Table I. Plasmid construction.

Plasmid	Template	5′-primer	3′-primer	Restriction enzyme site	Plasmid inserted
pGADGHx-HIPK1β	EST clone (Ac. BC033012)	hHIPK1 ATG	HIPK1β stop	EcoRI, XhoI	pGADGHx
pGADGHx-HIPK1β(sc)	EST clone (Ac. BC033012)	hHIPK1sc	HIPK1β stop	EcoRI, XhoI	pGADGHx
pGADGHx-HIPK1α(sc)	EST clone (Ac. BC036057)	hHIPK1sc	HIPK1α stop	EcoRI, XhoI	PGAD-GHx
pGADGHx-HIPK2	pcDNA-F-HIPK2	hHIPK2 ATG	hHIPK2 BglII anti	EcoRI, XhoI	PGAD-GHx
pGADGHx-HIPK1α	EST clone (Ac.BC033012)	hHIPK1 ATG	HIPK1β stop	EcoRI, XhoI	PCMV-Flag
pCMV-F-HIPK1α(SC)	EST clone (Ac.BC036057)	hHIPK1sc	HIPK1α stop	EcoRI, XhoI	pCMV-Flag
pCMV-F-HIPK1α	EST clone (Ac.BC033012)	hHIPK1 ATG	HIPK1β stop	EcoRI, XhoI	pCMV-Flag

Combinations of plasmids constructed, primers, templates and restriction enzyme sites and plasmids inserted were shown.

RT–PCR

Total testis RNAs were prepared from human tissues (OriGene) or cultured cells by the acid guanidine thiocyanate–phenol–chloroform method, and cDNA was synthesized using the oligo dT primer and BcaBEST polymerase (Takara Co., Ltd). The first strand of cDNA products of HIPK1α and HIPK1β was amplified with specific primers for the first 5 min at 94°C and then for 34–38 cycles of 1 min at 94°C, 1 min at 62°C and 1.5 min at 72°C. The first strand of cDNA products of DJ-1 was amplified with specific primers for the first 5 min at 94°C and then for 28 cycles of 1 min at 94°C, 2 min at 58°C and 3 min at 72°C.

Nucleotide sequences of the oligonucleotide used for PCR primers were as follows:

GAPDH-sense, 5′-TGAAGGTCGGAGTCAACG-GATTTGGT-3′; GAPDH-antisense, 5′-CATGT GGGCCATGAGGTCCACCAC-3′; HIPK1-RT-sense, 5′-TCTTTGAGCAGCCCTTATTCCACT-3′; HIPK1-1-RT-antisense, 5′-GGGAGAAAAGATGAG-CAATGGAGC-3′; HIPK1-2-RT-antisense, 5′-GGG-GCTTCATTTGGCTTTATTCAG-3′; Human DJ-1-sense, 5′-GGTGCAGGCTTGTAAACATATA AC-3′; Human DJ-1-antisense, 5′-CTCTAAGTGAT CGTCGCAGTTCGC-3′. The amplified products were separated on a 2% agarose gel and stained with ethidium bromide.

Indirect immunofluorescence

H1299 cells were transfected with 5 μg each of pcDNA3-DJ-1 and pEGFP-HIPK1α by the calcium phosphate precipitation method [50]. Forty-eight hours after transfection, the cells were fixed with a solution containing 4% paraformaldehyde and reacted with a combination of a mouse anti-Flag-monoclonal antibody (M2, Sigma) and an anti-DJ-1 polyclonal antibody [1]. The cells were then reacted with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG and observed under a confocal laser fluorescent microscope.

Knockdown of genes by siRNA and cell viability assay

H1299 cells were cultured in a 96-well plate and transfected with 80 pmole/1.2 × 10^6 cells of siRNAs targeting DJ-1 and HIPK1 genes using Lipofectamine 2000 (Invitrogen) according to the supplier's manual. At 24 h after transfection, the cells were treated with various amounts of hydrogen peroxide for 24 h and cell viability was then measured by an MTT assay using a cell counting kit-8 (DOJINDO). For RT–PCR and Western blot analysis, H1299 cells in a 6-cm dish were transfected with 80 pmole of siRNAs, and total RNAs and proteins were prepared at 72 and 48 h after transfection, respectively. The nucleotide

sequences for siRNAs targeting human DJ-1, human HIPK1 and luciferase genes were DJ-1: 5′-UGGAG-ACGGUCAUCCCUGUdTdT-3′ (upper strand) and 3′-dTdTACCUCUGCCAGUAGGGACA-5′ (lower strand) HIPK1: 5′-GUUUCAACUGGACUACAA-GCAACA-AG-3′ (upper strand) and 3′-UACAAA-GUUUGACCUGAUGUUCGUUGU-5′ (lower strand), and luciferase: 5′-CGUACGCGGAAUA-CUUCGAdTdT-3′ (upper strand) and 3′-dTdTGC-AUGCGCCUUAUGAAGCU-5′ (lower strand). The siRNAs were synthesized by Greiner, Japan.

Two-hybrid assay

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 [10].

Results and discussion

Identification of HIPK1 as a DJ-1-binding protein

We have reported that DJ-1 binds to PIASxα and DJBP and that it regulates AR transcription activity [10,11]. In addition to PIASxα and DJBP, cDNA spanning amino acids 776–1210 of human homeodomain-interacting protein kinase 1 (HIPK1) was obtained by a yeast twohybrid screening. Mouse HIPK1 has been identified to be a protein interacting with the homeoproteins NKx-1.2 and NK-1 to act as a transcriptional cofactor (repressor) [51]. After a search of genomic and cDNA databases, we found two splicing isoforms of human HIPK1 and tentatively named them HIPK1α and HIPK1β. The human HIPK1 gene comprises 16 exons, and HIPK1α and HIPK1β are comprised of 1210 and 1075 amino acids corresponding to 1–16 and 1–15 exons, respectively (Figure 1A). After a nucleotide database search, EST clones of accession Nos. BC036057 and BC033012 were found to have the entire sequences of human HIPK1α and HIPK1β, respectively. HIPK1α and HIPK1β are composed of several domains, including a protein kinase domain, homeoprotein-interaction domain and PEST domain (Figure 1A) [51]. Since there is no report on human HIPK1β, the expressions of HIPK1 mRNAs were first examined by RT–PCR using various RNAs extracted from human tissues (Figure 1B and C). The results showed that human HIPK1α mRNA, like human DJ-1 mRNA, was expressed ubiquitously in all of the tissues tested. HIPK1β was, on the other hand, found to be strongly expressed in the testis (Figure 1C).

