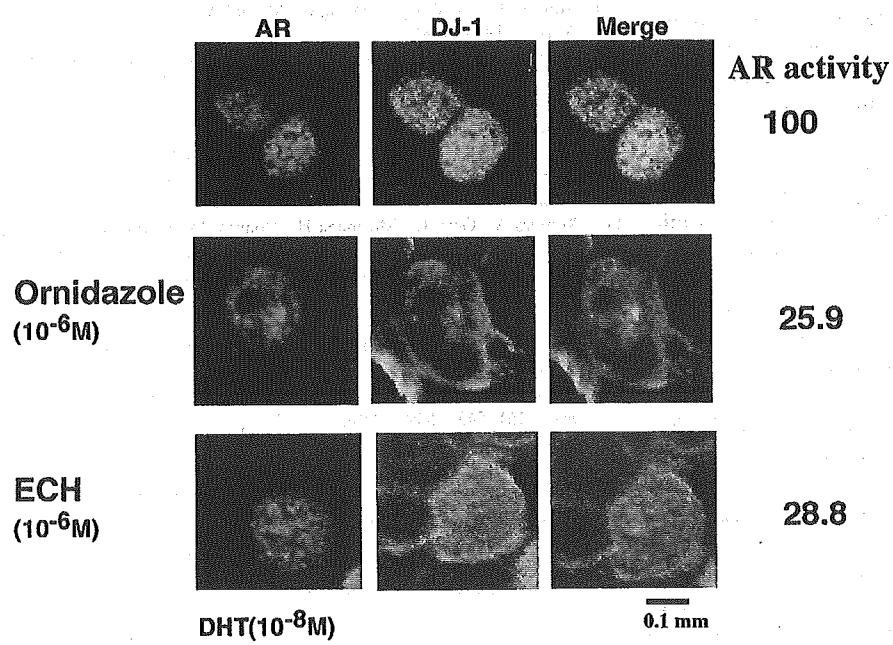

Fig. 3. Abrogation of Co-localization of DJ-1 with the AR by Ornidazole and ECH

Cos7 cells were transfected with an expression vector for FLAG-AR together with $pARE_2$-TATA-Luc, a reporter plasmid for the AR transcription activity. At 36 to 48 h after transfection, 10^{-6} M ornidazole and ECH were added to the cells. The cells were then fixed, reacted with an anti-FLAG monoclonal antibody (M2, Sigma) and an anti-DJ-1 polyclonal antibody,[1] and visualized with a rhodamine-conjugated anti-mouse IgG and an FITC-conjugated anti-rabbit IgG, respectively. The two figures have been merged. The average AR activities determined by the luciferase assay as described in the legend to Fig. 1 are indicated on the right sides of the figures.

cells treated with 10^{-6} M tamoxifen decreased, DJ-1 and the AR were found to be still co-localized. In cells treated with 10^{-6} M OHF, vinclozolin and nitrofen, the localization of the AR changed from dot-like spots to diffuse spaces in the nucleus as described previously.[14] The localization of DJ-1 in cells treated with these chemicals, on the other hand, was found to be dramatically changed; both OHF and nitrofen pushed DJ-1 into the cytoplasm and co-localization of the AR with DJ-1 was abrogated; p,p′-DDE localized DJ-1 to the diffuse spaces in the nucleus but these proteins were still co-localized. These results suggest that the proper localization of the AR in the nucleus as dot-like spots is not sufficient for the AR to exert its full transcription activity and that co-localization of the AR with DJ-1 is important.

These possibilities were supported by the results of experiments in which Cos7 cells were treated with 10^{-6} M ornidazole and ECH, and the typical results were shown of three independent experiments (Fig. 3). Although these two chemicals repressed the AR transcription activities to degrees similar to those with OHF, nitrofen and p,p′-DDE, the AR was found to be still localized as dot-like spots in the nucleus. Localizations of DJ-1, on the other hand, were changed to the cytoplasm or both the cytoplasm and the diffuse spaces in the nuclei of cells treated with ornidazole or ECH, respectively, thereby resulting in loss of co-localization of the AR with DJ-1 (Fig. 3). Since the transfection efficiency of plasmids into the cells were 10—20% and almost all of the cells transfected showed the tendency of the localization of DJ-1 and the AR, these results clearly indicate that the co-localization of the AR with DJ-1 as dot-like spots in the nucleus is essential for the AR to exert its full transcription activity.

Several studies have shown that the AR is complexed with other proteins, including coactivators and corepressors and that the proper localization of the AR with these proteins in the nucleus is necessary for its transcription activity. TIF2/GRIP and SRC1 of p160 cofactors and CBP of a general coactivator were found to be co-localized with the AR as dot-like spots in the nucleus, and abrogation of this localization of the AR with an anti-androgen agent or androgen antagonists led to the repression of AR transcription activity.[12—14] We reported that DJ-1 played a role as a positive regulator of the suppressed AR but did not respond to the AR that had already been activated.[3] In this study, we further characterized the relationship between DJ-1 and the AR by using chemicals as tools, and we found that ornidazole and ECH, which are known to be sperm or epididymis toxicants, also repressed AR transcription activity to the degrees similar to those of other androgen antagonists, thereby indicating that they can be classified as androgen antagonists. While OHF, vinclozolin, p,p′-DDE and nitrofen changed the localization of the AR to diffuse spaces in the nucleus, ornidazole and ECH kept the AR in dot-like spots in the nucleus. Moreover, the AR was co-localized with DJ-1 in diffuse spaces but not in dot-like spots in the nucleus in cells treated with vinclozolin and p,p′-DDE, and other chemicals were found to localize DJ-1 to the cytoplasm, thereby resulting in a loss of co-localization of DJ-1 with the AR. These results clearly indicate that the localization of the AR in dot-like spots in the nucleus is not sufficient and that the co-localization with DJ-1 in proper compartments in the nucleus is necessary for the AR to exert its full transcription activity, at least in these systems. What are the mechanisms of translocation of DJ-1 from the nucleus to the cytoplasm? DJ-1 has been reported to be induced in cells treated with paraquat or lipopolysaccharide, which induce the production of activated oxygen species,[20,21] and DJ-1 was strongly expressed and secreted from cells to serum in about half of breast cancer patients.[22] Since some chemicals are known to induce the production of activated oxygen species in cells, it is possible that the chemicals used in this study also produce the activated oxygen species, leading to a change in the components of the DJ-1–protein complex that determine the localization of DJ-1 in cells. Alternatively, proteins associated with DJ-1, which determines the localization of DJ-1 in cells, may change adter treatment of the chemicals.

Taken together, our findings indicate that DJ-1 is an essential component in determination of AR activity.

