Abnormal oxidative forms of DJ-1 have been found in some patients with sporadic forms of PD [20]. We previously reported that DJ-1 plays a role in the anti-oxidative stress reaction, in which reactive oxygen species (ROS) were eliminated *in vitro* and *in vivo* by oxidizing DJ-1 itself, and that mutations of DJ-1, including various mutations found in PD patients, lead to cell death [31,32]. Other groups also reported anti-oxidative activity of DJ-1 [33–35]. Oxidative stress is caused by ROS. Oxidative stress is an event in which large amounts of ROS are produced by alternative activities of scavenger proteins or by dysfunction of the mitochondrial respiratory chain pathway with reduction of complex I activity. We and other groups have shown that some DJ-1 is located in mitochondria in addition to the cytoplasm and nucleus [34,36]. In addition to its transcriptional activity as a coactivator, loss of an anti-oxidative stress function is therefore, thought to lead to the onset of PD. Recently, translocation of a part of DJ-1 after oxidative stress has been reported [37,38], but the mechanism of translocation is not yet clear.

In this study, we found that DJ-1 was associated with several chaperones, including Hsp70, mitochondrial chaperone mtHsp70/Grp75 and cochaperone CHIP and that DJ-1 mutants found in PD patients were associated with chaperones stronger than wild-type DJ-1. Furthermore, association of DJ-1 with mtHsp70/Grp75 in mitochondria was enhanced after oxidative stress to cells.

Materials and methods

Cells

Human 293T cells and mouse NIH3T3 cells harboring FLAG-tagged wild-type DJ-1 and various DJ-1 mutants [32,39] were cultured in Dulbecco's modified Eagle's medium with 10% calf serum.

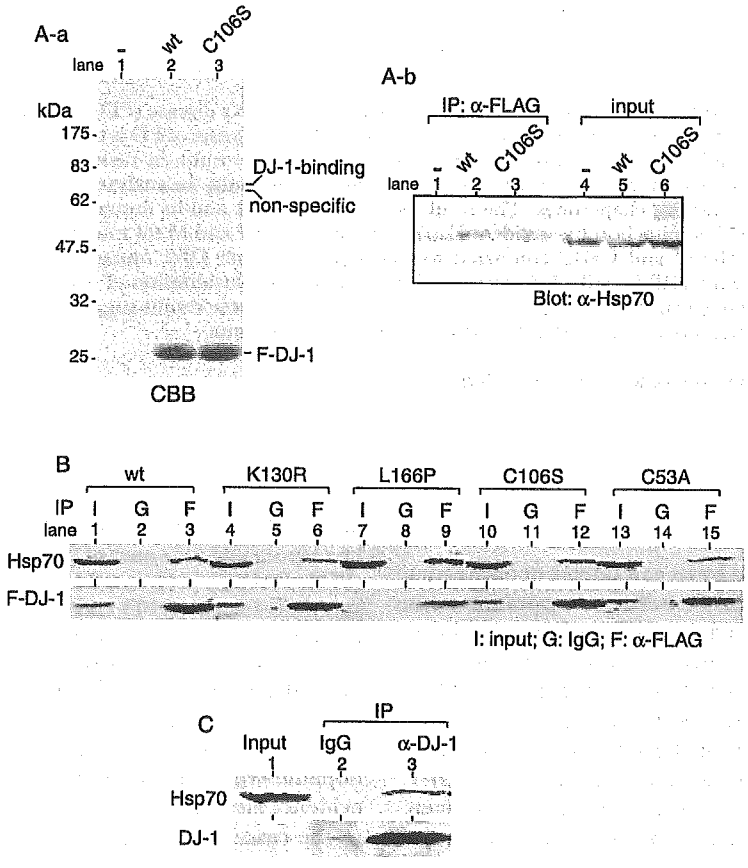

Figure 1. Association of DJ-1 with Hsp70. (A) Human 293T cells were transfected with pcDNA3-F-DJ-1 or pcDNA3-F-C106 by the calcium phosphate precipitation technique. Forty-eight hours after transfection, cell extracts were prepared and immunoprecipitated with an anti-FLAG antibody (M2, Sigma). The precipitates were then separated on a 12% polyacrylamide gel and stained with coomassie brilliant blue (CBB) (A-a). Aliquots of the precipitates were separated in the same gel as that in A-a and analyzed by Western blotting with an anti-Hsp70 polyclonal antibody (SR-B810, MBL) (A-b). (B) Human 293T cells were transfected with pcDNA3-F-DJ-1 or pcDNA3-F-DJ-1 mutants by the calcium phosphate precipitation technique. Forty-eight hours after transfection, proteins in the cell extracts were subjected to Western blotting with anti-Hsp70 (MBL) and anti-FLAG antibodies (M2, Sigma) as described in A. (C) Proteins prepared from 293T cells were immunoprecipitated with the anti-DJ-1 antibody, and the precipitates were analyzed by Western blotting with the anti-Hsp70 antibody.

Indirect immunofluorescence

NIH3T3 cells harboring FLAG-tagged wild-type DJ-1 and various DJ-1 mutants were treated with 10 μM MG132 for 12 h or 100 μM H_2O_2 for 4 h. The cells were then fixed with a solution containing 4% paraformaldehyde and reacted with a combination of rabbit anti-FLAG polyclonal (F7425, Sigma), mouse anti-Hsp70 monoclonal (SR-B810, MBL) or mouse antimtHsp70 monoclonal (ab13529, Abcam) antibodies. In the case of staining, both FLAG-DJ-1 and CHIP, anti-FLAG monoclonal (M2, Sigma) and rabbit anti-CHIP polyclonal (PC711, Oncogene) antibodies were used. The cells were also stained with DAPI. The cells were then reacted with a FITC-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG and observed under a confocal laser fluorescent microscope.

Western blotting

Human 293T cells cultured in 10-cm dish were transfected with 2 μg of expression vectors for FLAG-DJ-1 and FLAG-mutants of DJ-1 by the calcium phosphate precipitation technique. Forty-eight hours after transfection, 1 mg of proteins were extracted from cells with a 0.5% NP-40-containing buffer as described previously [10], immunoprecipitated with 2 μg of an anti-FLAG monoclonal antibody (M2, Sigma) or non-specific IgG, transferred onto a nitrocellulose membrane, and blotted with a 1/2000 dilution of anti-FLAG, 1/1000 dilutions of anti-CHIP (PC711, Oncogene), anti-Hsp70 (SR-B810, MBL) or anti-mtHsp70 (sc-13967, Santa Cruz) polyclonal antibodies. Immunoprecipitated proteins were then reacted with a HRP or IRDye800-conjugated second antibody and visualized by an ECL system or an infrared imaging system (Odyssey, LI-COR), respectively. Approximately, 1.4 mg of proteins extracted from mouse NIH3T3 cells harboring wild-type DJ-1 or mutants of DJ-1 were immunoprecipitated with 2 μg of an anti-FLAG antibody or non-specific IgG and then analyzed by Western blotting with a 1/250 dilution of anti-DJ-1 (ab4150, Abcam), 1/1000 dilutions of anti-FLAG, anti-CHIP, anti-Hsp70 or anti-mtHsp70 antibodies. Four micrograms of proteins prepared from 293T cells were also immunoprecipitated with 2 μg of an anti-DJ-1 polyclonal antibody [1], and the precipitates were analyzed by Western blotting with a 1/1000 dilution of the anti-Hsp70 antibody.