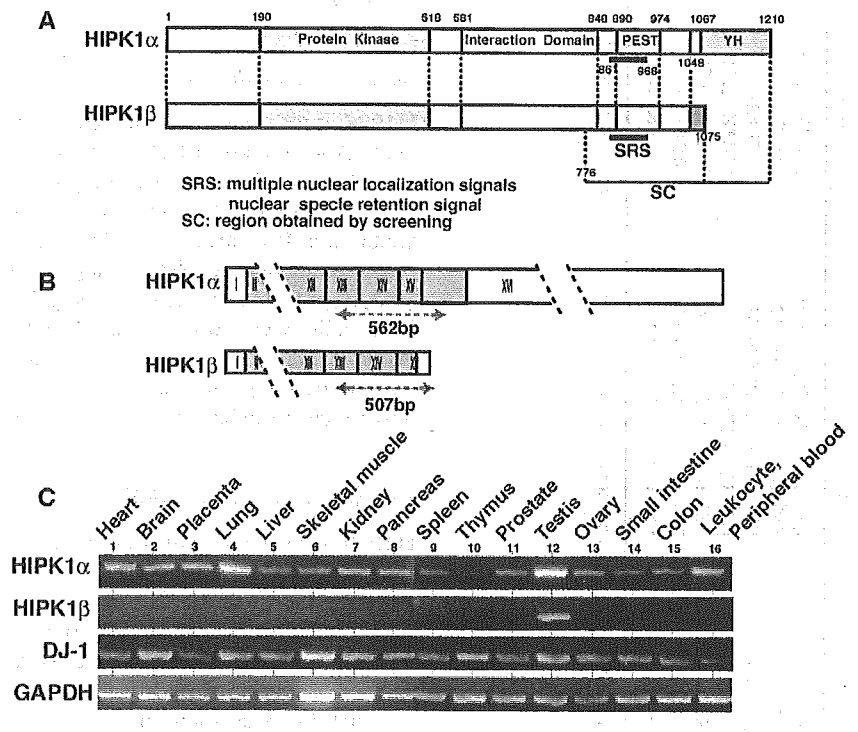

Figure 1. Expression of HIPK1 mRNA in human tissues. **(A)** Structures of HIPK1α and HIPK1β are shown. "YH" indicates the domain rich in tyrosine and histidine. **(B)** Positions of primers used for RT–PCR to amplify DNAs of HIPK1α and HIPK1β were shown. Numbers written in Greece letters indicate the numbers of corresponding exons. Sizes of PCR products corresponding to HIPK1α and HIPK1β to be amplified are 562 and 507 base pairs, respectively. **(C)** Human total RNAs were obtained from OriGene Technologies, and the expressions of HIPK1α and HIPK1β were analyzed by RT–PCR with specific primers for each HIPK1 isoform, DJ-1 and GAPDH on total RNA as substrates. The amplified DNAs were separated in 2% agarose gels and visualized under UV light.

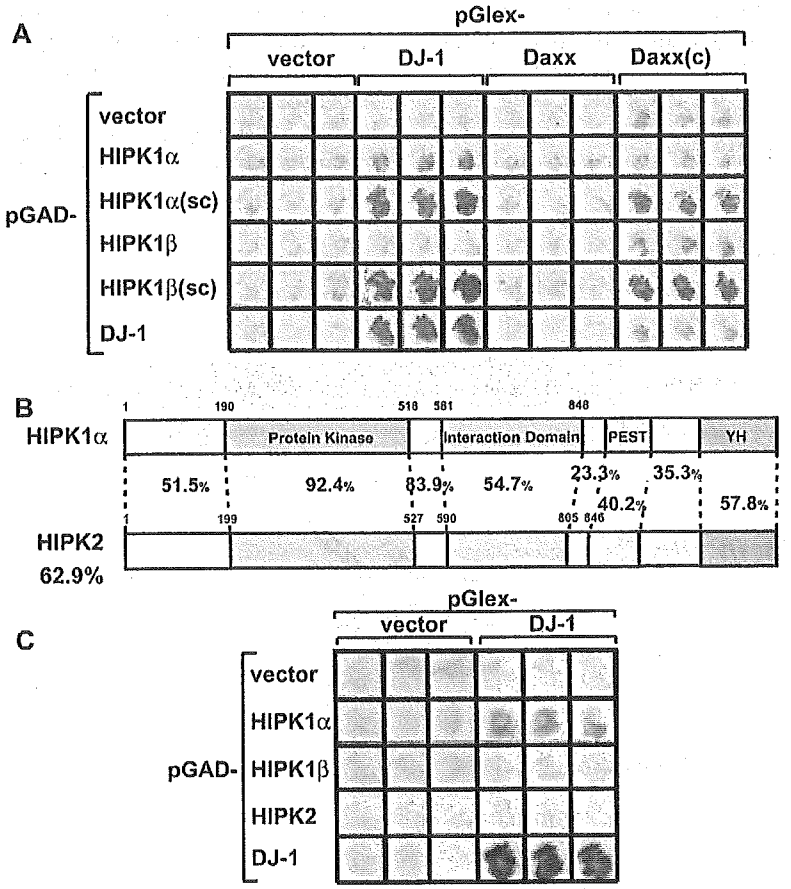

Figure 2. Associations of DJ-1 with HIPK1α and HIPK1β. **(A)** Interactions between DJ-1 with HIPK1α and HIPK1β were analyzed in a yeast twohybrid assay using a DJ-1 fusion protein with the LexA DNA-binding domain as bait and HIPK1-fusion proteins with the GAL4-activation domain as prey as described in "Materials and methods". Results obtained by using fusion proteins of the full-sized and C-terminal region of Daxx, Daxx and Daxx (c), respectively, with the LexA DNA-binding domain and the LexA DNA-binding domain alone as bait are also shown. HIPK1α(sc) and HIPK1β(sc) indicate the regions of HIPK1α and HIPK1β obtained by the screening. **(B)** Structures of HIPK1α and HIPK2 are schematically shown, and identity of amino acids in each domain is also shown. **(C)** Interactions of DJ-1 with HIPK1α and HIPK2 were analyzed in a yeast two-hybrid assay using a DJ-1 fusion protein with the LexA DNA-binding domain as bait and HIPK-fusion proteins with the GAL4-activation domain as prey as described in A. Results obtained by using fusion proteins of DJ-1 with the GAL4-activation domain and with the LexA DNAbinding domain as bait are also shown.

Associations of DJ-1 with HIPK1s

Associations of DJ-1 with HIPK1s were then examined by using a yeast two-hybrid assay, where DJ-1 and HIPK1s were fused to the LexA DNA-binding domain and GAL4 activation domain, respectively (Figure 2A). Since the C-terminal domain of a death domain-associated protein (Daxx) has been reported to associate with HIPK1 [45,49] and DJ-1 made a homodimer, the full-sized and C-terminal region of Daxx and DJ-1 were used as positive controls. While the C-terminal fragments of HIPK1α and HIPK1β (HIPK1α(sc) and HIPK1β(sc)), which were obtained in a screening of DJ-1-binding proteins, were found to strongly bind to both DJ-1 and the C-terminal region of Daxx (Daxx(C)), full-sized HIPK1α but not HIPK1β was found to bind to DJ-1. Binding activities of DJ-1, full-sized HIPK1α and HIPK1β to Daxx were not clear in this two-hybrid assay. HIPK1 is a member of the HIPK family of proteins comprised of HIPK1, HIPK2 and HIPK3. Since HIPK2, whose amino acid composition is 62.9% homologous to that of HIPK1α (Figure 2B), has been well characterized, interaction of DJ-1 with HIPK2 was also examined by the yeast two-hybrid assay (Figure 2C). The results showed that DJ-1 bound to full-sized HIPK1α but not HIPK2. We therefore concentrated on HIPK1α in this study.