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Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide-mediated oxidation in vivo in human umbilical vein endothelial cells☆

Tomoya Kinumi,[a,*] Junko Kimata,[a,b] Takahiro Taira,[c,d] Hiroyoshi Ariga,[c,d] and Etsuo Niki[a]

[a] *Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 1-8-31 Midorigaoka, Osaka 563-8577, Japan*
[b] *Thermo Electron K.K., C-2F, Moriya-cho, Kanagawa-ku, Yokohama 221-0022, 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

Mutation in DJ-1 gene is the cause of autosomal recessive Parkinson's disease, however, its physiological function remains unclear. The isoelectric point of DJ-1 shows an acidic shift after cells are treated with hydrogen peroxide. This suggests that DJ-1 is modified in response to oxidative stress. Here we report the structural characterization of an acidic isoform of DJ-1 using a proteomic approach with nanospray interface liquid chromatography-electrospray ionization/linear ion trap mass spectrometer. When human umbilical vein endothelial cells were exposed to hydrogen peroxide, all three cysteines in DJ-1 were oxidized to cysteine sulphonic acid. Although a small part of the Cys-46 and Cys-53 were oxidized, Cys-106 was oxidized completely at any hydrogen peroxide concentration used here. These results suggest that Cys-106 is the most sensitive among three cysteine residues to oxidative stress, and that DJ-1 function is regulated, in terms of the intracellular redox state, by oxidation of Cys-106.

Keywords: DJ-1; Proteome; Oxidative stress; Cysteine sulphonic acid; Two-dimensional gel electrophoresis; Mass spectrometry

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), the hydroxyl radical ($HO^{\cdot}$), and the superoxide anion ($O_2^{-\cdot}$), can damage various cellular components such as unsaturated lipids, proteins, and nucleic acids. Oxidative stress can be defined as an imbalance between the ROS production and antioxidant capacity of a cell. Under conditions of the oxidative stress, dysfunction of these lipids, proteins, and nucleic acids molecules is implicated in various degenerative diseases such as neurodegenerative diseases, atherosclerosis, and cancer. A recent proteomic study revealed that on two-dimensional gel electrophoresis (2-D gel) of mammalian cells, the isoelectric points (p*I*s) of several protein spots showed an acidic mobility shift under oxidative stress [1,2]. In these studies, DJ-1 was identified as an oxidative stress responsive protein that showed an acidic p*I* shift on exposure of a human endothelial cell line (ECV304) to H_2O_2 and the ROS generating compound, paraquat [3]. DJ-1 was originally identified as an oncogene product that transforms NIH3T3 cells in cooperation with H-Ras [4]. Another research group independently identified an RNA-binding protein composed of an RNA-binding subunit and a regulatory subunit (RS) in FTO-2B rat hepatoma cells [5]. CAP1 is a rat sperm protein related to male fertility

☆ *Abbreviations:* HUVEC, human umbilical vein endothelial cells; p*I*, isoelectric point; 2-D gel, two-dimensional gel electrophoresis; LC, liquid chromatography; MS/MS, tandem mass spectrometry; IT/MS, ion trap mass spectrometer; nanospray LC-IT/MS, nanospray interface capillary LC-electrospray ionization/ion trap mass spectrometer; SIM, selected ion monitoring; DTT, dithiothreitol; TFA, trifluoroacetic acid.

* Corresponding author. Fax: +81-72-751-9964.
E-mail address: t.kinumi@aist.go.jp (T. Kinumi).

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[6]. Both the RS and CAP1 are identical to DJ-1, which is preferentially expressed in testis. Although DJ-1 is broadly distributed in various human tissues, the function of DJ-1 remains unclear, it appears to act as a multifunctional enzyme. However, Bonifati et al. [7] reported that point (L166P) and deletion mutants of DJ-1 correlated with the early onset of familial Parkinson's disease, characterized as the PARK7 form. To understand the relation between the possible molecular function of DJ-1 and the pathogenesis of Parkinson's disease, the X-ray crystal structure of DJ-1, including the L166P mutation form, has been reported by several independent groups [8–11]. These studies reveal that DJ-1 forms a dimeric structure both in the crystal and in buffer solution and that the L166P mutation destabilises this dimer structure. A recent report shows that DJ-1 has H_2O_2 eliminating activity and knock down of DJ-1 expression rendered a neuroblastoma cell line susceptible to H_2O_2 induced cell death [12,13]. We also found that the spots of DJ-1, together with those of the peroxiredoxins, were shifted to the acidic side on 2-D gel by exposure of primary cultured human umbilical vein endothelial cells (HUVEC) to H_2O_2. The structure of the acidic satellite spot of DJ-1 was expected to be an oxidized isoform by a post-translational process, as was also expected for the peroxiredoxins. In the present study, the DJ-1 spots isolated by 2-D gel were analysed by nanospray interface capillary liquid chromatography-electrospray ionization/linear ion trap mass spectrometer (nanospray LC-IT/MS). Here, we report the structural characterization of DJ-1 modified in vivo, which is responsible for oxidative stress.

Materials and methods

Cell culture and two-dimensional gel electrophoresis. HUVEC were purchased from BioWhittaker and cultured in EGM-2 (BioWittaker) containing endothelial cells growth factors with 2% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. All experiments were performed after four passages. After treatment with H_2O_2, the cells were washed with phosphate-buffered saline (PBS) and harvested, and then dissolved in isoelectric focusing sample buffer consisting of 9 M urea, 2% (3-[{3-cholamidopropyl}dimethylammonio]-1-propanesulphonate) (Chaps), 65 mM dithioerythritol (DTE), and 0.5% carrier ampholyte (pH 4–7; Amersham Biosciences). The sample solution was applied to an immobilized pH gradient gel (13 cm, pH 4–7; Amersham Biosciences) and rehydrated for 12 h. Isoelectric focusing was performed for a total of 48,990 Vh at a maximum voltage of 8000 V. Each strip was equilibrated in two steps, in 50 mM Tris–HCl (pH 8.8), 6 M urea, 2% sodium dodecyl sulphate (SDS), and 30% glycerol, supplemented with 10 mg/ml dithiothreitol (DTT) and 40 mg/ml iodoacetamide, respectively, for 20 min each. The second dimension was carried out by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were stained with SyproRuby (Molecular Probes) and scanned by Molecular Imager FX (Bio-Rad Laboratories).