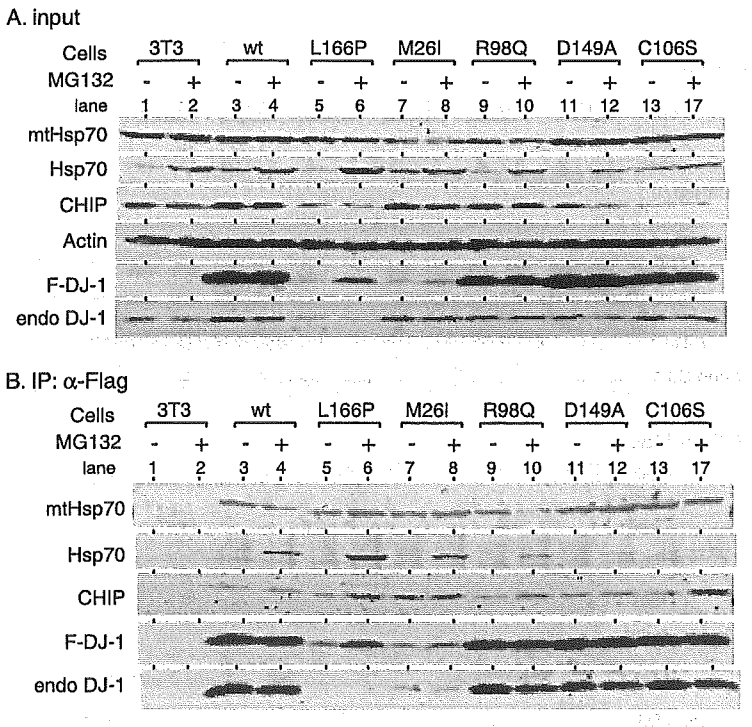

Figure 2. Association of wild-type and mutants of DJ-1 with chaperones in cells cultured in the presence of MG132. (A) Mouse NIH3T3 cells harboring FLAG-wild-type DJ-1 and its mutants were cultured in the presence of 10 μM MG132 for 12 h. Proteins extracted from cells were analyzed by Western blotting with anti-FLAG, anti-DJ-1 (ab4150, Abcam), anti-mtHsp70 (sc-13967, Santa Cruz), anti-Hsp70 (SR-B812, MBL), anti-CHIP (PC711, Oncogene) and anti-actin (PC711, Oncogene) antibodies. 3T3 indicates parental NIH3T3 cells. (B) Proteins extracted from mouse NIH3T3 cells that had been cultured in the presence of 10 μM MG132 for 12 h were immunoprecipitated with an anti-FLAG antibody. The precipitates were then analyzed by Western blotting with anti-FLAG, anti-DJ-1, anti-mtHsp70, anti-Hsp70, anti-CHIP and anti-actin antibodies.

Results

Association of DJ-1 with Hsp70

To identify DJ-1-binding proteins, human 293T cells were transfected with expression vectors for FLAG-tagged wild-type DJ-1 and C106S mutant DJ-1, which is a mutant with a cysteine-toserine substitution corresponding to a catalytic site for putative protease activity [40] and also a mutant lacking anti-oxidative stress activity [32,39]. Forty-eight hours after transfection, protein extracts prepared from transfected cells were immunoprecipitated with an anti-FLAG antibody and the precipitates were stained with coomassie brilliant blue (CBB) after separation of proteins on a polyacrylamide gel (Figure 1A-a). In addition to a band corresponding to FLAG-DJ-1, protein bands that had been coimmunoprecipitated with both wild-type and C106S DJ-1 were observed above non-specific bands (Figure 1A-a, lanes 2 and 3). Since the molecular masses of these bands were approximately 70 kDa and since DJ-1 is thought to have chaperone-like activity [41], we suspected that the 70-kDa protein is Hsp70. To examine this possibility, proteins immunoprecipitated with the anti-FLAG antibody were analyzed by Western blotting with an anti-Hsp70 antibody (Figure 1A-b). The results showed that Hsp70 was co-immunoprecipitated with both FLAG-tagged wild-type and C106S mutant DJ-1.

293T cells were then transfected with expression vectors for FLAG-tagged wild-type and various mutants of DJ-1. In addition to the C106S mutant, three mutants of DJ-1 were used: C53A, a mutant that disrupts the dimer formation of DJ-1; K130R, a mutant with a lysine-toarginine substitution corresponding to a major sumoylation site [10,36]; and L166P, a mutant found in PD patients [16]. Forty-eight hours after transfection, cell extracts were immunoprecipitated with an anti-FLAG antibody and the immunoprecipitates were analyzed by Western blotting with anti-Hsp70 and anti-FLAG antibodies (Figure 1B). FLAG-DJ-1 was first confirmed to be immunoprecipitated with the anti-FLAG antibody (Figure 1B, lanes 3, 6, 9, 12 and 15). It was noted that the amount of FLAG-L166P DJ-1 was less than the amounts of wild-type DJ-1 and other DJ-1 mutants as described previously [39] (Figure 1B, lane 7). Endogenously expressed Hsp70 was found to be coprecipitated with FLAG-DJ-1 in cells that had been transfected with all the expression vectors for DJ-1 and its mutants, and L166P DJ-1 was found to be associated more strongly with Hsp70 than were other DJ-1 mutants (Figure 1B, lane 9), suggesting that Hsp70 plays a role in

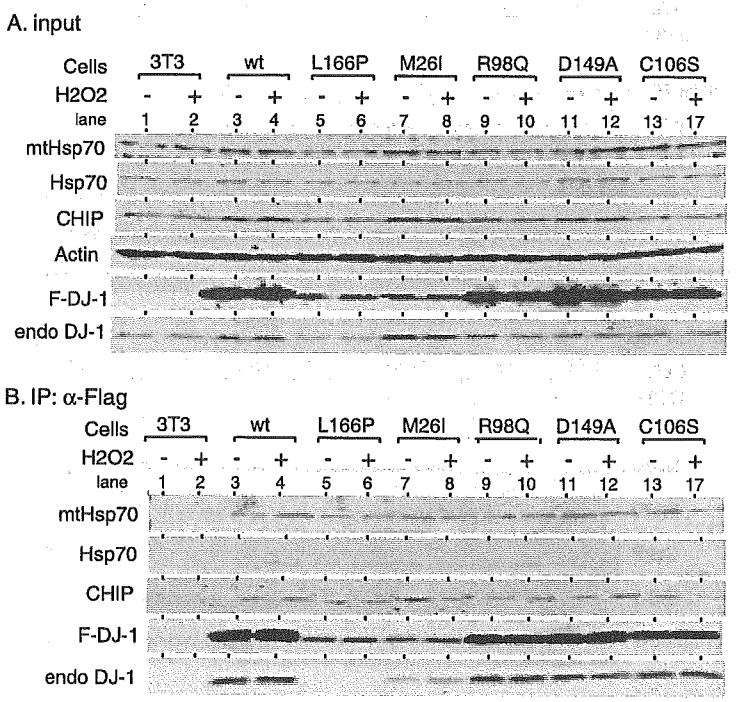

Figure 3. Association of wild-type and mutants of DJ-1 with chaperones in H_2O_2-treated cells. (A) Mouse NIH3T3 cells harboring FLAG-wild-type DJ-1 and its mutants were reacted with 100 μM of H_2O_2 for 4 h. Proteins extracted from cells were analyzed by Western blotting with anti-FLAG, anti-DJ-1, anti- mtHsp70, anti-Hsp70, anti-CHIP and anti-actin antibodies as described in the legend of Figure 2A. 3T3 indicates parental NIH3T3 cells. (B) Proteins extracted from mouse NIH3T3 cells that had been treated with 100 μM of H_2O_2 for 4 h were immunoprecipitated with an anti-FLAG antibody. The precipitates were then analyzed by Western blotting with anti-FLAG, anti-DJ-1, anti-mtHsp70, anti-Hsp70, anti- CHIP and anti-actin antibodies.