Association of DJ-1 with HIPK1α in cells was then examined. To do this, Flag-tagged HIPK1α was transfected into human H1299 cells. Forty-eight hours after transfection, cell extracts were prepared from transfected cells, proteins in the extracts were immunoprecipitated with an anti-DJ-1 antibody or nonspecific IgG, and the precipitates were analyzed by Western

blotting with anti-Flag or anti-DJ-1 antibody (Figure 3A). The anti-DJ-1 antibody, but not IgG, was first confirmed to precipitate endogenously expressed DJ-1 (Figure 3A, lower part). The results also showed that Flag-HIPK1α was co-immunoprecipitated with DJ-1 (Figure 3A, upper part), indicating that DJ-1 is associated with HIPK1α in H1299 cells. Association of DJ-1 with Flag-HIPK1α was also observed in human 293T cells (data not shown). *In vitro* binding assays were then carried out using 35S-labeled HIPK1α(sc) that had been synthesized in a reticulocyte lysate and GST-DJ-1 or GST that had been

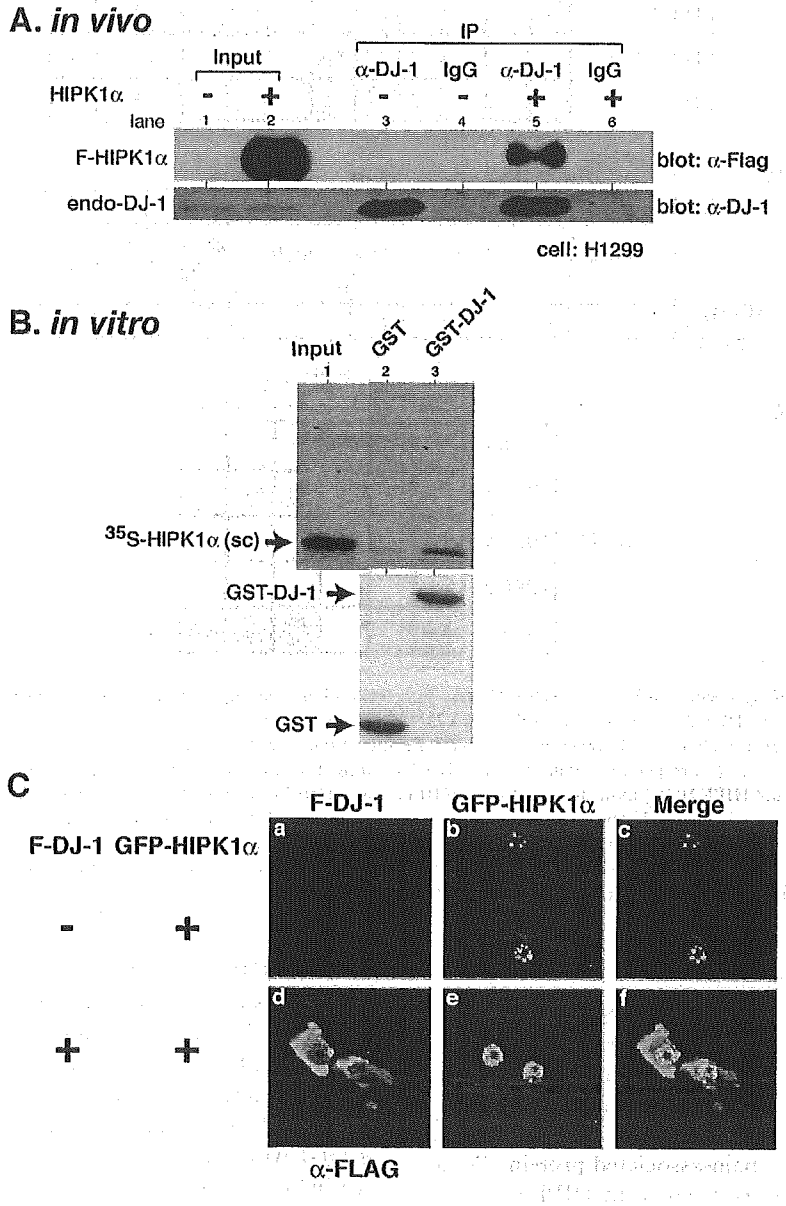

Figure 3. Interaction and colocalization of DJ-1-1 with HIPK1α, a splicing variant of HIPK1. (**A**) Human H1299 cells were transfected with an expression vector for Flag-HIPK1α. Fortyeight hours after transfection, whole cell extracts were prepared and then immunoprecipitated with an anti-DJ-1 antibody or non-specific IgG. Immunoprecipitates were then analyzed by Western blotting with an anti-Flag or anti-DJ-1 antibody. (**B**) GST or GST-DJ-1 was expressed in *E. coli* BL21(DE3) and applied to glutathione-Sepharose 4B. 35S-HIPK1α(sc) synthesized *in vitro* in a coupled transcription/translation system was then applied to the column. The labeled proteins that had bound to the column were separated in a gel and visualized by fluorography. 1/50 volumes of the labeled S- HIPK1α(sc) used for the binding reaction were applied to the same gel (lane 2 in the upper part of figure). GST and GST-DJ-1 used in this experiment were separated on the gel and stained with coomassie brilliant blue (lower part). (**C**) H1299 cells were transfected with expression vectors for Flag-DJ-1 with or without GFPHIPK1α by the calcium phosphate precipitation technique. Forty-eight hours after transfection, the cells were fixed, reacted with an anti-Flag monoclonal antibody (M2, Sigma), and visualized with a rhodamine-conjugated anti-mouse IgG. GFP-HIPK1α was visualized by self-fluorescence of GFP. The two figures have been merged.

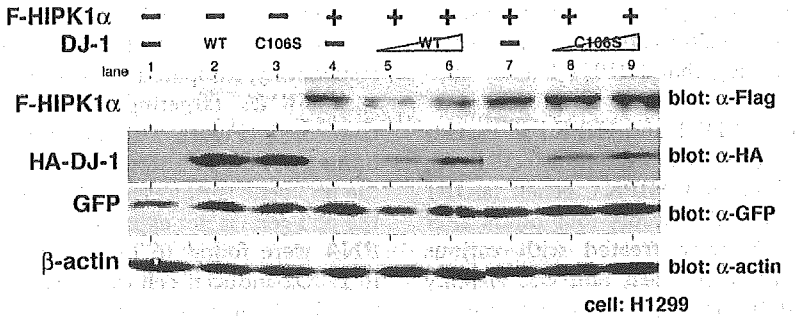

Figure 4. Degradation of HIPK1 by DJ-1. H1299 cells in a 10-cm dish were transfected with 15 μg of pcDNA3-Flag-HIPK1α, 2.5 μg of pEGFP-C1 and various amounts of pcDNA3-HA-DJ-1 or pcDNA3-HA-C106S DJ-1 by the calcium phosphate precipitation technique. Forty-eight hours after transfection, proteins in the cell extracts were subjected to Western blotting with anti-HA (MBL), anti-Flag antibodies (M2, Sigma), anti-GFP (MBL) and anti-actin (Chemicon) antibodies. The amounts of transfected pcDNA3-HA-DJ-1 or pcDNA3-HA-C106S DJ-1 were 1.25 μg (lanes 4 and 7), 2.5 μg (lanes 5 and 8) and 6.25 μg (lanes 6 and 9).

expressed in and purified from *E. coli* (Figure 3B). The results showed that GST-DJ-1 bound to HIPK1α(sc), suggesting that DJ-1 directly binds to HIPK1α.