In-gel enzymatic digestion and mass spectrometry. Excised spots were washed twice with 100 mM ammonium bicarbonate (pH 8.8) and then dehydrated with acetonitrile. The gel pieces were rehydrated in 10 mM DTT ammonium bicarbonate solution and alkylated with 50 mM iodoacetamide. After dehydration with acetonitrile, the gel pieces were rehydrated on ice for 10 min in 20 μl of 20 mM ammonium bicarbonate containing 50 ng/μl sequence grade modified trypsin (Promega). The rehydrated gel pieces were replaced with 20 μl of 20 mM ammonium bicarbonate, and in-gel digestion was performed for 15 h at 37 °C. The resulting peptides were extracted twice with 20 μl of 20 mM ammonium bicarbonate and then three times in 20 μl of 0.5 % trifluoroacetic acid (TFA) in 50% acetonitrile. The extracts were concentrated to 20 μl using a SpeedVac concentrator (Thermo Electron). The sample was injected into a reversed phase trap cartridge (CapTrap, Michrom BioResources) equipped in an autosampler (HTC PAL, CTC Analytics). The cartridge was washed with 0.1% TFA/H_2O and then automatically switched to a LC flow line by a 10-port switching valve. A capillary LC system (Paradigm, Michrom BioResources) was coupled on-line to a nano-electrospray ionization (nano-ESI) linear ion trap mass spectrometer (LTQ, Thermo Electron) through the autosampler equipped with the trap cartridge. In some cases, a 3-D ion trap mass spectrometer (LCQ Deca, Thermo Electron) with a capillary LC system (MAGIC2002, Michrom BioResources) was used for protein identification instead of LTQ and Paradigm. Chromatographic separations were conducted on a reversed phase capillary column (MAGIC C18, 0.2 mm i.d., 50 mm length; Michrom BioResources) at a flow rate of 2 μl/min which was reduced from the 50 μl/min LC flow with a precolumn flow splitter. The gradient profile consisted of a linear gradient from 5% solvent B (H_2O/acetonitrile/formic acid, 10/90/0.1, v/v/v) to 65% B in 40 min against solvent A (H_2O/acetonitrile/formic acid, 98/2/0.1, v/v/v). Nano-ESI was performed using 20 mm length and 20 μm i.d. fused silica spray tip (PicoTip, New Objective) by applying 1.8 kV spray voltage. Data dependent MS/MS spectra were acquired and converted into DTA file format to be submitted to the MASCOT (Matrix Science) search software. NCBI and SWISS-PROT were the protein databases used.

Western blot analysis. Proteins separated by 2-D gel were transferred to polyvinylidene fluoride (PVDF) membrane (ProBlott, Applied Biosystems), blocked with BlockAce (Dainippon Pharmaceutical), and incubated with a polyclonal anti DJ-1 antibody in TBST (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20). The membranes were washed three times with TBST and incubated with peroxidase-conjugated secondary antibody. The specific spot was visualized with an ECL Western blotting detection system (Amersham Biosciences).

Results and discussion

We first undertook to identify the proteins differentially expressed in control cells and oxidative stress loaded cells. Fig. 1 shows the 2-D gel of A: HUVEC (control) and B: HUVEC treated with 100 μM H_2O_2 for 1 h. Several spots disappeared or were reduced in the density in cells exposed to H_2O_2, and new spots appeared simultaneously on the acidic side of each of these spots in the range of 0.3–0.5 in the p*I*. Ten of these spots were identified as pairs of five proteins: DJ-1, and peroxiredoxins 2, 3, 4, and 6 as summarized in Table 1. DJ-1 gave a single spot under control conditions, whereas the acidic spot appeared as a doublet spot under conditions of oxidative stress, which were generated by exposure of the cells to H_2O_2 (in Fig. 1, acidic DJ-1 spot overlaps with the acidic peroxiredoxin 3 spot). The p*I* of the basic DJ-1 spot on 2-D gel was 6.4 which is in good agreement with the theoretical p*I* (6.3), whereas the acidic satellite of DJ-1 had a p*I* of 6.0. The sensitivity of

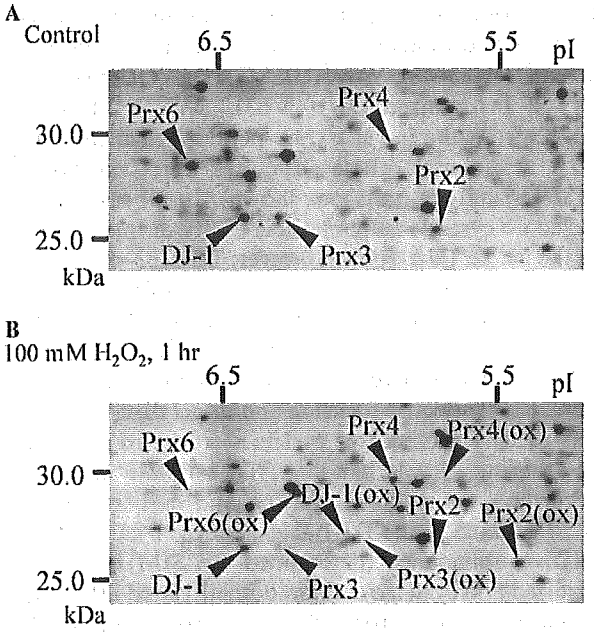

Fig. 1. 2-D gel image of HUVEC whole cell extract (A, control; B, cells exposed to 100 μM H_2O_2 for 1 h). The 2-D gels were visualized by fluorescent staining with SyproRuby. Peroxiredoxins 2, 3, 4, and 6 are abbreviated to Prx 2, 3, 4, and 6, respectively. The spots that appeared after exposure of cells to H_2O_2 are indicated as DJ-1(ox), Prx 2(ox), Prx 3(ox), Prx 4(ox), and Prx 6(ox).

this acidic shift of DJ-1 to the exposure of HUVEC to H_2O_2 was examined by Western blot analysis. Fig. 2 shows the dose dependence of spot migration after 1 h exposure of HUVEC to H_2O_2 concentrations in the range 100 μM–1 mM. The acidic spot shift occurred at concentrations greater than 100 μM H_2O_2, although peroxiredoxin 3 had already shifted at the same concentration. The spots of peroxiredoxins 2, 3, 4, and 6 also shifted to acidic satellites under oxidative stress conditions as shown in Fig. 1. This result indicates that the in vivo response of DJ-1 to H_2O_2 is not as sensitive as the response of peroxiredoxin 3. Upon exposure to H_2O_2, DJ-1 appeared as a pair of spots, not in a multiple spot pattern. Mitsumoto et al. [3] speculated that the acidic p*I* shift is due to a post-translational process that causes the oxidation of a cysteine residue to cysteine sulphinic acid (Cys-SO_2H). Therefore, the acidic spot shift of DJ-1 was expected to result from the oxidation of an active cysteine residue, as is the case for the peroxiredoxins. LC-MS and tandem mass spectrometry analysis revealed that in in vivo oxidized peroxiredoxin 2 (the acidic satellite spot of peroxiredoxin 2), the active-site cysteine was oxidized to cysteine sulphonic acid (Cys-SO_3H) [14]. Later, Wagner et al. [15] reported that the acidic peroxiredoxin 2 spot contained a cysteine sulphinic acid entity in addition to cysteine sulphonic acid. They also characterized the acidic spot of peroxiredoxins 1, 3, 4, and 6 as containing an oxidized cysteine at the active site, using a combination of an enzyme digestion method specific for oxidized cysteine residues and mass spectrometry. By analogy, it was assumed that the acidic spot of DJ-1 contained oxidized cysteine residues such as cysteine sulphinic acid or cysteine sulphonic acid. To examine this hypothesis, we characterized the structure of the acidic DJ-1 spot using LC-MS and LC-MS/MS analysis with nanospray LC-IT/MS. DJ-1 has three cysteines at residues 46, 53, and 106 amino acid from the N-terminal. In this analysis, a tryptic peptide containing modified cysteine-106 was observed from the acidic DJ-1 spot by selected ion monitoring (SIM) LC/MS chromatogram, which was selectively monitored at $m/z = 1129.6$. This ion corresponds to the doubly charged ion of the oxidized DJ-1 tryptic fragment, DJ-1(100–122), GLIAAIC*AGPTALLAHEIGFGSK in Fig. 3A, where DJ-1(100–122) represents the peptide containing residues 100–122 of DJ-1, and C* represents cysteine sulphonic acid. Oxidation of the cysteine in this fragment of DJ-1 (2210.2 Da) to cysteine sulphonic acid adds 48 Da to the molecular mass of the peptide, resulting in a final mass for oxidized DJ-1(100–122), of 2258.2 Da. This doubly charged chromatographic peak at $m/z = 1129.6$ was observed only in the acidic satellite spot, and not in the