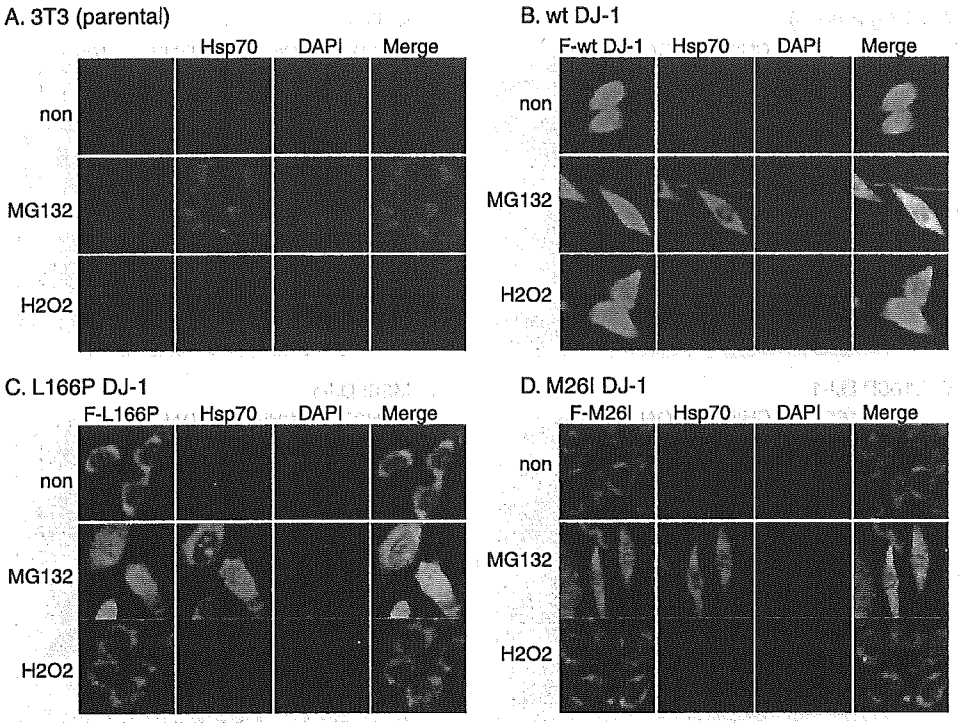

Figure 4. Colocalization of Hsp70 with DJ-1 and its mutants. Mouse NIH3T3 cells harboring FLAG-wild-type DJ-1 and its mutants were reacted with 10 μM MG132 for 12 h or with 100 μM of H_2O_2 for 4 h. The cells were then fixed with paraformaldehyde and reacted with a combination of a rabbit anti-FLAG polyclonal antibody (F7425, Sigma) and mouse anti-Hsp70 (SR-B810, MBL) antibody. Nuclei were stained with DAPI. Cells were visualized under a confocal laser fluorescent microscope as described in "Materials and methods".

refolding or stimulating degradation of L166P DJ-1 due to its aggregated form [36]. Furthermore, endogenously expressed Hsp70 in 293T cells was found to be co-immunoprecipitated with an anti-DJ-1 antibody (Figure 1C), indicating that DJ-1 and Hsp70 are associated in cells.

Effects of a proteasome inhibitor and oxidative stress on association of DJ-1 with chaperones

We and others previously reported that two mutants of DJ-1, L166P [39,42–46] and M26I [38,39] that had been found in PD patients were stabilized by a proteasome inhibitor, MG132 and Hsp70 and CHIP have been shown to bind to unfolded or aggregated proteins to facilitate following protein degradation by the ubiquitin-proteasome system [47]. Furthermore, we and others have also shown that some DJ-1 is located in mitochondria [33,36]. We therefore examined the expression and association of DJ-1 with chaperones, including Hsp70, CHIP and mtHsp70/Grp75, a mitochondria-resident chaperone, in mouse NIH3T3 cells harboring wildtype and various mutants of DJ- that had been treated with MG132 for 12 h (Figure 2). The expression of Hsp70 was found to be induced in all of the cells after MG132 treatment, while expressions of mtHsp70 and CHIP did not change (Figure 2A). As described previously [38,39], reduced levels of exogenously added human FLAG-L166P and M26I DJ-1 were increased after the addition of MG132 (Figure 2A, lanes 6 and 8). It is interesting that the level of endogenously expressed mouse DJ-1 was up- and down-regulated in cells harboring wild-type and L166P DJ-1, respectively (Figure 2A, lanes 3–6), suggesting that expression of the *DJ-1* gene or DJ-1 protein is regulated by DJ-1 itself. To explore associations of DJ-1 with chaperons, cell extracts were immunoprecipitated with an anti-FLAG antibody and the precipitates were analyzed by Western blotting with antibodies against each protein and with an anti-mouse DJ-1 antibody (Figure 2B). Formation of homodimers between endogenous DJ-1 and all of the FLAG-DJ-1 except for L166P was observed as described previously [39,44]. mtHsp70 was found to form complexes with wild-type and all of the DJ-1 mutants, indicating that some DJ-1 is localized in mitochondria. Although associations of Hsp70 with wild-type DJ-1 and all of the mutants of DJ-1 were observed in the presence of MG132, L166P and M26I DJ-1 were found to be strongly associated with Hsp70 (Figure 2B, lanes 6 and 8). Furthermore, CHIP was also found to be associated strongly with L166P and M26I. These results suggest that L166P and M26I, whose genes have been found as homozygous mutations in PD patients [16,19], may easily be aggregated, thereby facilitating association

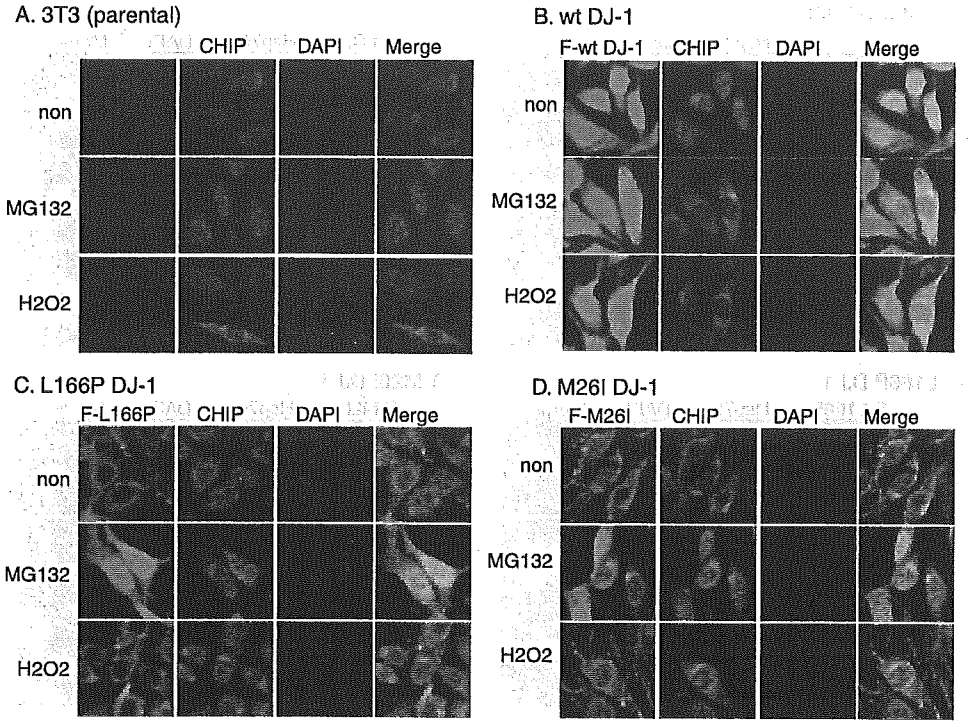

Figure 5. Colocalization of CHIP with DJ-1 and its mutants. Mouse NIH3T3 cells harboring FLAG-wild-type DJ-1 and its mutants were reacted with 10 μM MG132 for 12 h or with 100 μM of H_2O_2 for 4 h. The cells were then fixed with paraformaldehyde and reacted with a combination of a mouse anti-FLAG monoclonal antibody (M2, Sigma) and rabbit anti-CHIP (PC711, Oncogene) antibody. Nuclei were stained with DAPI. Cells were visualized under a confocal laser fluorescent microscope as described in "Materials and methods".

with Hsp70 and CHIP, as compared to other PD-derived mutants, R98Q and D149A, whose genes have been found as heterozygous mutations [17,18].

To examine the effect of oxidative stress on complex formation between DJ-1 and chaperones, cells were treated with 100 μM H_2O_2 for 4 h and analyzed as described above (Figure 3). Under this condition, expression levels of all of the proteins examined were found not to change drastically (Figure 3A). While associations of various DJ-1 mutants with Hsp70 and CHIP were observed, the levels of association did not change after addition of H_2O_2. Association of wildtype DJ-1, but not DJ-1 mutants, with mtHsp70 was, however, found to be significantly stimulated by treatment of cells with H_2O_2 (Figure 3B, lanes 3 and 4), suggesting that some DJ-1 is translocated to mitochondria to bind to mtHsp70 after oxidative stress.

Colocalization of DJ-1 with chaperons

Mouse 3T3 cells harboring FLAG-tagged wild-type, L166P and M26I DJ-1 were treated with MG132 or H_2O_2 for 12 or 4 h, and FLAG-DJ-1 and chaperones were stained with anti-FLAG and anti-chaperone antibodies. After cells were reacted with FITC- and rhodamine-conjugated second antibodies, cells were visualized under a confocal laser fluorescent microscope (Figures 4–6). Nuclei in cells were also stained with DAPI. As shown by the Western blotting data, expression of Hsp70 in parental NIH3T3 cells was hardly observed without treatment of MG132 (Figure 4A). DJ-1 has been shown to be localized both in the cytoplasm and nucleus and to be translocated to the nucleus after exposure to mitogen [1] or UV irradiation [36]. In NIH3T3 cells harboring exogenously added FLAG-wild-type DJ-1, a large proportion of FLAG-DJ-1 was found to be localized in the nucleus (Figure 4B). FLAG-L166P and -M26I DJ-1 were, on the other hand, found to be preferentially localized in the cytoplasm (Figure 4C, D).