DJ-1 has been shown to be localized both in the cytoplasm and nucleus in human HeLa cells and is translocated from the cytoplasm to nucleus during the S-phase of the cell cycle upon induction by mitogen [1]. Recent findings have also shown that some DJ-1 is localized in mitochondria [33,37,40] and that mitochondrial localization of DJ-1 is stimulated by oxidative stress [39,41,42]. To determine the cellular localization of HIPK1α and DJ-1, an expression vector for GFP-tagged HIPK1α was cotransfected with Flag-tagged DJ-1 into human H1299 cells. Two days after transfection, the cells were stained with anti-Flag antibody, and Flag-DJ-1 and GFP- HIPK1α were detected by a rhodamine-conjugated second antibody and self-fluorescence of GFP, respectively, and then visualized under a confocal laser microscope (Figure 2C). As previously described [47,49], HIPK1α (green) was localized in the nucleus as speckles, and HIPK1α and DJ-1 (red) were found to be colocalized in the nucleus as shown by the yellow color (Figure 3C, Merge).

Degradation of HIPK1 by DJ-1

During this study, we often observed that the expression of ectopically transfected HIPK1α was not detected by Western blotting when DJ-1 was also transfected into various cell types. Since structural and biochemical analyses of DJ-1 suggest that DJ-1 is a protease [52,53], though controversial results have also been reported [54–56], various amounts of an expression vector for HA-tagged wild-type DJ-1 or C106S mutant DJ-1 were cotransfected with a constant amount of Flag-HIPK1α into H1299 cells. Forty-eight hours after transfection, the expression levels of Flag-HIPK1α and HA-DJ-1 were examined by Western blotting with anti-Flag and anti-HA antibodies (Figure 4). Cysteine at amino acid number 106 of DJ-1 (C106) is not only a putative catalytic center for protease activity of DJ-1 [52] but also an amino acid to be oxidized after oxidative stress [30,33]. C106S mutant DJ-1, a mutant in which there is substitution from cysteine to serine, has been shown to lose activity of DJ-1 against oxidative stress [32–35]. pCMV-EGFP was also cotransfected with DJ-1 and HIPK1α to normalize transfection efficiencies. The results showed that the expression level of Flag-HIPK1α was decreased by transfected HA-wild-type DJ-1, but not by C106S mutant DJ-1, in a dosedependent manner. Since RT-PCR analysis showed that the expression level of mRNA of transfected Flag-HIPK1α was not changed after cotransfection of HA-DJ-1 (data not shown), these results suggest that DJ-1 directly or indirectly degrades HIPK1α.

Effect of reduced expression levels of DJ-1 and HIPK1 on H_2O_2-induced cell death

We and others have shown that DJ-1 protects cells against oxidative stress-induced cell death [31–35]. Pro- and anti-apoptotic functions of HIPK1 have, on the other hand, been reported. When cells were exposed to reagents that damage DNA, nuclear-localized HIPK1 phosphorylated p53 tumor suppressor, leading to activation of p53 transcription activity to trigger apoptosis [45]. When cells were deprived of glucose or receive oxidative stress, HIPK1 phosphorylated nucleus-localized Daxx, and phosphorylated Daxx was then translocated to the cytoplasm to associate with and activate an (ASK-1), resulting in activation of an apoptosis signal [45,49]. Alternatively, after stimulation of apoptosis by tumor necrosis factor (TNF) alpha, sumoylated HIPK1 in the nucleus was desumoylated and translocated to the cytoplasm to associate with ASK1-interacting protein (AIP1), a signal transducer in TNFalpha-induced ASK1 activation, resulting in an apoptosis signal [48]. HIPK1 (−/−) cells that had been established from

HIPK1-knockout mice were, however, more susceptible than HIPK1 (+/+) cells to apoptosis induced by DNA damage, suggesting that HIPK1 has an anti-apoptotic function [46].

To investigate roles of DJ-1 and HIPK1 in H2O2-induced apoptosis, H1299 cells were transfected with siRNAs targeting DJ-1 and HIPK1 genes (siDJ-1 RNA and siHIPK1 RNA). Twenty-four hours after transfection, the cells were treated with various concentrations of H2O2 for 24 h and cell viability was then measured by an MTT assay (Figure 5). The expression levels of HIPK1 and DJ-1 were first examined by RT–PCR, and less than 10% and about 30% of expression levels of DJ-1 and HIK1 mRNAs, respectively, compared to the levels in cells transfected with siRNA targeting the luciferase gene, were confirmed (Figure 5A). As previously described in human SH-SY5Y and mouse NIH3T3 cells, in which the p53 gene is expressed [32], H1299 cells, in which the p53 gene is not expressed, transfected with siDJ-1 RNA were found to be rendered more susceptible to H2O2-induced cell death (Figure 5B), suggesting that DJ-1 protects cells from H2O2-induced cell death in a p53-independent manner. H1299 cells

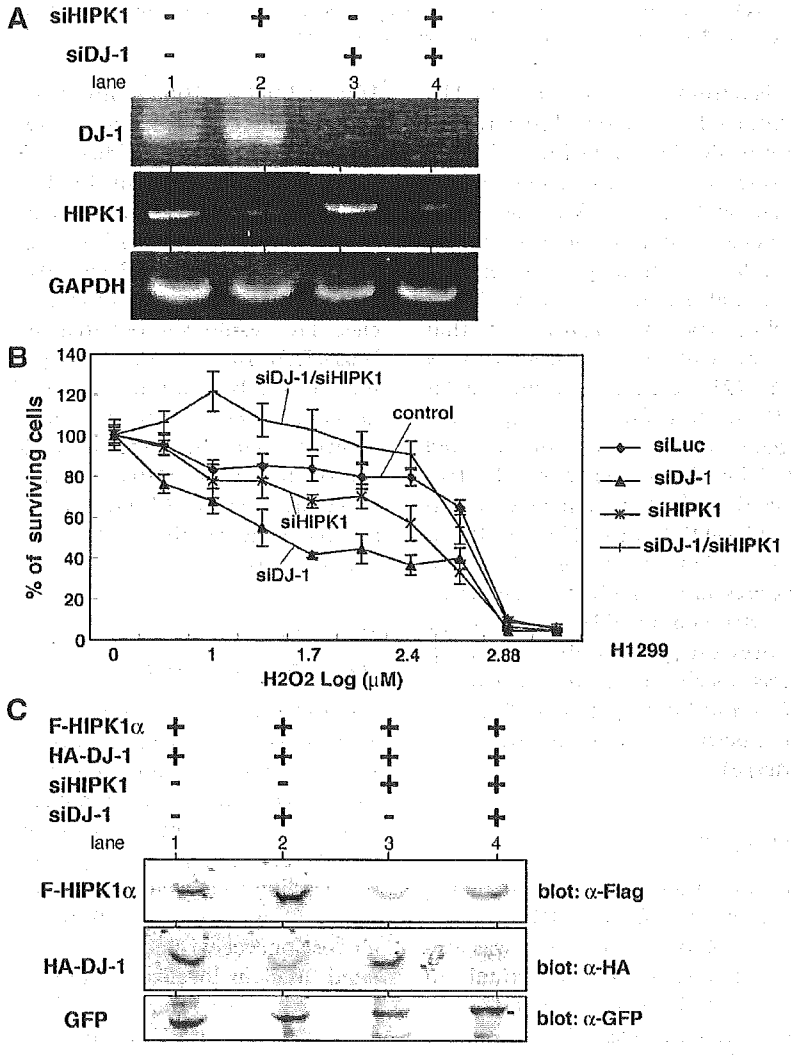

Figure 5. Effect of HIPK1 on H2O2-induced cell death. **(A)** H1299 cells were transfected with siRNAs targeting DJ-1 and HIPK1 genes alone or together using Lipofectamine 2000. At 72 h after transfection, total RNAs were extracted and the expression levels of mRNAs of DJ-1, HIPK1 and GAPDH were examined by RTPCR as described in Materials and methods. **(B)** H1299 cells were transfected with siRNAs targeting DJ-1 and HIPK1 genes alone or together using Lipofectamine 2000. At 24 h after transfection, cells were treated with various amounts of H2O2 for 24 h and cell viability was then measured by an MTT assay using a cell counting kit-8. The amounts of H2O2 used were 5, 10, 25, 50, 100, 250, 500, 750 and 1000 μM. **C** H1299 cells in a 6-cm dish were transfected with 3 μg each of pcDNA3-Flag-HIPK1α, pcDNA3-HA-DJ-1 and pEGFP-C1 together with 80 pmol of siRNAs targeting DJ-1 and HIPK1 genes alone or together using Lipofectamine 2000. At 48 h after transfection, proteins in the cell extracts were subjected to Western blotting with anti-HA (MBL), anti-Flag antibodies (M2, Sigma) and anti-GFP (MBL) antibodies.