Table 1
Proteins identified by LC-MS/MS corresponding to the spots that showed acidic shift on 2-D gel

Name of Protein	Observed		Theoretical		Accession No.	Sequence coverage (%)
	p*I*	M.W. (kDa)	p*I*	M.W. (Da)		
DJ-1	6.4	26.2	6.3	19,878	gi_31543380	68
DJ-1 (acidic)	6.0	26.2				93
Peroxiredoxin 2	5.8	25.3	5.7	22,049	gi_2507169	48
Peroxiredoxin 2 (acidic)	5.4	25.3				66
Peroxiredoxin 3	6.2	26.2	6.1	28,047	gi_14250063	10[a]
Peroxiredoxin 3 (acidic)	6.0	26.5				31
Peroxiredoxin 4	5.9	29.7	5.9	30,749	gi_5453549	19[a]
Peroxiredoxin 4 (acidic)	5.7	29.7				43
Peroxiredoxin 6	6.6	28.7	6.0	25,133	gi_4758638	13[a]
Peroxiredoxin 6 (acidic)	6.2	28.7				91

[a] These spots were identified using LCQ mass spectrometer rather than LTQ mass spectrometer.

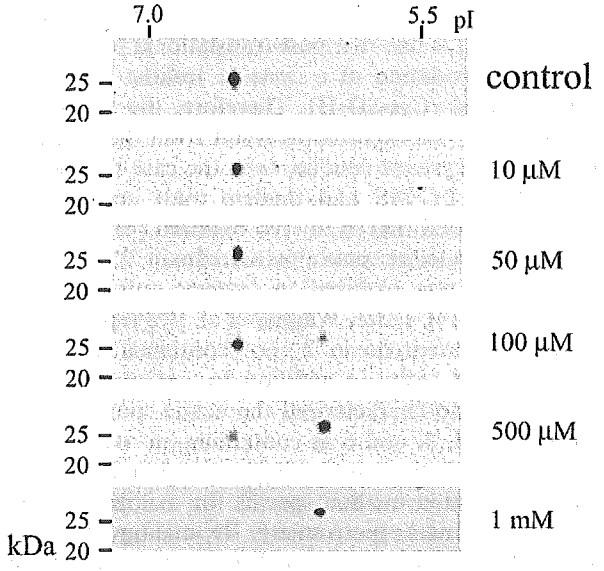

Fig. 2. Western blot analysis of 2-D gel separated proteins obtained from HUVEC exposed to H_2O_2 at the indicated concentrations for 1 h. DJ-1 spots were detected with a DJ-1 specific polyclonal antibody.

native (basic) spot (Fig. 3B). Only the reduced form of DJ-1(100–122) was found in the native DJ-1 spot (data not shown). The structure of the $m/z = 1129.6$ peak in Fig. 3A, possibly oxidized DJ-1(100–122), was determined by LC-MS/MS analysis. The MS/MS spectrum of oxidized DJ-1(100–122) is shown in Fig. 4. A mass difference between y16 and y17 of 151 Da shows the presence of cysteine sulphonic acid at position of Cys-106 in oxidized DJ-1(100–122). Using the same method, we identified cysteine sulphonic acids at Cys-46 in oxidized DJ-1(33–48), VTVAGLAGKDPVQC*SR, and at Cys-53 in oxidized DJ-1(49–63), DVVIC*PDASLED AKK, from the in-gel tryptic peptide fragments of the DJ-1 acidic spot. These structures were confirmed by MS/MS analysis (data not shown). In all cases, we detected no cysteine sulphinic acid entity or intact cysteine in the acidic DJ-1 spot. However, DJ-1(33–48) and DJ-1(49–63) isolated from the preparation of the acidic DJ-1 spot also contained carbamidomethylated cysteine (Cys-CAM), resulting from the iodoacetamide modification of free sulfhydryl groups during the equilibration step after isoelectric focusing and in-gel digestion, which was used to protect free cysteines from artificial oxidation during sample handling. To estimate the relative occurrence of oxidized cysteine and carbamidomethylated cysteine in the DJ-1 acidic spot, we calculated the relative abundance (determined by SIM) of cysteine oxidized and cysteine carbamidomethylated DJ-1 tryptic peptides obtained from the acidic DJ-1 spot, DJ-1(33–48), DJ-1(49–63), and DJ-1(100–122), expressed as ratios in Fig. 5. In this figure, the ratios of relative ion abundance for each peptide are shown for each H_2O_2 concentration, which correspond to the amount of H_2O_2 applied to HUVEC. The ionization efficiency may be different between the oxidized and carbamidomethylated peptides, so it is difficult to quantify these values absolutely. The oxidation of peptide and phosphorylation of peptide are expected to be similar in that both are decreasing the ionization efficiency in same extent,