After addition of MG132 to cells, elevated expressions of both L166P and M26I DJ-1 in the nucleus but not cytoplasm were observed (Figures 4–6, C and D). Furthermore, expression and colocalization of Hsp70 with wild-type, L166P and M26I DJ-1 in the cytoplasm were found (Figs. 4B–D). After addition of H_2O_2 to cells, on the other hand, FLAG-wild-type DJ-1 appeared to be translocated from the nucleus to cytoplasm (Figures 4B, 5B and 6D). Localizations of L166P and M26I DJ-1 did not change (Figures 4–6, C and D). CHIP was found to be localized both in the nucleus and cytoplasm before or after addition of MG132 or H_2O_2 and to be colocalized with wild-type, L166P and M26I DJ-1 (Figure 5). Furthermore, while localizations of wild-type, L166P and M26I DJ-1 were different from each other before or after addition of

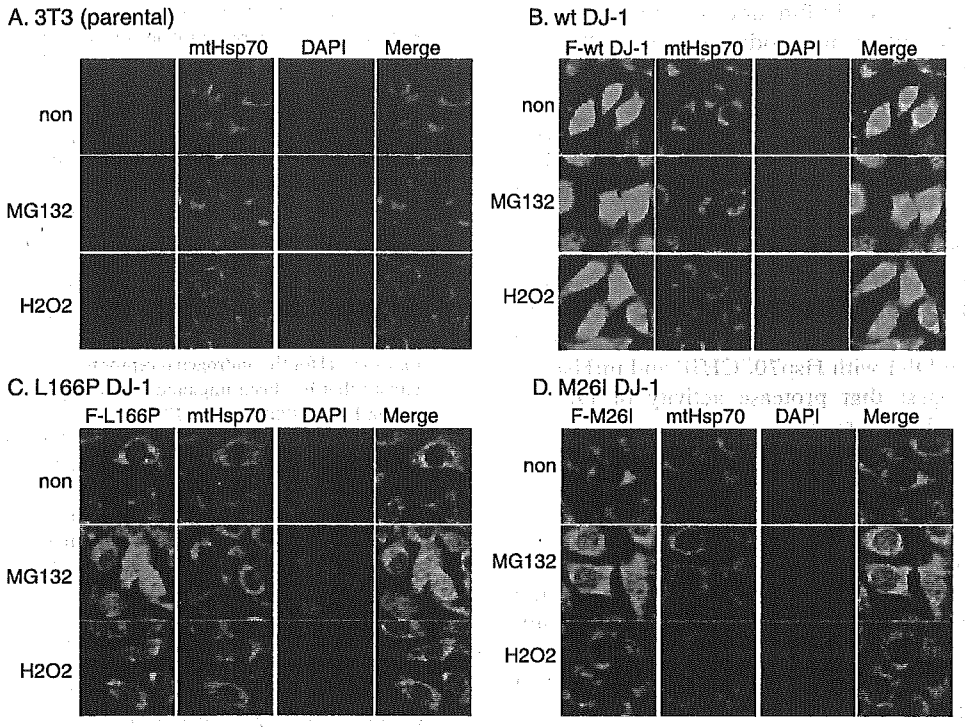

Figure 6. Colocalization of mtHsp70 with DJ-1 and its mutants. Mouse NIH3T3 cell lines harboring FLAG-wild-type DJ-1 and its mutants were reacted with 10 μM MG132 for 12 h or with 100 μM of H_2O_2 for 4 h. Cells were then fixed with paraformaldehyde and reacted with a combination of a rabbit anti-FLAG polyclonal antibody (F7425, Sigma) and mouse anti-mtHsp7 (ab13529, Abcam) antibodies. Nuclei were stained with DAPI. Cells were visualized under a confocal laser fluorescent microscope as described in "Materials and methods".

MG132 or H_2O_2, wild-type, L166P and M26I DJ-1 were found to be colocalized with mtHsp70, a mitochondria-resident Hsp70, only in the cytoplasm, indicating that some DJ-1 is localized in mitochondria and that wild-type DJ-1 is translocated to mitochondria after addition of H_2O_2 (Figure 6B).

Discussion

In this study, we showed that DJ-1 and its mutants were associated and colocalized with chaperones, including Hsp70, CHIP and mtHsp70, and that L166P and M26I mutants, which have been found in PD patients, had stronger affinity to Hsp70 and CHIP than did wild-type DJ-1. Furthermore, oxidative stress caused by addition of H_2O_2 to cells resulted in enhancement of association of wild-type DJ-1 with mtHsp70 and translocation of wild-type DJ-1 to mitochondria. Alternatively, since some DJ-1 was located in mitochondria in cells that had not been treated with H_2O_2, H_2O_2 treatment may enhance the binding of mitochondrial Hsp70 to mitochondrial DJ-1. Compared to wild-type DJ-1, L166P and M26I mutants were not translocated to the cytoplasm or mitochondria after treatment of cells with H_2O_2. These phenomena might result in no or reduced activities against oxidative stress of these mutants. Alternatively, drastic changes of the structure of these mutants, especially L166P of DJ-1, may loose susceptibility to oxidative stress, resulting in no translocation to mitochondria. Since L166P DJ-1 and perhaps M26I, too, became susceptible to degradation due to their misfiled structures, chaperones are thought to easily access these DJ-1 mutants.

Although during preparation of this manuscript, DJ-1 has been reported to associate with Hsp70 and CHIP [48] and to be localized, in part, in mitochondria under the condition of oxidative stress [37,38], colocalization of DJ-1 with these chaperones and associations of DJ-1 mutants with chaperones were not investigated. Since DJ-1 was colocalized with Hsp70 and CHIP in the cytoplasm and with mtHsp70 in mitochondria, it is therefore, thought that translocation of DJ-1 from the cytoplasm to mitochondria is triggered by conversion of associated chaperones. Since ROS are mainly produced in mitochondria and DJ-1 plays a role in anti-oxidative stress through elimination of ROS [32], it is reasonable that DJ-1 is translocated to mitochondria after oxidative stress. DJ-1 has been reported to be translocated from the cytoplasm to nucleus after UV irradiation, resulting in injury of DNA in the nucleus [36]. DJ-1 is therefore thought to translocate to a place where stress occurs. Since the onset of PD is thought

to be triggered by dysfunction of mitochondrial complex I, resulting in production of ROS, the findings in this study will help to understand the roles of DJ-1 in the onset of PD.

What is the meaning of association of DJ-1 with chaperones? Hsp70 and CHIP have been reported to facilitate degradation of unfolded or aggregated proteins by the ubiquitin-proteasome system [47]. Protease activity of DJ-1 has been suggested by structural and biochemical analyses [40,45], though controversial results have also been reported [45,48,49,50]. The results of this study showing association of DJ-1 with Hsp70, CHIP and mtHsp70, therefore suggest that protease activity of DJ-1 is facilitated by these chaperons.

Acknowledgements

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Expression profiles of genes in DJ-1-knockdown and L166P DJ-1 mutant cells

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Abstract

DJ-1 is a novel oncogene and a causative gene for the familial form of Parkinson's disease (PD). DJ-1 has been shown to play roles in anti-oxidative stress by eliminating reactive oxygen species and in transcriptional regulation of genes. Loss of these functions of DJ-1 is thought to trigger the onset of PD. In this study, to identify genes for which expressions are regulated by DJ-1, DNA microarray analyses were carried out using two mouse NIH3T3 cell lines, DJ-1-knockdown cells and cells harboring an exogenously added L166P DJ-1 mutant found in PD patients. In both cell lines, drastic changes in expressions of genes, including genes related to stress, apoptosis, oxidative stress and neurotoxicity, were observed and changes in expressions were confirmed by RT-PCR. Of the genes identified, expression level of the extracellular superoxide dismutase (SOD3) gene was found to decrease in DJ-1-knockdown cells, while expressions of SOD1 and SOD2 genes did not change. Furthermore, expression of the tau gene, a gene whose product gives cells neurotoxicity by aggregation, was found to increase at its promoter level in L166P DJ-1 cells. These findings suggest that DJ-1 regulates expressions of genes for which functions are thought to be related to cell death or neurodegeneration.