transfected with siHIPK1 RNA were found to be susceptible to H2O2-induced cell death, but the extent of susceptibility was less than that after transfection of siDJ-1 RNA into cells, suggesting that HIPK1 also plays a role in anti-oxidative stress and that weak susceptibility of siHIPK1-transfected cells to H2O2-induced cell death is affected by low knock-down efficiency of siHIPK1 RNA. H1299 cotransfected with siDJ-1 and siHIPK1 RNAs were, however, found to be more resistant to H2O2-induced cell death. Since DJ-1 degrades HIPK1α as shown in Figure 4, it is thought that the elevated level of HIPK1 induced by DJ-1-knockdown protected H1299 cells from H2O2-induced cell death. We tried several times to make antibodies against HIPK1 using GST-HIPK1 or a synthetic peptide corresponding to the amino acids of HIPK1 as immunogens, but we did not succeed in establishing an anti-HIPK1 antibody that detects endogenous HIPK1. Commercially available anti-HIPK1 antibodies also did not work. To evaluate the effect of DJ-1-knockdown on the amount of HIPK1, therefore, Flag-tagged HIPK1α, HA-tagged DJ-1 and GFP were cotransfected with various combinations of siRNAs targeting HIPK1 and DJ-1. Forty-eight hours after transfection, cell extracts were prepared and proteins in the extracts were analyzed by Western blotting with anti-Flag and HA antibodies (Figure 5C). GFP was used as an internal control. As shown in the levels of endogenous mRNAs, the expression levels of Flag-HIPK1α and DJ-1-HA were reduced by transfection of respective siRNA alone (Figure 5C, lanes 2 and 3). When both siHIPK1 and siDJ-1 RNAs were transfected together, the expression level of DJ-1 was decreased but the extent of decrease was more than that after transfection of siDJ-1 RNA alone into cells, and the expression level of HIPK1α was increased, suggesting that degradation of HIPK1α was inhibited by DJ-1-knockdown (Figure 5C, lane 4). The results also suggest that HIPK1 protects H1299 cells against H2O2-induced cell death when the expression level of DJ-1 is extremely low stronger than when the expression level of DJ-1 is high. It is thought that both DJ-1 and HIPK1 play a role in anti-oxidative stress in H1299 cells under this condition and that the balance of the expression levels of DJ-1 and HIPK1 determine susceptibility to H2O2-induced cells death.

In this study, we identified HIPK1 as a DJ-1-binding-protein and found that HIPK1 was degraded by wild-type DJ-1 but not by C106S mutant DJ-1, a mutant lacking a putative catalytic amino acid for protease. Since protease activity of DJ-1 with C106 as a catalytic amino acid has been shown by structural and biochemical analyses [52,53], though controversial results have also been reported [54–56], it is likely that HIPK1 was degraded by DJ-1 directly or indirectly via other proteases that had been activated by DJ-1. We further found that HIPK1, like DJ-1, prevented H1299 cells from H2O2-induced cells death and that knockdown of DJ-1 expression by siRNA resulted in enhancement of its protective activity, due to the elevated expression level of HIPK1. As described above, pro- and anti-apoptotic functions of HIPK1 have been reported [45–51]. These activities have been examined in various cell types that had been deprived of glucose or treated with TNFalpha and DNAdamaging reagents [45–51]. Addition of H2O2 to cells has not been reported. These results suggest that pro- and anti-apoptotic functions of HIPK1 are determined in cell type- and inducer of cell death stimuli-dependent manners.

A number of studies have shown activity of DJ-1 against oxidative stress-induced cell death using various cell types, and at least three mechanisms underlying the anti-oxidative stress function of DJ-1 have been proposed: DJ-1 scavenges oxidative stress-induced ROS [32], DJ-1 regulates expressions of genes encoding proteins that play a role in preventing cell death [15,57], and interaction of DJ-1 with Daxx sequesters Daxx to the nucleus in order to leave ASK-1 in an inactive form [43]. On the other hand, it has been reported that Daxx phosphorylated by HIPK1 was translocated to the cytoplasm to activate ASK-1, leading to activation of the apoptosis pathway [49]. It is thought that when the high expression level of DJ-1 degrades HIPK1, Daxx is not phosphorylated to be sequestered to the nucleus, resulting in inactivation of the apoptosis pathway. DJ-1, therefore, plays a role in anti-oxidative stress by using various pathways.

Acknowledgements

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Roles of distinct cysteine residues in *S*-nitrosylation and dimerization of DJ-1

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Abstract

A significant proportion of early onset parkinsonism is inherited as an autosomal-recessive trait (AR-EP). DJ-1 was identified as one of the causative genes for AR-EP (PARK7), and DJ-1 protein has been implicated in oxidative stress response through oxidation of one of the three cysteine residues (i.e., Cys106). However, the individual roles of these cysteine residues remained unclear. We show by a systematic mutagenesis analysis that Cys46 and Cys53 of DJ-1, but not Cys106, are susceptible to *S*-nitrosylation in vitro as well as in cultured cells. Furthermore, alanine substitution of Cys46 diminished dimerization of DJ-1, a fundamental feature of this protein. These results indicate that distinct cysteine residues of DJ-1 harbor differential roles in relation to its structure and function.

Keywords: DJ-1; Parkinson's disease; *S*-nitrosylation; Dimerization; Neurodegeneration

Parkinson's disease (PD) is one of the most common neurodegenerative diseases affecting substantia nigra and presenting extrapyramidal symptoms including rigidity, tremor, and akinesia. A subset of PD patients manifest the symptoms early in their life (early onset PD: EP), and a significant proportion of EP is inherited as an autosomal-recessive trait (AR-EP). Subsequent to the discovery of parkin as the initial and major responsible gene for AR-EP (PARK2) [1], DJ-1 was identified as a causative gene for another type of AR-EP (PARK7) [2]. A number of studies have so far suggested that DJ-1 is a multi-functional protein, which is involved in transforming process of tumor cells [3], positive modulation of the transcriptional activity of androgen receptor [4,5], molecular chaperoning for α-synuclein [6], or cell survival by antagonizing with PTEN [7]. Notably, the demonstration of an increase in the protein level of DJ-1 upon oxidative stress [8,9], as well as the anti-oxidative stress activity of DJ-1 shown in cultured cells that overexpress DJ-1 [10,11], treated by RNAi for DJ-1 [12], or in DJ-1-deficient mice [13] or flies [14–17], strongly suggested that loss of anti-oxidative activity in the nigral neurons in PARK7 patients may cause the premature degeneration of these neurons. These findings also strongly supported the notion that oxidative stress may play a major role in the pathogenesis of PD and parkinsonism, based on genetics of familial PD.