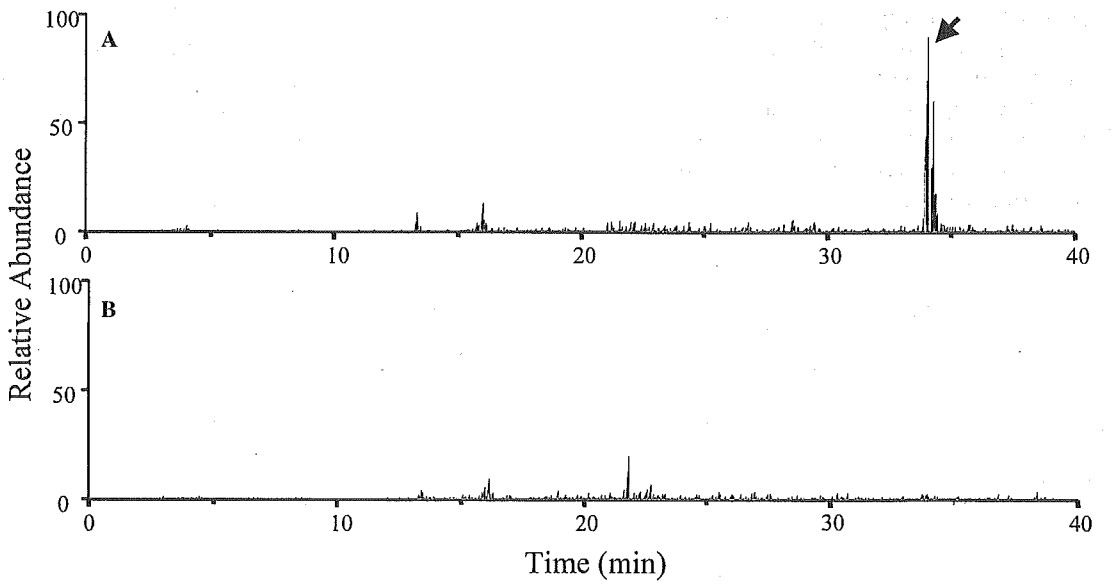

Fig. 3. Selected ion monitoring (SIM) LC/MS chromatogram for $m/z = 1129.6$. Arrowhead indicates the $m/z = 1129.6$ peak, which corresponds to the doubly charged ion of oxidized DJ-1(100–122). SIM of (A) in-gel tryptic digest of the acidic spot and (B) in-gel tryptic digest of the native spot of DJ-1 from 2-D gel separated HUVEC proteins after exposure of the cells to 100 μM H_2O_2 for 1 h. Relative ion abundances (y-axis) for both (A) and (B) were adjusted to the same intensity.

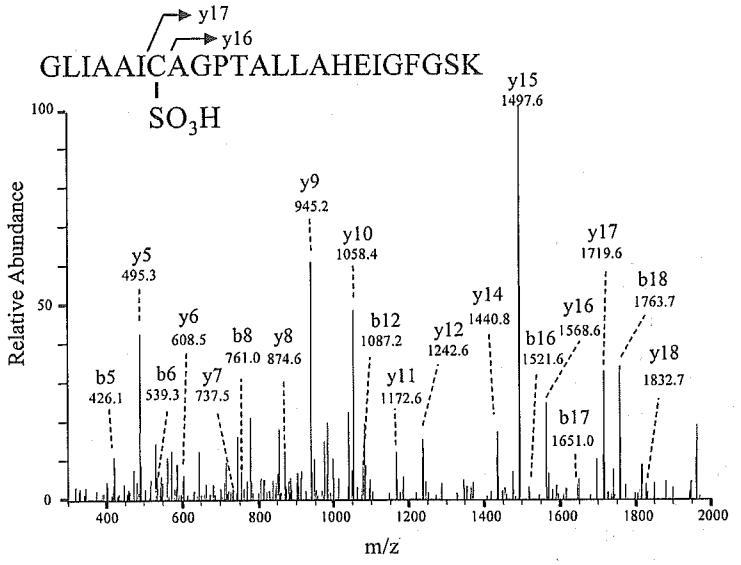

Fig. 4. MS/MS spectrum of the doubly charged $m/z = 1129.6$ ion. This MS/MS spectrum provides sequence data for unequivocal assignment to oxidized DJ-1(100–122), the cysteine of which was oxidized to cysteine sulphonic acid.

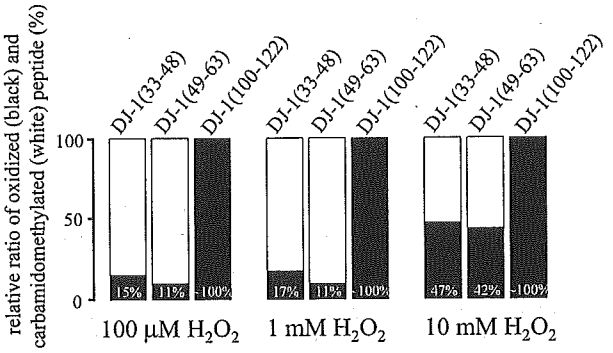

Fig. 5. Relative amount of cysteine oxidized and cysteine carbamidomethylated DJ-1(33–48), DJ-1(49–63), and DJ-1(100–122) prepared from the acidic DJ-1 spot from HUVEC exposed to 100 μM, 1 mM, or 10 mM H_2O_2 for 1 h. Black and white areas represent the relative ratios of oxidized peptide and carbamidomethylated peptide, respectively. These ratios were calculated as percentages, based on the intensity of SIM for each peptide. The relative ratios of oxidized peptide are indicated in white letters in the black bars. DJ-1(33–48), DJ-1(49–63), and DJ-1(100–122) contain Cys-46, Cys-53, and Cys-106, respectively. Free cysteine was detected as carbamidomethylated cysteine due to the modification with iodoacetamide between isoelectric focusing and SDS–PAGE in the second dimension. Carbamidomethylated Cys-106 was not found at any H_2O_2 concentration.

because the both of modifications lead to similar acidic p*I* shift. It has been reported that peptide phosphorylation exhibits 0.5- to 0.7-fold decrease in the ionization efficiency of electrospray ionization [16,17]. However, as long as we estimate the relative levels of oxidation of these DJ-1 tryptic peptides by comparing relative ion abundance, such a difference in ionization efficiency between cysteine carbamidomethylated and cysteine oxidized peptide is not a significant problem. The data show 11–17% of Cys-46 and Cys-53 were oxidized to cysteine sulphonic acid, when 100 μM and 1 mM H_2O_2 were applied to HUVEC. With 10 mM H_2O_2 treatment, 42–47% of these cysteines were oxidized. However, Cys-106 was fully oxidized to cysteine sulphonic acid at any H_2O_2 concentration between 100 μM and 10 mM. Possibly Cys-46 and Cys-53 were directly oxidized by excess H_2O_2, whereas Cys-106 was specifically oxidized even at low H_2O_2 concentrations. This result indicates that Cys-106 is remarkably sensitive to H_2O_2 mediated oxidation in vivo.