Keywords: DJ-1; Transcriptional regulation; Oxidative stress; Parkinson's disease

DJ-1 was first identified by us as a novel oncogene [17], and elevated expression of DJ-1 has been reported in breast cancer, smoker-derived lung adenocarcinoma and prostate cancer [6,13,16]. DJ-1 was later found to be related to infertility of rats and mice [12,20,29–31]. Recently, DJ-1 has been shown to be responsible for the onset of familial Parkinson's disease (PD), PARK7 [3], and 11 mutations in familial and sporadic forms of PD have been reported [1,7,8]. DJ-1 has been shown to be expressed in almost all brain tissues in healthy men and patients with neurodegenerative diseases, including PD, Pick's disease and multiple system atrophy [2,18,22]. DJ-1 has been found to play a role in anti-oxidative stress, reactive oxygen species being eliminated in vitro and in vivo by oxidation of DJ-1 itself, and mutations of DJ-1, including various mutations found in PD patients, that lead to cell death have been found [26,33]. Other groups have also reported anti-oxidative activity of DJ-1 [14,33]. DJ-1 has also been reported to play a role in transcriptional regulation of genes. DJ-1 has been shown to be a positive regulator of the androgen receptor [19,25,27] and p53 [24] and to be a negative regulator of PTEN tumor suppressor [10] and pyrimidine tract-binding protein-associated splicing factor (PSF) [32]. DJ-1 has been shown to protect PSF-induced neuronal apoptosis in concert with a co-activator, p54nrb [32]. These findings suggest that two functions of DJ-1, anti-oxidative stress and transcriptional regulation, contribute to the onset of PD.

In this study, we identified genes for which expressions change in DJ-1-knockdown or L166P DJ-1-carrying cells

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by DNA microarray analysis. Genes identified include genes related to apoptosis, oxidative stress and neurotoxicity.

D2 cells, which are mouse NIH3T3 cells in which expression of DJ-1 has been knocked down by siRNA targeting the DJ-1 gene, and L166P cells, which are mouse NIH3T3 cells harboring exogenously added FLAG-tagged human DJ-1, were described previously [26,28]. L166P DJ-1 mutant has been found in PD patients [3] and its loss of anti-oxidative and transcription activities has been reported [4,14,23,26,32,33]. Total RNAs were prepared from D2, L166P and parental NIH3T3 cells and were used for DNA-microarray analyses using Genechip (Affymetrix Mouse Genome MOE430A). Expression levels of genes in D2 cells or L166P cells were then compared to those in parental NIH3T3 cells. The results showed that expression levels of 49 and 93 genes in D2 cells and expression levels of 70 and 12 genes in L166P cells increased and decreased, respectively, by more than 3.5 times compared to the levels in parental cells. The genes were classified genes related to apoptosis, oxidative stress and neurotoxicity, and all of the genes were shown in Supplementary Table S1 (Table S1). It is interesting that number of genes with the decreased expression levels are larger than those with the increased expression levels in D2 cells, while number of genes with the increased expression levels are larger than those with the decreased expression levels in L166P cells. Of genes with the increased expression levels, five genes for glutathione *S*-transferase, serine/cysteine protease inhibitor and zinc finger protein 59 and pleiomorphic adenoma gene-like 1 were found in both D2 and L166P cells, while no common genes with the decreased expression levels were found (Table S1). Since wild-type DJ-1 acts as a transcriptional coactivator, it is reasonable that larger number of genes with the decreased expression levels were observed in D2 cells. The results that there were small numbers of common genes with the increased or decreased expression levels in both D2 and L166P cells suggest that L166P DJ-1 gained transcriptional activity different from that of wild-type DJ-1.

We then chose genes for which functions are thought to be related to DJ-1 and carried out RT-PCR to confirm different expressions of genes in two cells (Figs. 1 and 3). The genes chosen and the nucleotide sequences of primers used are shown in Table S2. In D2 DJ-1-knockdown cells, decreased expression of DJ-1 mRNA was first examined by RT-PCR (Fig. 1A-l and B-e). Of 11 genes selected by DNA microarray as genes with increased expression levels in DJ-1-knockdown cells versus parental cells, expression levels of four genes encoding neuronal cell death-induced protein kinase (NIPK), stress-induced CHOP target (Car 6), stress-induced cell cycle modulator (gadd45a) and p53 target (Ogn) were found to be increased (Figs. 1A-a, c, k and h). Of four genes selected by DNA microarray as genes with decreased expression levels in D2 cells versus parental cells, on the other hand, the expression level of the extracellular superoxide dismutase (SOD3) gene was found to be decreased (Fig. 1B-b). Expression levels of the three known genes of the superoxide dismutase gene family, SOD1, SOD2 and SOD3, in D2 and parental cells were further examined by RT-PCR (Fig. 2). Only the expression level of the SOD3 gene was found to be decreased in DJ-1-kockdown cells.

Of 10 genes selected by DNA microarray as genes with increased expression levels in L166P cells versus parental cells, expression levels of only two genes, one encoding MBL-associated serine protease 3 (masp3) and one encoding tau were found to be increased (Fig. 3A-d and j). Of two

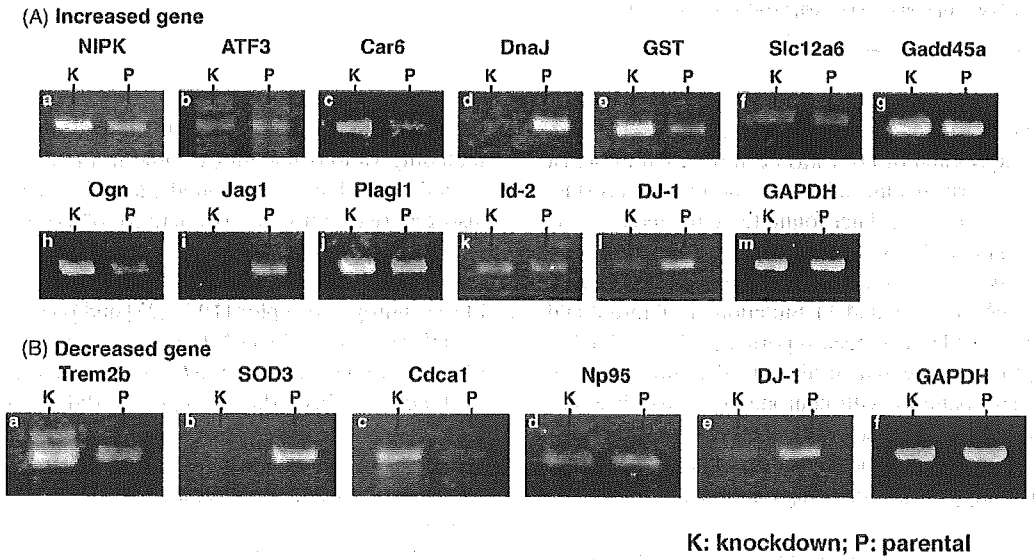

Fig. 1. (A and B) Expressional changes in genes in DJ-1-knockdown cells. Total RNAs were extracted from D2 and NIH3T3 cells by using Isogen (Nippon Gene), and cDNA was synthesized using the oligo dT primer and BcaBEST polymerase (Takara). The first strand of cDNA products was amplified with specific primers for the first 2 min at 94 °C and then for 22–26 cycles of 0.5 min at 94 °C, 0.5 min at 60 °C and 0.5 min at 72 °C. The nucleotide sequences of the sense and antisense primers are shown in Table S2. The amplified products were separated on a 2% agarose gel and stained with ethidium bromide.