X-ray crystallographic analyses have shown that DJ-1 forms a homodimer and that cysteine (Cys) 106 is selectively oxidized under an oxidative stress condition [18,19]. The specific oxidation of Cys106 upon oxidative stress was also confirmed by a rigorous protein chemical analysis [20]. Crystallographic analyses also showed that YDR533Cp and YajL/ThiJ, homologues of DJ-1 in *Saccharomyces cerevisiae* and *Escherichia coli*, respectively [21,22], form dimers, and the amino acid sequences located at the predicted dimer interface of DJ-1 and its homologues are highly conserved [23]. It has also been shown that L166P mutant DJ-1 linked to PARK7 fails to form a dimer and does not confer resistance to oxidative stress [10,24]. Based

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on these findings, it is widely believed that dimerization is an important feature of DJ-1 that underlies its proper structure and functions.

Besides oxidation, protein *S*-nitrosylation is known as one of the reversible post-translational modifications that target the thiol group of Cys residues in proteins by the attack of nitrogen oxide (NO) [25], the latter being physiologically generated by NO synthase. It has been shown that the biological activities of a wide variety of proteins, e.g., thioredoxin [26], matrix metalloprotease-9 [27], and GAPDH [28], are modulated by *S*-nitrosylation. Protein S-nitrosylation is shown to be facilitated under an oxidative stress condition [29,30]. Notably, it has recently been shown that parkin is *S*-nitrosylated in the brains of patients with sporadic PD, leading to a decrease in its ubiquitin ligase activities [29,30].

These findings led us to examine the individual roles of the three distinct Cys residues in human DJ-1. In this report, we show that DJ-1 also is a target protein of *S*-nitrosylation in vitro and in cultured cells, and that Cys46 and Cys53, but not Cys106, are susceptible to this modification, using the biotin-switch assay, an efficient method to specifically detect protein *S*-nitrosylation [31]. We also show that Cys46 is involved in the dimerization of DJ-1.

Materials and methods

Construction of expression plasmids. A cDNA encoding full-length human DJ-1 fused with an N-terminal FLAG tag in pcDNA3 vector (Invitrogen) was cloned as described (FLAG-DJ-1) [4]. cDNAs encoding mutant DJ-1 harboring Leu166Pro mutation (DJ-1/L166P), DJ-1/C46A, DJ-1/C53A, DJ-1/C106S, and C46A/C53A double mutation (DJ-1/C46/53A), DJ-1/C46/106A, DJ-1/C53/106A, or C46A/C53A/C106A triple mutation (Cys (−)) with N-terminal FLAG tags were generated by the long-PCR protocol using cDNAs encoding FLAG-DJ-1 as a template, and the following oligonucleotides were used as PCR primers: 5′-GGACC AGCTTCGAATTCGCGCCTGCAATTG-3′ for DJ-1/L166P, 5′-CCCA GTACAGGCTAGTCGCGATGTGGTCATTTG-3′ for DJ-1/C46A, 5′-GTGGTCATTGCTCCTGATGCTAGCCTTGAA-3′ for DJ-1/C53A, 5′-CTGATAGCCGCGATCGCTGCAGGTCCTAC-3′ for DJ-1/C106A, 5′-CTGATAGCCGCGATATCTGCAGGTCCTAC-3′ for DJ-1/C106S as forward primers, 5′-CAATTGCAGGCGCGAATTCGAAGCTGGTC C-3′ for DJ-1/L166P, 5′-CAAATGACCACATCGCGACTAGCCTGTA CTGGG-3′ for DJ-1/C46A, 5′-TTCAAGGCTAGCATCAGGAGC AATGACCAC-3′ for DJ-1/C53A, 5′-GTAGGACCTGCAGCGATCGC GGCTATCAG-3′ for and DJ-1/C106A, 5′-GTAGGACCTGCAGATA TCGCGGCTATCAG-3′ for DJ-1/C106S as reverse primers. A cDNA encoding N-terminally myc-tagged DJ-1 (myc-DJ-1) was generated by PCR using *Platinum* Pfx (Invitrogen), and the following oligonucleotides were used as PCR primers: 5′-CCCAAGCTTCCACCATGGAACAAAA ACTCATCTCAGAAGAGGATCTGATGGCTTCCAAAAGAGCTC-3′ as a forward primer and 5′-CCGCTCGAGC TAGTCTTTAAGAACAAG-3′ as a reverse primer. The amplified cDNA was subcloned into pcDNA3 vector. cDNAs encoding DJ-1/L166P, DJ-1/C46A, DJ-1/C53A, and DJ-1/C106S fused with N-terminal myc tags were generated by the long-PCR protocol using a cDNA encoding myc-DJ-1 in pcDNA3 as the template, using primer pairs described above.

Cell culture and transfection. Monkey COS-1 cells and human SH-SY5Y neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C in 5% CO_2 atmosphere. Transient expression in COS-1 cells was performed by the DEAE–dextran method, and stable SH-SY5Y cell lines were generated by transfecting the cDNAs in pcDNA3 vector using LipofectAMINE (Invitrogen) and selection in DMEM containing G418 (Calbiochem) at 500 μg/ml.

Antibodies and immunochemical analyses. The rabbit polyclonal antibody against the C-terminal region of human DJ-1 was generated by Nakagawa as described [8]. Other antibodies were purchased from Sigma (anti-FLAG M2 monoclonal, anti-FLAG polyclonal), Cell Signaling Technology (anti-myc 9B11 monoclonal), and Santa Cruz Biotechnology (anti-myc polyclonal), respectively. Sample preparations and analyses, including immunoblot analysis and co-immunoprecipitation, were performed as previously described [32,33], except for using 0.1% Triton X-100 as a detergent.

Biotin-switch assay. S-Nitrosylated proteins were detected using the biotin-switch assay as described by Jaffrey et al. [31] with some modifications. Briefly, samples that were prepared in HEN buffer (250 mM Hepes–NaOH, 1 mM EDTA, and 0.1 mM neocuproine, pH 7.7) containing 1% Triton X-100 were first incubated with *S*-nitrosoglutathione (GSNO; Sigma) or glutathione (GSH; WAKO) in darkness for 1 h at room temperature. The samples were then subjected to acetone precipitation to remove the NO donor and re-solubilized with 200 μl HENS buffer (HEN buffer containing 1% SDS) followed by addition of 2 μl of 2 M methyl methane thiosulfonate (MMTS; Pierce) to block the free thiol group. The samples were incubated at 50 °C for 20 min with frequent agitation by vortexing. MMTS was then removed by acetone precipitation, and the resultant precipitates were washed once with ice-cold acetone and then re-solubilized in 200 μl HENS buffer. The samples were then incubated with 2 mM ascorbic acid (Sigma) to release the NO from the *S*-nitrosylated thiol group, which was subsequently biotinylated by incubation with 400 μM biotin–HPDP (Pierce). The biotinylated proteins were then precipitated by incubating samples with 50 μl streptavidin–Sepharose beads High Performance (Amersham Biosciences). The streptavidin–Sepharose was then pelleted and washed 3 times with HEN buffer containing 0.5% SDS and 0.5% Triton X-100. The biotinylated proteins bound to the streptavidin beads were eluted by SDS–PAGE sample buffer and subjected to immunoblot analysis.