Using nanospray LC-IT/MS, we identified the in vivo modification site and the structure of the acidic satellite spot of DJ-1 that was generated by exposure of HUVEC to H_2O_2. This on-line combination of capillary LC and linear ion trap mass spectrometer via nanospray ionization made possible this highly sensitive structural analysis and the relative quantification of the 2-D gel separated proteins. There are three cysteine residues in DJ-1. Of these, MS/MS analysis and SIM chromatography revealed that Cys-106 was completely oxidized to cysteine sulphonic acid. On the other hand, less than half of the Cys-46 and Cys-53 residues were oxidized to cysteine sulphonic acid. These oxidized cysteines were found only as cysteine sulphonic acid, and not as sulphinic acid, probably because cysteine sulphinic acid is autoxidized to cysteine sulphonic acid [18]. Although only two of DJ-1 spots (the native spot and acidic satellite spot) were apparent on SyproRuby stained 2-D gel and Western blot analysis after application of H_2O_2 concentrations from 100 μM to 1 mM, we identified a mixture of three cysteines in different oxidation states in the DJ-1 acidic spot. This can be explained in the following two ways. First, these data suggest that Cys-106

contributes strongly to the apparent p*I* shift on 2-D gel, whereas the two other cysteines may not alter the p*I* on 2-D gel. DJ-1 denatured in isoelectric focusing buffer containing 9 M urea seems to form a specific structure(s), in which Cys-46 and Cys-53 are not exposed on the protein surface. Second, to prevent any artificial oxidation, we performed cysteine alkylation at the equilibration step between isoelectric focusing and SDS–PAGE in the second dimension. A previous report concluded that it is hard to induce artificial cysteine oxidation by residual ammonium persulphate in the gel matrix during electrophoresis [19]. However, our highly sensitive analysis could detect trace amounts of DJ-1 tryptic fragments containing artificially oxidized cysteine residues. It is possible that a small fraction of Cys-46 and Cys-53 were directly oxidized by excess H_2O_2 or oxidized during sample handling under the aerobic denaturing conditions due to incomplete cysteine carbamidomethylation [20]. Unlike these two cysteines, Cys-106 has a highly specific character. This residue is well conserved in DJ-1 homologs, including the PfpI family (a family of intracellular cysteine proteases), ThiJ family (which catalyses the phosphorylation of hydroxymethyl pyrimidine [HMP] to HMP monophosphate) [9]. This cysteine is essential for the catalytic activity of these enzymes and may act as a nucleophile in their catalytic reactions. The GAT superfamily (class I glutamine amidotransferase-like superfamily, which includes catalase HPII, the subunit of anthranilate synthase TrpG, and a domain of GMP synthetase) is also found as structurally similar proteins to DJ-1 [8]. Furthermore, in the case of the GAT superfamily, the conserved cysteine residue confers hydrolase activity, except in catalase HPII. It has been reported that DJ-1 possesses cysteine protease like activity, which was abolished by mutation of Cys-106 to Ala [21], suggesting that Cys-106 plays an important role in this protease activity. During X-ray crystal structure analysis of DJ-1, Cys-106 was shown to be very sensitive to radiation damage, which could result in structural modifications such as the partial elimination of the thiol group or cleavage of the peptide backbone [9]. This specific cysteine residue appears to act as a redox-active cysteine, like a catalytic site cysteine of peroxiredoxins [22]. Those observations suggest that the Cys-106 is located at a characteristic redox environment compared to other two cysteine residues, and we can conclude that Cys-106 is the cysteine most sensitive to in vivo H_2O_2 treatment. Although the function of DJ-1 is still unclear, these observations suggest that Cys-106 is a key residue for DJ-1 function and that oxidative modification of Cys-106 leads, in some cases, to loss of function. DJ-1 forms dimeric structure as demonstrated by crystal structure analysis and gel exclusion chromatography. The dimeric structure appears to correlate with the function of DJ-1 under physiological conditions. Cys-53 is located on the dimer interface, which consists of identical β-strands from each DJ-1 monomer [8]. It may be hard to occur in physiological conditions, however oxidation at Cys-53 causes disruption of the dimer structure, which can also regulate the physiological function. Cookson [23] has remarked that both oxidative stress and proteasome inhibition are common to several neurodegenerative diseases, including Parkinson's disease. We assume that dysfunction of DJ-1 leads to an imbalance in the intracellular redox state or alter the turnover of the protein, which results in Parkinson's disease. DJ-1 may act as a multifunctional enzyme. However, in terms of the intracellular redox state, it functions as an antioxidant protein or oxidative stress sensor, together with its cysteine protease activity, are possibly regulated by oxidative modification of Cys-106.

Acknowledgments

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Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients

Kazuko Takahashi-Niki,[a] Takeshi Niki,[a] Takahiro Taira,[a,b] Sanae M.M. Iguchi-Ariga,[a,c] and Hiroyoshi Ariga[a,b,*]

[a] *CREST, Japan Science and Technology Corporation, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*
[b] *Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060-0812, Japan*
[c] *Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan*

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Abstract

DJ-1 is a multi-functional protein that plays roles in transcriptional regulation and anti-oxidative stress, and loss of its function is thought to result in onset of Parkinson's disease. We have previously reported that L166P, a mutant DJ-1 found in Parkinson's disease patients, had no activity to prevent hydrogen peroxide (H_2O_2)-induced cell death. In this study, we analyzed other mutants of DJ-1 found in Parkinson's disease patients, including M26I, R98Q, and D149A, as well as L166P. We first found that all of the mutants made heterodimers with wild-type DJ-1, while all of the mutants except for L166P made homodimers. We then found that M26I and L166P, both of which are derived from homozygous mutations of the DJ-1 gene, were unstable and that their stabilities were recovered, in part, in the presence of proteasome inhibitor, MG132. NIH3T3 cell lines stably expressing these mutants of DJ-1 showed that cell lines of L166P and C106S, a mutant for protease activity (−) of DJ-1, had no activity to scavenge even endogenously producing reactive oxygen species. These cell lines also showed that all of the mutants had reduced activities to eliminate exogenously added H_2O_2 and that these activities, except for that of D149A, were parallel to those preventing H_2O_2-induced cell death.

Keywords: DJ-1; Parkinson's disease; Oxidative stress; Reactive oxygen species

DJ-1 was first identified by our group as a novel candidate of the oncogene product that transformed mouse NIH3T3 cells in cooperation with activated *ras* [1]. The human DJ-1 gene is mapped at chromosome 1p36.2-p36.3, where a hot spot of chromosome abnormalities has been reported in several tumors [2]. DJ-1 has been reported to be a circulating tumor antigen in breast cancer [3] and to be overexpressed in smoker-derived lung adenocarcinoma [4]. DJ-1 has also been reported to be a regulatory component of an RNA-binding protein complex [5] and to be related to infertility [6–11]. We have shown that DJ-1 acts as a positive regulator for the androgen receptor (AR) by sequestering PIASxα or DJBP [12–14]. PIASxα, a member of the PIAS family of proteins, and DJBP, a novel DJ-1-binding protein, function as negative regulators for AR by preventing AR DNA-binding activity and by recruiting histone deacetylase complex, respectively [12,13]. These findings indicate that DJ-1 is a multi-functional protein in somatic cells and sperm.