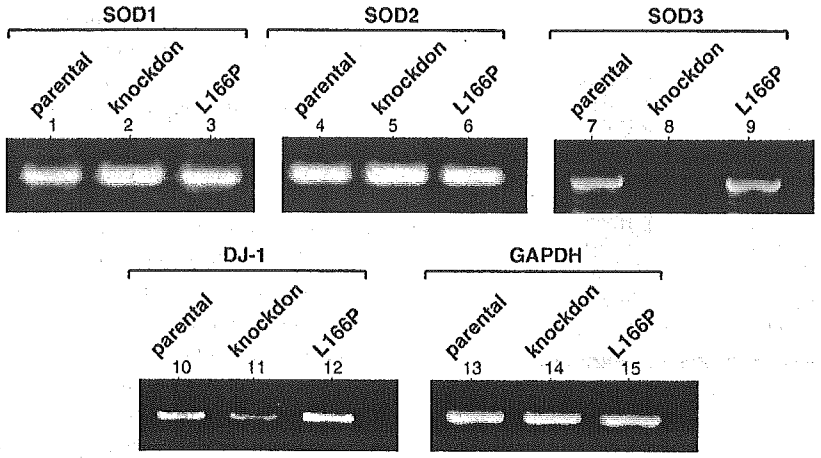

Fig. 2. Expression of mRNAs of SOD family members. Total RNAs were extracted by D2 and NIH3T3 cells, and RT-PCR was carried out using the specific primers for genes of SOD1, SOD2 and SOD3 as shown in Table S2.

genes selected by DNA microarray as genes with decreased expression levels in L166P cells versus parental cells, on the other hand, the expression levels of the RNA-binding protein with multiple splicing gene (Rbpms) was found to be decreased (Fig. 3B-b). Furthermore, to confirm that change in the expression of the tau gene is at the transcription level, a plasmid containing the promoter of the mouse tau gene linked to the luciferase gene was transfected into L166P and parental cells, and its luciferase activity was measured at 48 h after transfection (Fig. 4A). The results showed that luciferase activity level was increased in L166P cells compared to that in parental cells. To further examine the effect of L166P DJ-1 on promoter activity of the tau gene, expression vectors for wild-type DJ-1 and L166P DJ-1 were cotransfected with the tau promoter-luciferase construct into D2 cells and their luciferase activity levels were measured (Fig. 4B). To reduce the effect of endogenous DJ-1 in cells on luciferase activities, D2 cells were used. The results showed that while wild-type DJ-1 decreased luciferase activity, L166P DJ-1 increased luciferase activity in a dose-dependent manner, suggesting that DJ-1 negatively regulates tau gene expression at the transcription level and that L166P DJ-1 rather stimulates tau gene expression.

In this study, we identified genes for which expressions changed in DJ-1-knockdown cells or PD-derived mutant L166P-carrying cells by DNA microarray analyses. Of the genes identified, genes whose functions are thought to be related to that of DJ-1 were selected and changes in the

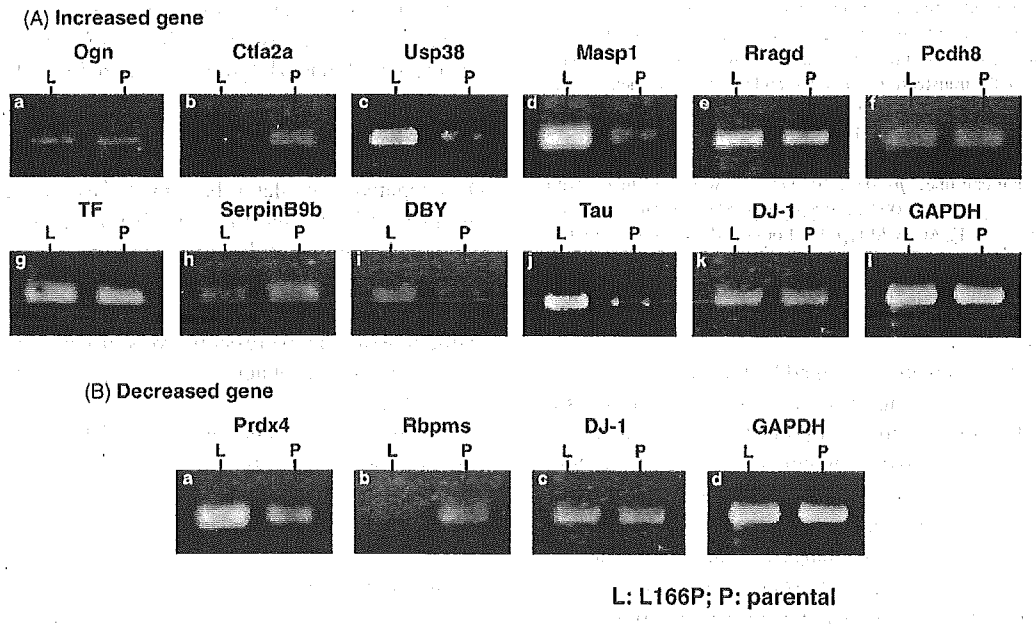

Fig. 3. (A and B) Expressional changes in genes in L166P cells. Total RNAs were extracted from L166P and NIH3T3 cells, and RT-PCR was carried out using the specific primers for genes as shown in Table S2.

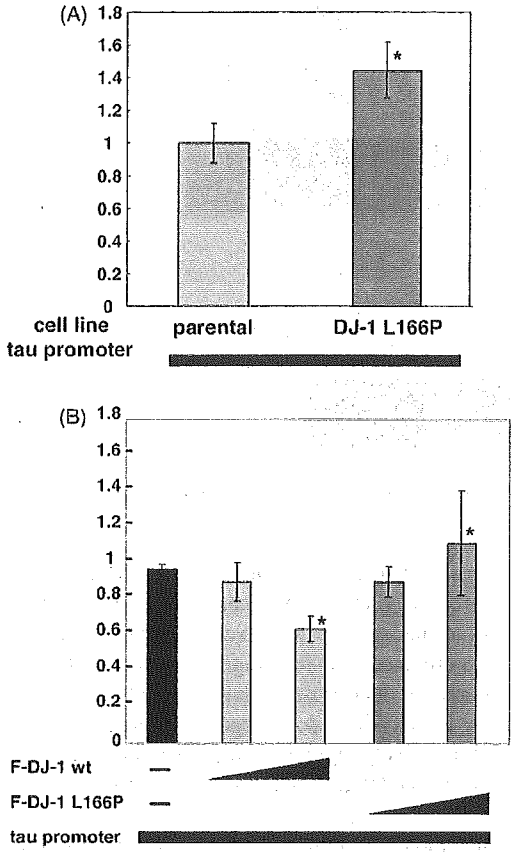

Fig. 4. Effect of the L166P DJ-1 mutant on promoter activity of the mouse tau gene. The promoter region in pCAT3-B-tau promoter 1000 [5] was inserted into a pGVB luciferase vector and named pGVB-tau promoter 1000. (A) NIH3T3 and L166P cells in a 6-cm dish were transfected with 1 μg of pGVB-tau promoter 1000 and 1 μg of pCMV-β-gal. Forty-eight hours after transfection, whole cell extracts were prepared by the addition of the Triton X-100-containing solution from a Pica gene kit (Wako Pure Chemicals) to the cells. About a one-fifth volume of the extract was used for β-galactosidase assay to normalize the transfection efficiency, 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 3–5 times. Asterisks indicate significant difference from luciferase activity of parental cell line; $^{*}p < 0.05$. (B) D2 cells were transfected with 0.5 μg of pGVB-tau promoter 1000 and 0.5 μg of pCMV-β-gal together with 0.1 or 0.5 μg of pCMV-FLAG-wild-type DJ-1 or f pCMV-FLAG-L166P DJ-1 [26]. Forty-eight hours after transfection, luciferase assays were carried out as described in part (A). Asterisks indicate significant difference from luciferase activity of parental cell line without transfection of DJ-1; $^{*}p < 0.05$.

expressions of genes were confirmed by RT-PCR. The results that there were small numbers of common genes with the increased or decreased expression levels in both DJ-1-knockdown cells and L166P-carrying cells suggest that loss of function and gain of function occurred in DJ-1-knockdown cells and L166P-carrying cells, respectively. Gain of function of L166P DJ-1 might include change of transcription factors that bind to DJ-1, resulting in improper transcriptional regulation of DJ-1. In DJ-1-knockdown cells, expression levels of genes for Car6 and Gadd45a, both of which are induced by stress, for NIPK, which is a kinase induced by neuronal cell death, and for Ogn, which is a transcriptional target of p53, were found to be increased, indicating that DJ-1 negatively regulates expressions of these genes. We and others have reported that expression of DJ-1 was induced by several stresses, including addition of paraquat [15], H_2O_2 [11,26] and UV irradiation to cells [24], that DJ-1 prevents cell death [26], and that DJ-1 is a positive regulator of p53 [24]. Although the molecular mechanisms underlying decreased expression levels of genes induced by DJ-1 are not clear, it is important to elucidate the relationship between DJ-1 and these genes in terms of the onset of cancer or PD. Of the three superoxide dismutase (SOD) genes, only the expression level of the extracellular superoxide dismutase (SOD3) gene was decreased in DJ-1-knockdown cells. SOD is a key enzyme for scavenging reactive oxygen species. We previously reported that activity of a mixture of SOD1 and SOD2, which are a cytosolic Cu/Zn-dependent SOD and mitochondrial Mn-dependent SOD, respectively, did not change in cells into which siRNA targeting DJ-1 was transfected [26]. Although the activity of SOD3 was not measured in that study, the present results showing that expression levels of SOD1 and SOD2 genes did not change in DJ-1-knockdown cells are consistent with the previous results of our study. Since DJ-1 has been shown to be exposed to serum in breast cancer patients [13] and since we found that DJ-1 shuttles between the inside and outside of cells (data not shown), DJ-1 is thought to scavenge reactive oxygen species mutually with SOD3 in the extracellular space.