Crosslinking assays. Cells were rinsed with PBS once and harvested in PBS containing 0.1% Triton X-100. The samples were subsequently treated with 2.5 mg/ml of disuccinimidyl suberate (DSS) and incubated for 1 h at room temperature followed by dialysis against PBS for 8 h twice according to the manufacturer's instructions. The samples were then immunoprecipitated with anti-tag antibodies as indicated and subjected to an immunoblot analysis.

Results

DJ-1 is S-nitrosylated at Cys46 and Cys53 in vitro and in cultured cells

We first examined in vitro *S*-nitrosylation of DJ-1 using *S*-nitrosoglutathione (GSNO) as an NO donor by the biotin-switch assay. Basically, this method enables detection of *S*-nitrosylated thiol group of a Cys residue, by blocking the free thiol moiety by MMTS and then selectively biotinylating the originally *S*-nitrosylated moiety after reduction by ascorbate (covalently oxidized thiol group is not affected throughout the reactions). In the presence of 0.25 or 1 mM GSNO, we observed an extensive *S*-nitrosylation of FLAG-tagged (arrows) and endogenous (arrowheads) DJ-1 in lysates of stably transfected SH-SY5Y cells as revealed by a robust recovery of FLAG- or free DJ-1 by the biotin-switch assay (Fig. 1A, lanes 3 and 4). We next explored whether DJ-1 can be *S*-nitrosylated in vivo in cultured cells. For this purpose, we treated SH-SY5Y cells stably overexpressing FLAG-DJ-1 with GSNO and assessed the extent

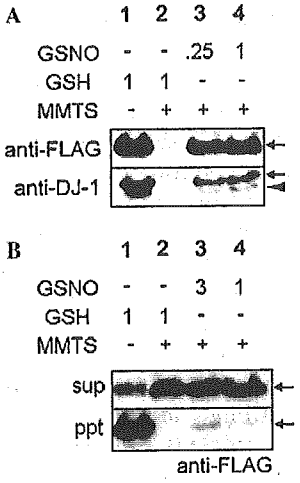

Fig. 1. DJ-1 is *S*-nitrosylated in vitro and in cultured cells in vivo. (A) Lysates from SH-SY5Y cells stably overexpressing wild-type DJ-1 were subjected to in vitro *S*-nitrosylation and analyzed by the biotin-switch assay. Bound fractions to avidin beads were analyzed by immunoblotting with anti-FLAG (upper row) or anti-DJ-1 (lower row) antibodies. (B) SH-SY5Y cells stably overexpressing wild-type DJ-1 were treated with GSNO for 6 h at the indicated concentrations above the panels (mM). Bound ("ppt," lower row) and unbound ("sup," upper row) fractions to streptavidin beads were analyzed by immunoblotting with anti-FLAG antibody. Glutathione (GSH, lanes 1 and 2) was used as a negative control for the NO donor, *S*-nitrosoglutathione (GSNO, lanes 3 and 4). The numbers above each lane for GSNO and GSH indicate the concentration of reagents (mM). Note that lack of DJ-1 pulled down by streptavidin beads by MMTS treatment only (lane 2) indicates complete blocking of free thiol groups by this treatment, ensuring the specificity of detection of *S*-nitrosylated proteins. Arrow, FLAG-DJ-1; arrowhead, endogenous DJ-1.

of *S*-nitrosylation of DJ-1 by the biotin-switch assay. FLAG-tagged DJ-1 was biotinylated and pulled down by streptavidin beads (Fig. 1B, ppt, lanes 3 and 4, arrow) upon prior exposure to 1 or 3 mM GSNO, suggesting that DJ-1 can also be *S*-nitrosylated in vivo in cultured cells.

Previous reports have shown that DJ-1 is preferentially oxidized at Cys106 [18–20]. To examine which of the three Cys residues in DJ-1 is targeted to *S*-nitrosylation, we systematically mutated Cys residues of DJ-1 in various combinations, and overexpressed the Cys mutant (mt) DJ-1 in COS cells. We then treated the cell lysates by GSNO to induce *S*-nitrosylation of DJ-1 in vitro and detected *S*-nitrosylation of Cys mt DJ-1 by the biotin-switch assay. All the DJ-1 proteins harboring a single Cys substitution (i.e., at C46A, C53A or C106S) were *S*-nitrosylated, the C53A mt being least *S*-nitrosylated, followed by the C46A mt (Fig. 2A, lane 6). We then examined *S*-nitrosylation of the double Cys mt DJ-1. DJ-1 substituted at both Cys46 and Cys53 to alanine (C46/53A) was not *S*-nitrosylated (Fig. 2B, lane 6), whereas DJ-1 harboring either Cys46 or Cys53 (C46/106A, C53/106A) was *S*-nitrosylated, the C46/106A mt being less *S*-nitrosylated compared to C53/106A (Fig. 2B, lane 6). Taken together, we concluded that DJ-1 is *S*-nitrosylated at Cys46 and Cys53, but not at Cys106.

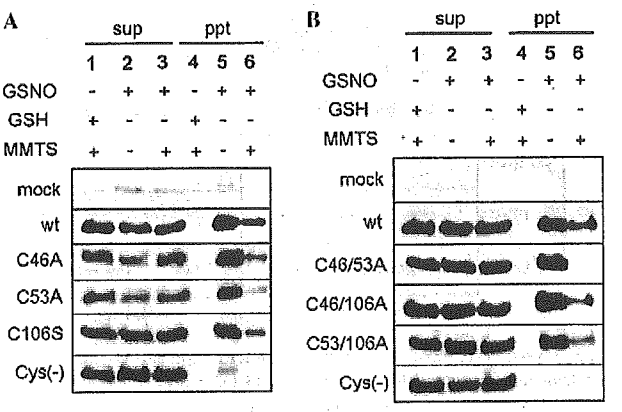

Fig. 2. DJ-1 is *S*-nitrosylated at Cys46 and Cys53. COS cells were transiently transfected with FLAG-DJ-1 harboring Cys substitutions at the indicated residues (A, single mutants; B, double mutants; Cys (−), triple mutant lacking all Cys residues), and the cell lysates were treated with GSNO or GSH in vitro as indicated above each lane. Bound ("ppt," lanes 4–6) and unbound ("sup," lanes 1–3) fractions to streptavidin beads were analyzed by immunoblotting with anti-FLAG antibody. Note that lane 6 represents the results of the biotin-switch assay. A faint band in lane 5 of Cys (−) is a non-specific one.