Deletion and point (L166P) mutations of DJ-1 have recently been shown to be responsible for onset of familial Parkinson's disease, PARK7 [15], and other homozygous and heterozygous mutations of DJ-1 have been identified in patients with familial or sporadic Parkinson's disease [16–18]. DJ-1 was shown to be expressed in almost all brain tissues in healthy men and patients with neurodegenerative diseases, including Parkinson's disease, Pick's disease, and multiple system atrophy [19–21]. Various lines of evidence also suggest that oxidative stresses contribute to the cascade leading

* Corresponding author. Fax: +81-11-706-4988.
E-mail address: hiro@pharm.hokudai.ac.jp (H. Ariga).

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to dopaminergic cell degeneration in PD ([22–24]; see recent review, [25]), and expression of DJ-1 was shown to be induced by oxidative stresses [26–29]. A p*I* shift of DJ-1 towards a more acidic isoform has been observed in PD patients [19]. We previously reported that DJ-1 plays a role in the anti-oxidative stress reaction, in which reactive oxygen species were eliminated in vitro and in vivo by oxidizing DJ-1 itself, and that mutations of DJ-1, including L166P, lead to cell death [30,31].

In this study, we analyzed properties and protective activities toward H_2O_2-induced cell death of various mutants found in Parkinson's disease patients and found reduced activities for both eliminating H_2O_2 and protecting cell death.

Materials and methods

Cells. Human 293T and mouse Flp-InNIH3T3 cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium with 10% calf serum.

*Establishment of cell lines harboring wild-type DJ-1 or mutants of DJ-1. Kpn*I–*Xho*I fragments containing the CMV promoter and Flag-tagged wild-type or mutant DJ-1s were inserted into *Kpn*I–*Xho*I sites of pcDNA5/FRT. These plasmids were cotransfected with pOG44, an expression vector for Flp recombinase, into Flp-InNIH3T3 cells (Invitrogen) by the calcium phosphate precipitation method, and the cells were cultured in the medium in the presence of 100 μg/ml Zeocin and 100 μg/ml hygromycin for 14 days. The cells that were resistant to both drugs were then selected, and expression of Flag-DJ-1 was examined by Western blotting with an anti-Flag antibody (M2, Sigma).

Establishment of a DJ-1-knockdown cell line. The nucleotide sequence of the upper strand of the oligonucleotide used for construction of an siRNA vector targeting mouse DJ-1 gene is 5′-GGATCCC GCCTTGCTAGTAGAATAAACTTCAAGAGAGTTTATTCTAC TAGCAAGGTTTTTTCCAAAAGCTT-3′. After annealing oligonucleotides corresponding to the upper and lower strands of DNA, they were inserted into *Bam*HI–*Hin*dIII sites of pRNA-U6.1/Neo (GenScript). These plasmids were transfected into mouse Flp-In3T3 cells (Invitrogen) by the calcium phosphate precipitation method, and the cells were cultured in the medium in the presence of 200 μg/ml G418 for 14 days. The cells that were resistant to the drug were then selected, and expression of DJ-1 was examined by Western blotting with an anti-mouse DJ-1 antibody [10].

Cell viability assay. Cells were cultured in a 96-well plate and treated with various amounts of hydrogen peroxide for 12 or 24 h. Cell viability was then measured by an MTT assay using a cell counting kit-8 (Dojindo).

Formation of homodimers or heterodimers. Human 293T cells were transfected with expression vectors for FLAG-DJ-1 and HA-DJ-1 by the calcium phosphate precipitation technique. Forty-eight hours after transfection, proteins were extracted from cells with 0.5% NP-40-containing buffer, immunoprecipitated with an anti-FLAG antibody (M2, Sigma) or non-specific IgG, transferred onto a nitrocellulose membrane, and blotted with anti-HA and anti-FLAG antibodies. Immunoprecipitated proteins were then reacted with HRP or IRDye800-conjugated second antibody and visualized by an ECL system or an infrared imaging system (Odyssey, LI-COR), respectively. For a two-hybrid assay in yeast, wild-type DJ-1 and mutants of DJ-1 were inserted into the pGLex or pGAD-GHx vector and subjected to a two-hybrid assay as described previously [12]. Proteins extracted from mouse NIH3T3 cells harboring wild-type DJ-1 or mutants of DJ-1 were immunoprecipitated with an anti-FLAG antibody or non-specific IgG and then blotted with anti-DJ-1 [10] and anti-FLAG antibodies.

Results

Dimer formation of DJ-1 mutants found in Parkinson's disease patients

The crystal structure of DJ-1 suggests that DJ-1 forms a homodimer [32–36], and wild-type DJ-1 but not the L166P mutant of DJ-1 has been shown to form a dimer [37,38]. We have further shown that disruption of dimer formation of DJ-1 abrogates anti-oxidative stress function [31]. To confirm this and further examine dimer-forming activities of DJ-1 mutants found in Parkinson's disease patients, 293T cells were transfected with various combinations of expression vectors for FLAG-tagged DJ-1 and HA-tagged DJ-1. Forty-eight hours after transfection, cell extracts were immunoprecipitated with an anti-FLAG antibody and the immunoprecipitates were blotted with anti-HA and anti-FLAG antibodies (Fig. 1A). FLAG-DJ-1 was first confirmed to be immunoprecipitated with the anti-FLAG antibody (Fig. 1A, lanes 1 and 3). HA-DJ-1 was found to be coprecipitated with FLAG-DJ-1 only in cells that had been transfected with both expression vectors (Fig. 1A, lane 3), clearly indicating that wild-type DJ-1 forms a homodimer.

Formation of a homodimer or heterodimer between wild-type DJ-1 and mutants of DJ-1 was then examined by a yeast two-hybrid assay (Fig. 1B). Four mutants of DJ-1, M26I, R98Q, D149A, and L166P, which were found in Parkinson's disease patients [15–17], two mutants, C46S and C53A, that are unable to form a homodimer, and the mutant C106S, which is a mutant with a cysteine-to-serine substitution corresponding to a catalytic site for protease activity, were used for this study. R98Q is, however, likely to be a polymorphism [16]. When mutants of DJ-1 and wild-type DJ-1 alone were used as bait and prey, respectively, all of the mutants of DJ-1, like wild-type DJ-1, were found to be bound to wild-type DJ-1 (Fig. 1B, left part). This heterodimer formation between mutants and wild-type DJ-1 was also found in experiments in which wild-type DJ-1 and mutants were used as bait and prey, respectively (Fig. 2B, left part). All of the mutants, except for L166P, on the other hand, were found to form homodimers (Fig. 2B, left part).