By using L166P cells, expression of the tau gene was found to be positively regulated by L166P DJ-1. Furthermore, promoter activity of the tau gene was found to be inhibited and to be activated by ectopic expressions of wild-type DJ-1 and L166P DJ-1, respectively. Microtubule-associated protein tau is the major component of filamentous neurofibrillary lesions of Alzheimer's disease and other tauopathies. Since it has been reported that DJ-1 was colocalized with tau in patients with PD, Pick's disease and multiple system atrophy [18,22] and that protease activity of DJ-1 was implicated by results of structural [9] and biochemical analyses [21], it is thought that DJ-1 negatively regulates the expression of tau at both mRNA and protein levels and that L166P DJ-1 reversely regulates the expression of tau due to its gain of function. Although the expression level of the tau gene has not been shown in PARK7 patients harboring the L166P mutation [3], it is interesting to examine expression levels of genes that are related to the pathogenesis of neurodegenerative disorders, including the tau gene.

The findings of this study should be useful for understanding the roles of DJ-1 in the onset of neurodegenerative disorders, including PD.

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Induction of Reactive Oxygen Species by Bisphenol A and Abrogation of Bisphenol A-Induced Cell Injury by DJ-1

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DJ-1 was first identified as an activated *ras*-dependent oncogene. DJ-1 is related to male fertility, and its expression in sperm decreases in response to exposure to a number of reproductive toxicants. DJ-1 has been associated with the onset of familial Parkinson's disease (PD) in humans, and has been found to have activity against oxidative damage by eliminating reactive oxygen species (ROS). In this study, we investigated the role of DJ-1 in oxidative stresses by administration of bisphenol A (BPA), which has been reported to induce oxidative stress in rodents, to male mice and cultured cells. In male mice, we found that BPA significantly increased the expression level of DJ-1 in the sperm and brain. In cultured Neuro2a and GC1 cells, we found that BPA induced ROS production and significantly compromised mitochondrial function concomitant with elevated expression and oxidization of DJ-1. DJ-1 was found to maintain the complex I activity against BPA-induced oxidative stress after the localization in mitochondria. The results showed that DJ-1 plays a role in the prevention of mitochondrial injury-induced cell death.

Key Words: **bisphenol A; reactive oxygen species; DJ-1; cell death; oxidative stress; mitochondria.**

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* (Nagakubo *et al.*, 1997). DJ-1 is 20-kDa protein comprising 189 amino acid residues that is expressed in various tissues with particularly high levels of expression in the testis, sperm, and brain (Bandopadhyay *et al.*, 2004; Wagenfeld *et al.*, 2000). DJ-1 was later found to be a positive regulator of the androgen receptor (Niki *et al.*, 2003; Taira *et al.*, 2004a; Takahashi *et al.*, 2001) and p53 (Shinbo *et al.*, 2005a) and to be a negative regulator of PTEN tumor suppressor (Kim *et al.*, 2005). DJ-1 has also been reported to be related to infertility of rats and mice and to participate in fertilization for sperm to penetrate into the zonae pellucida of eggs (Klinefelter *et al.*, 2002; Okada *et al.*, 2002; Wagenfeld *et al.*, 1998, 2000; Welch *et al.*, 1998; Yoshida *et al.*, 2003). Recently, DJ-1 has been shown to be responsible for onset of familial Parkinson's disease (PD), PARK7 (Bonifati *et al.*, 2003), and 11 mutations in familial and sporadic forms of PD have been reported (Abou-Sleiman *et al.*, 2003; Hague *et al.*, 2003).

Reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO^-), damage various cell components such as unsaturated lipids, proteins, and nucleic acids. Oxidative stress is caused by ROS in cells in which large amounts of ROS are produced by alternative activities of scavenger proteins or by dysfunction of the mitochondrial respiratory chain pathway with reduction of complex I activity. Complex I (NADH:ubiquinone oxidoreductase) catalyses the first step in the mitochondrial electron transport chain, by which electrons from the oxidation of NADH are used to convert oxygen to water, the energy liberated being trapped in ATP formation and ultimately used as the body's energy source. Oxidation of nucleic acid, lipid, and protein is thought to result in the onset of various diseases, including cancer, infertility, and neurodegenerative diseases such as PD (Agarwal *et al.*, 2003; Benhar *et al.*, 2002; Golden *et al.*, 2002; Rego and Oliveira, 2003). Expression of DJ-1 and oxidation of DJ-1 have been shown to be induced in cells that had been administered ROS-inducing chemicals (Kinumi *et al.*, 2004; Mitsumoto *et al.*, 2001; Mitsumoto and Nakagawa, 2001), and abnormal oxidative forms of DJ-1 have been found in some patients with sporadic forms of PD (Bandopadhyay *et al.*, 2004). We and other groups have shown that some DJ-1 is located in mitochondria in addition to the cytoplasm and nucleus (Shendelman *et al.*, 2004; Shinbo *et al.*, 2005a; Zhang *et al.*, 2005) and that translocation of DJ-1 to mitochondria was stimulation by oxidative stress (Blackinton *et al.*, 2005; Jin *et al.*, 2005; Li *et al.*, in press). Precise functions of DJ-1 in mitochondria are, however, not clear.

In addition to its transcriptional activity as a coactivator, 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 various mutations found in PD patients, lead to oxidative stress-induced cell death (Taira *et al.*,

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2004b; Yokota *et al.*, 2003). Other groups also reported anti-oxidative activity of DJ-1 (Canet-Aviles *et al.*, 2004; Martinat *et al.*, 2004; Shendelman *et al.*, 2004). It has been shown that, of three cysteines at amino acid numbers 46, 53, and 106 in DJ-1, C106 was first oxidized by addition of SO_3H or SO_2H, followed by oxidation of C46 and C53 with a dose of H_2O_2 added to cultured cells (Kinumi *et al.*, 2004), and that C106 was required for DJ-1 to exert activity against oxidative stress (Canet-Aviles *et al.*, 2004; Martinat *et al.*, 2004; Shendelman *et al.*, 2004; Taira *et al.*, 2004b; Takahashi-Niki *et al.*, 2004). In addition to oxidation of DJ-1, DJ-1 has been reported to be conjugated with SUMO-1 on a lysine residue at amino-acid number 130 (K130) (Shinbo *et al.*, in press). Sumoylation of DJ-1 at K130 has been found to essential for DJ-1 to exert its full activities, including activities for *ras*-dependent transformation, cell growth stimulation, and anti-oxidative stress (Shinbo *et al.*, in press). Loss of these functions of DJ-1 is therefore thought to lead to the onset of PD.

Some endocrine disruptors (EDs) such as lindane, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and bisphenol A (BPA) have been shown to induce oxidative stress in the brain, liver, kidney, testis, and epididymal sperm in rodents (Bindhumol *et al.*, 2003; Chitra *et al.*, 2003; Junqueira *et al.*, 1988; Kabuto *et al.*, 2003, 2004; Latchoumycandane *et al.*, 2002; Stohs *et al.*, 1991). Furthermore, BPA and some bisphenols have been reported to reduce mitochondrial function (Nakagawa and Toyama, 2000), but the precise mechanisms underlying these phenomena have not been elucidated.

In this study, we analyzed the effect of DJ-1 on BPA-induced cell death. The results showed that BPA induced oxidative stress in mitochondria and that DJ-1 plays a role as a scavenger of ROS to prevent cell death.