Dimer formation of DJ-1 is compromised by the C46A mutation

Based on the X-ray crystallographic analysis, wild-type (wt) DJ-1 has been shown to form a dimer: Cys53 residue was found to be located at the interface of the two DJ-1 proteins and Cys46 also resided close to the interface [18,19,23]. To examine the individual roles of these Cys residues on dimer formation of DJ-1, we generated wt FLAG- or myc-DJ-1 as well as C46A, C53A or C106S mt FLAG- or myc-DJ-1, and subjected these mutants to a co-immunoprecipitation analysis. Wt FLAG-DJ-1 and myc-DJ-1 doubly expressed in COS-1 cells were co-immunoprecipitated, whereas L166P mt FLAG-DJ-1 and myc-DJ-1 linked to PARK7 were not co-immunoprecipitated with each other, as previously described [34] (Fig. 3). C53A mt FLAG-DJ-1 was immunoprecipitated with C53A mt myc-DJ-1, as did C106S mt FLAG-DJ-1 and myc-DJ-1. In sharp contrast, C46A mt FLAG-DJ-1 and myc-DJ-1 showed only a trace amount of co-immunoprecipitation compared to wt or other Cys mt DJ-1, implicating Cys46 in the dimer formation of DJ-1 (Fig. 3). We further investigated the association of C46A mt DJ-1 by a chemical crosslinking analysis, using disuccinimidyl suberate (DSS), a primary amine-specific crosslinker that covalently crosslinks proteins with its interactors. We treated wt DJ-1 with DSS and then immunoprecipitated the covalently linked protein complexes, and found a clear band shift of DJ-1 to a position of ~50 kDa on immunoblots, which was equivalent to the size of a DJ-1 dimer, consistent with the results of a simple immunoprecipitation analysis (Fig. 4, DSS (+), arrow). In contrast to the results of simple co-immunoprecipitation, the intensity of the shifted band of C46A mt DJ-1 upon DSS treatment was

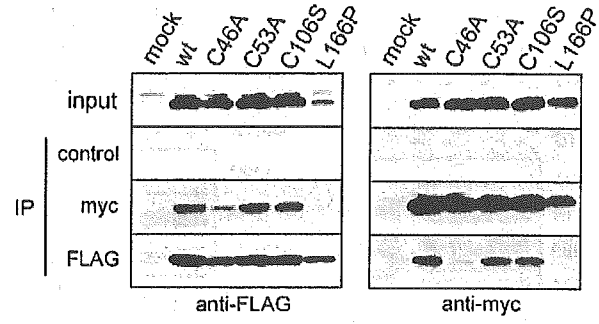

Fig. 3. Cys46 is required for the proper dimerization of DJ-1. COS-1 cells were transiently co-transfected with FLAG-DJ-1 and myc-DJ-1 of wild-type (wt), or those harboring corresponding Cys (C46A, C53A, and C106S) or L166P mutations. The cell lysates were immunoprecipitated with anti-myc or anti-FLAG antibodies (3rd and 4th rows from top, respectively), and then analyzed by immunoblotting with anti-FLAG (left panel) or anti-myc (right panel) antibodies, respectively. Control, immunoprecipitation using an irrelevant mouse IgG (LB509). Input shows the immunoblotting of fractions subjected to immunoprecipitation.

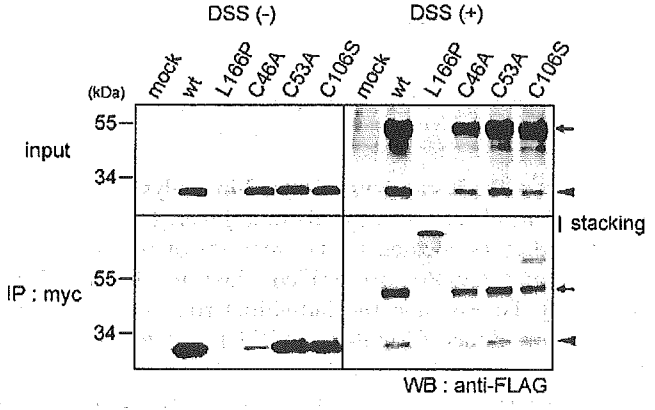

Fig. 4. Dimerization of DJ-1 assessed by co-immunoprecipitation followed by chemical crosslinking with DSS. Lysates of COS-1 cells co-transfected with wt or mt FLAG- and myc-DJ-1 (as in Fig. 3) were immunoprecipitated by anti-myc antibody, and analyzed by immunoblotting with anti-FLAG antibody, with (right panel) or without (left panel) crosslinking with DSS. The upper panel (input) shows the immunoblotting of fractions subjected to immunoprecipitation. Arrow, crosslinked DJ-1 dimer; arrowhead, DJ-1 monomer. Molecular mass standards are shown in kilodaltons at the left of the panel.

similar to that of wt DJ-1 (Fig. 4, DSS (+), arrow). These results suggest that substitution of Cys46 disrupts the physical interaction of two DJ-1 polypeptides, although the positioning of the two polypeptides may be relatively preserved, thus allowing the chemical crosslinking of the dimer in the presence of an excess amount of a crosslinker.

Discussion

Human DJ-1 protein harbors three Cys residues, two of which (i.e., Cys46 and Cys106) are evolutionarily highly conserved [23], suggesting the functional and structural importance of these residues. In the present study, we show that (i) Cys46 and Cys53 are susceptible to *S*-nitrosylation, and that (ii) Cys46 is required for the proper dimerization of DJ-1.

Protein chemical and crystallographic analyses have shown that Cys106 of DJ-1 is preferentially oxidized under an oxidative condition [18–20]. In this regard, the possibility that the exclusive detection of *S*-nitrosylation at Cys46 and Cys53 may reflect the complete and irreversible masking of the thiol group of Cys106 by an extensive oxidation upon overexpression in cultured cells, resulting in insusceptibility to *S*-nitrosylation of this residue. However, our observation that a significant amount of C46/53A double mt DJ-1 was biotinylated and pulled down upon omission of MMTS treatment that blocks the free thiol group (Fig. 2B, lane 5) strongly indicates that sufficient amount of free (i.e., non-oxidated) Cys106 was retained in the C46/53A mutant DJ-1 proteins, arguing against the liability of Cys106 to be *S*-nitrosylated. *S*-nitrosylation has been known to preferentially occur at Cys residues flanked by basic and acidic residues [35], although the amino acid sequences around Cys46 or Cys53 of DJ-1 do not fit with these characteristics. Therefore, the propensity of DJ-1 to be *S*-nitrosylated at Cys46 and Cys53 might be due to the three-dimensional dispositions of the Cys residues in DJ-1. It remains to be determined which of these two Cys residues is the major potential *S*-nitrosylation site: the extent of *S*-nitrosylation of these two Cys residues was apparently different between the single or double mutants, which also may be due to the minute differences in three-dimensional arrangements of the Cys residues. The effects of *S*-nitrosylation on the structure (e.g., dimerization) and function (e.g., anti-oxidative activity) of DJ-1 should further be clarified. Since protein *S*-nitrosylation may be facilitated under oxidative stress conditions [29,30], the latter being implicated in the pathophysiology of PD and related disorders [36], it is tempting to speculate that the function and structure of DJ-1 may be altered by *S*-nitrosylation in the brains of patients with sporadic or familial PD, like in the case of *S*-nitrosylation of parkin [29,30].

Cys46 has been predicted to be located close to the dimer interface [18,19,23]. Taken together with our observation that C46A substitution significantly affected the stable dimerization of DJ-1, we speculate that Cys46 plays a critical role in the maintenance of the structure of the DJ-1, especially at the dimer interface. However, the successful crosslinking of C46A mt DJ-1 as a dimer may suggest that the structural alteration of the dimer interface caused by this substitution may be a subtle one. Nonetheless, the fact that such a slight alteration led to a significant reduction in co-immunoprecipitation of dimer implicates Cys46 in the function and structure of DJ-1, which is in agreement with the phylogenetical conservation of Cys46. In contrast, Cys53, which is located at the dimer interface close to each other at a distance of 3.1 Å but not as highly conserved as Cys46 (e.g., unconserved in invertebrates), was not required for the dimerization of DJ-1.

Our observation that distinct Cys residues in DJ-1 harbor different functional and structural roles underscores the need for further investigations into the changes in DJ-1 in relation to oxidative and nitrosative stress conditions, especially in PD and related neurodegenerative conditions.

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