To further investigate heterodimer formation of mutants of DJ-1 with wild-type DJ-1, NIH3T3 cell lines harboring exogenously added Flag-tagged wild-type DJ-1 or various mutants of DJ-1 were established as described previously [31]. Proteins in cell extracts were immunoprecipitated with an anti-FLAG antibody and the precipitates were blotted with anti-DJ-1 and anti-FLAG antibodies (Fig. 1C). The results clearly showed that all of the mutants of DJ-1 were coimmunoprecipitated with endogenous DJ-1, though a smaller amount of DJ-1 was coprecipitated with L166P DJ-1 (Fig. 1C, lane 19).

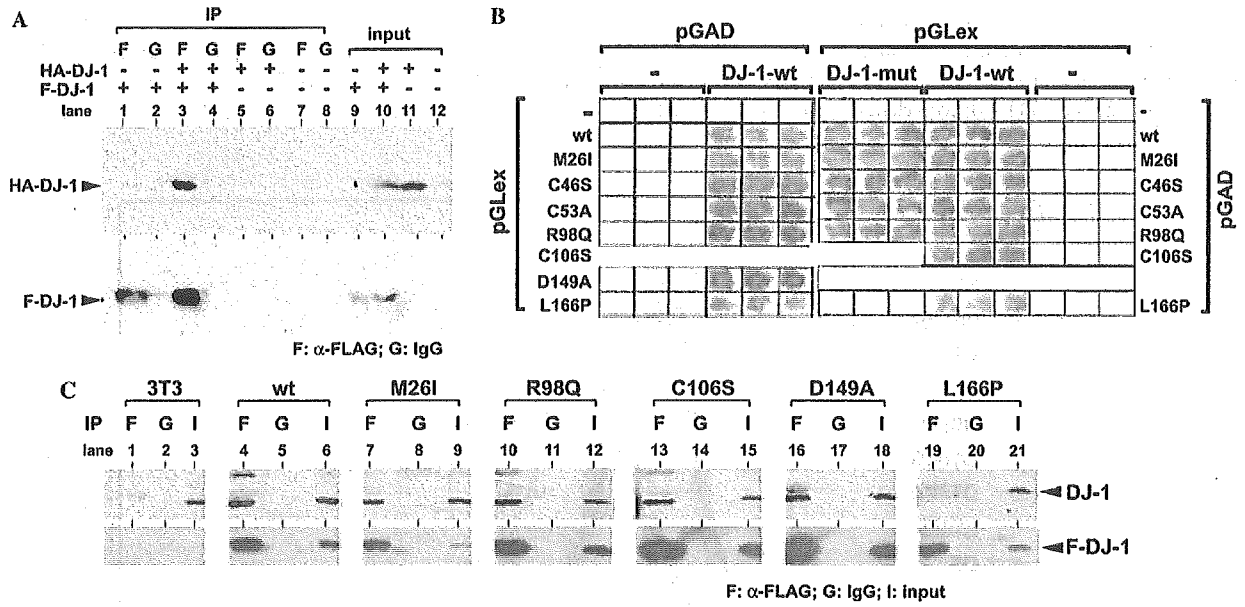

Fig. 1. Dimer formation of DJ-1 and mutants of DJ-1. (A) Human 293T cells were transfected with various combinations of expression vectors for HA-DJ-1 and FLAG-DJ-1. Forty-eight hours after transfection, proteins extracted from transfected cells were immunoprecipitated with an anti-FLAG antibody and blotted with the anti-FLAG antibody or an anti-HA antibody. (B) *Saccharomyces cerevisiae* L40 cells were cotransformed with pGLex-DJ-1 and various mutant constructs fused with the GAL4 activation domain, and the β-galactosidase activity of each colony was tested. Similarly, transformants with pGLex-DJ-1 mutants and pGAD-DJ-1 mutants were also used for binding assays. (C) Proteins extracted from mouse NIH3T3 cell lines harboring FLAG-wild-type DJ-1 and its mutants were immunoprecipitated with an anti-FLAG antibody and blotted with the anti-FLAG antibody or an anti-DJ-1 antibody.

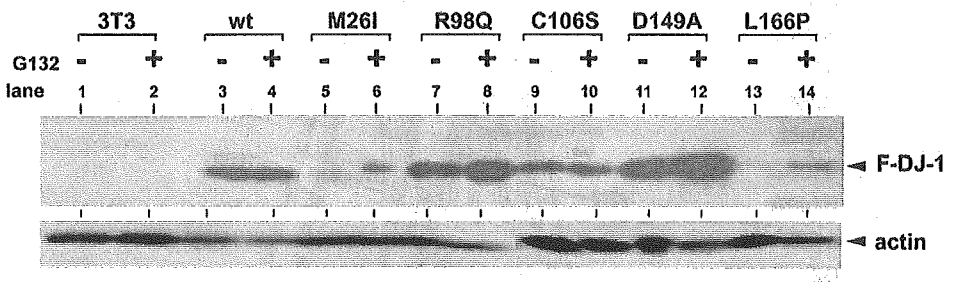

Fig. 2. Stability of DJ-1 and its mutants. Mouse NIH3T3 cell lines harboring FLAG-wild-type DJ-1 and its mutants were cultured in the presence of 10 mM MG132 for 12 h. Proteins extracted from cells were blotted with anti-FLAG and anti-actin antibodies. 3T3 indicates parental NIH3T3 cells.

Stabilities of mutants of DJ-1

The L166P mutant of DJ-1 has been shown to be unstable and to be degraded, in part, by the ubiquitin–proteasome system [38–41]. To examine stability of other mutants of DJ-1 in cells, NIH3T3 cell lines harboring exogenously added Flag-tagged wild-type or various mutants of DJ-1 were cultured in the presence or absence of proteasome inhibitor, MG132, and proteins extracted with these cell lines were blotted with anti-FLAG and anti-actin antibodies (Fig. 2). Proteins in parental NIH3T3 cells did not react with an anti-FLAG antibody (Fig. 2, lanes 1 and 2). In the absence of MG132, the amounts of M261 and L166P DJ-1 were found to be smaller than that of wild-type DJ-1, and amount of D149A was larger than that of wild-type DJ-1. In the presence of MG132, on the other hand, amounts of all of the mutants of DJ-1 found in Parkinson's disease patients increased, while the amounts of wild-type DJ-1 and C106S, a protease-minus mutant that was not found in Parkinson's disease patients, were not affected by MG132. These results suggest that all of the mutants of DJ-1 in Parkinson's disease patients were degraded, in part, by the proteasome system and that D149 DJ-1 is much more stable than is wild-type DJ-1.

Reduced activities of mutants of DJ-1 against H_2O_2-induced cell death

Reactive oxygen species (ROS) are constantly produced and their scavenging systems are also present in cells to maintain cell homeostasis against over-produced