MATERIALS AND METHODS

Animals and treatment. C57BL/6 male mice at 5 weeks of age were purchased from Sankyo Laboratory, Sapporo, Japan. The mice were acclimated to the laboratory for 1 week prior to the experiments and then divided into the following three groups: one group of mice at 6 weeks of age orally administered daily bisphenol A (BPA, Wako) dissolved in corn oil at 10 or 100 μg/kg body weight for 1 or 2 weeks, one group of mice given corn oil (5 ml/kg) alone for the same period, and one group of mice not treated. At 24 h after last administration, the mice were killed, and their brain and cauda epididymides were removed. To purify sperm, sperm was extracted from the cauda epididymes of male mice with a syringe, transferred to a tube containing 5% Ficoll in phosphate-buffered saline (PBS), and centrifuged at 14,000 rpm for 5 min. The pellet fraction containing purified sperm and brain were stored at −80°C until use. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Committee for Animal Research at Hokkaido University.

Cells and treatment. Mouse Neuro2a and GC1 cells were purchased from American Tissue Culture Collection. All the cells used in this study were cultured in Dulbecco's modified medium supplemented with 10% calf serum. Cells were then administered various concentrations of BPA dissolved in dimethyl sulfoxide (DMSO) for 24 or 48 h. As a vehicle control, 0.1% DMSO was added to the medium.

Western blotting and isoelectric focusing. Proteins were prepared from whole tissues or cultured cells for Western blot analysis as follows. The brain and sperm were homogenized in 20 mM phosphate buffer (pH 7.5) containing 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, and protease inhibitors. Cultured cells were homogenized in a buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM PMSF, and protease inhibitors. Extracts were then centrifuged at 12,000 rpm for 5 min, and their supernatants were used. To analyze proteins in mitochondria by Western blotting, cells were homogenized in a buffer containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.3), and 1 mM EDTA and then centrifuged at 1000 × *g* for 10 min. Their supernatant fractions were then centrifuged at 12,000 × *g* for 15 min, and the pellet fractions (mitochondrial-enriched fractions) were suspended in a buffer containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.3), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF. Concentration of mitochondria protein was measured by using a BCA protein assay kit (PERBIO), and 20 μg of mitochondria protein was used for Western blotting. For isoelectric focusing, cells were homogenized in PBS containing 2% NP-40 and centrifuged at 12,000 rpm for 5 min, and their supernatants were used. Proteins in these extracts were then separated in a 12.5% polyacrylamide gel containing SDS or in an isoelectric focusing gel of pH 5–8, transferred onto nitrocellulose membranes, and reacted with an anti-DJ-1 antibody, which was an affinity-purified rabbit anti-DJ-1 polyclonal antibody described previously (Nagakubo *et al.*, 1997), an anti-complex I monoclonal antibody cocktail (Mito Science), or an anti-actin antibody (Chemicon). The proteins on the membranes were then reacted with IRDye800- or Alexa Fluor680-conjugated secondary antibodies followed by visualization using an infrared imaging system (Odyssey, LI-COR).

Flow cytometric analysis. Nero2a and GC1 cells were mixed with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes) or 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) (Daiichi Kagaku) in Hank's solution containing 10 mM HEPES-KOH (pH 7.4) for 30 min in the dark, and their fluorescences were measured using a FACScan flow cytometer (Becton-Dickinson) with excitation at 488 and emission at 530 nm. The amounts of fluorescences were also quantified using a "CELL QUEST" program as a value of "median".

Indirect immunofluorescence. Cells were mixed with 500 nM MitoTracker-Green (Molecular Probes) for 40 min and then reacted with 5 μM MitoSOX Red (Molecular Probes) for 10 min. The cells were then fixed with a solution containing 4% paraformaldehyde and reacted with an affinity-purified rabbit anti-DJ-1 polyclonal antibody. The cells were then reacted with a Cy-5-conjugated anti-rabbit IgG and observed under a confocal laser fluorescent microscope.

siRNA. The nucleotide sequences for siRNAs targeting DJ-1 were 5′-CCUUGCUAGUAGAAUAAACdTdT-3′ and 3′-dTdTGGAACGAUCAUCU-UAUUUG-5′ for upper and lower strands, respectively. siRNA-targeting luciferase was purchased from Greiner (Japan). Twenty nM siRNA was transfected into cells using Lipofectamine 2000 (Invitrogen) according to the supplier's manual.

Complex I activity. A mitochondria-enriched fraction was prepared as described above. Seventy μg of mitochondria protein and 0.05 mM ubiquinone 1 were added to 35 mM phosphate buffer (pH 7.4) containing 2.65 mM sodium cyanide, 5 mM $MgCl_2$, 1 mM EDTA, 1 mg/ml bovine serum albumin, and 2 μg/ml antimycin in a final volume of 0.48 ml. After incubation of the mixture at 37°C for 2 min, 0.02 ml of 5 mM NADH solution was added to the reaction mixture, and decrease in absorbance was measured by a spectrophotometer at 340 nm for 4 min.

Cell viability assay. Cell viability was measured by a methyl thiazolyl tetrazolium (MTT) assay using a cell counting kit 8 (DOJINDO).

Statistical analyses. Data are expressed as means ± SD. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student's *t*-test.

RESULTS

Change in DJ-1 Expression in Male Mice and Cultured Cells Caused by BPA Administration

Since it has been reported that concentrations of extracted BPA from food such as anchovy, herring, and sardine and from saliva are 10–50 $\mu g/g_{liq}$ and 90–931 μg/h, respectively, (Biles *et al.*, 1999; Olea *et al.*, 1996), we chose 10 and 100 μg/kg body weight/day of BPA as the low and high concentrations, respectively. Although these concentrations of BPA to mice are relatively high, these concentrations have been used in several studies to examine the molecular mechanism of BPA action (see recent references, Al-Hiyasat *et al.*, 2004; Nikaido *et al.*, 2004; Toyama *et al.*, 2004). These two concentrations of BPA dissolved in corn oil were administered orally to C57BL/6N male mice for 1 or 2 weeks, and corn oil alone was administered to mice as a vehicle control. Another control group consisted of untreated mice. Expressions of DJ-1 in the brain and sperm after administration of BPA were then examined by Western blotting (Figs. 1A and 1B, upper panels), and intensities of bands were measured using an infrared imaging system (Figs. 1A and 1B, lower panels). The results showed that expression levels of DJ-1 in the brain and sperm were significantly increased over a period of 2 weeks by 1.5- to 2-fold compared to those in the vehicle control in the case of administration of high-dose BPA. In the case of administration of low-dose BPA, however, there was little change in DJ-1 expression. These results indicate that BPA stimulates DJ-1 expression in mice.

To investigate the effect of BPA on DJ-1 expression *in vitro*, BPA dissolved in DMSO was administered to cultured mouse Neuro2a and GC1 cells, cells that originate from neuronal cells and spermatogonia immortalized with p53, respectively. Viabilities of Neuro2a and GC1 cells in the presence of BPA were first determined by an MTT assay (Fig. 2A). At 48 h after BPA administration, viabilities of Neuro2a and GC1 cells had significantly decreased at concentrations of more than 50 μM and 100 μM BPA, respectively, suggesting that Neuro2a cells are more sensitive than GC1 cells to BPA. We therefore used BPA at concentrations less than 50 and 100 μM for administration to Neuro2a and GC1 cells, respectively, in the following experiments. Expression levels of DJ-1 after administration of BPA for 24 and 48 h in cells were then determined by Western blotting (Figs. 2B and 2C). Results showed that expressions of DJ-1 in Neuro2a and GC1 cells increased with BPA administration in time- and dose-dependent manners, and the expression levels were 2.5-fold higher than in vehicle control cells.

Production of ROS in Neuro2a and GC1 Cells Induced by BPA Administration

ROS levels in Neuro2a and GC1 cells after administration of BPA were measured by using flow cytometry (Fig. 3). At various times after administration of 50 or 100 μM BPA, cells were reacted with DCFH-DA, which detects various ROS (Kobzik *et al.*, 1990). BPA was found to induce the production of ROS in Neuro2a cells in a time-dependent manner (Fig. 3A). In GC1 cells, on the other hand, ROS production peaked at 12 h after BPA administration and decreased with time (Fig. 3B). Furthermore, to measure highly reactive oxygen species (hROS) such as hydoxyradical (·OH) and peroxynitrite ($ONOO^-$), cells that had been administered BPA were reacted with HPF, which only detects hROS (Setsukinai *et al.*, 2003). H_2O_2 added to both Neuro2a and GC1 cells at a concentration of 100 μM for 1 h was not detected by HPF (Figs. 3C and 3D). BPA, on the other hand, was found to generate hROS production in both Neuro2a and

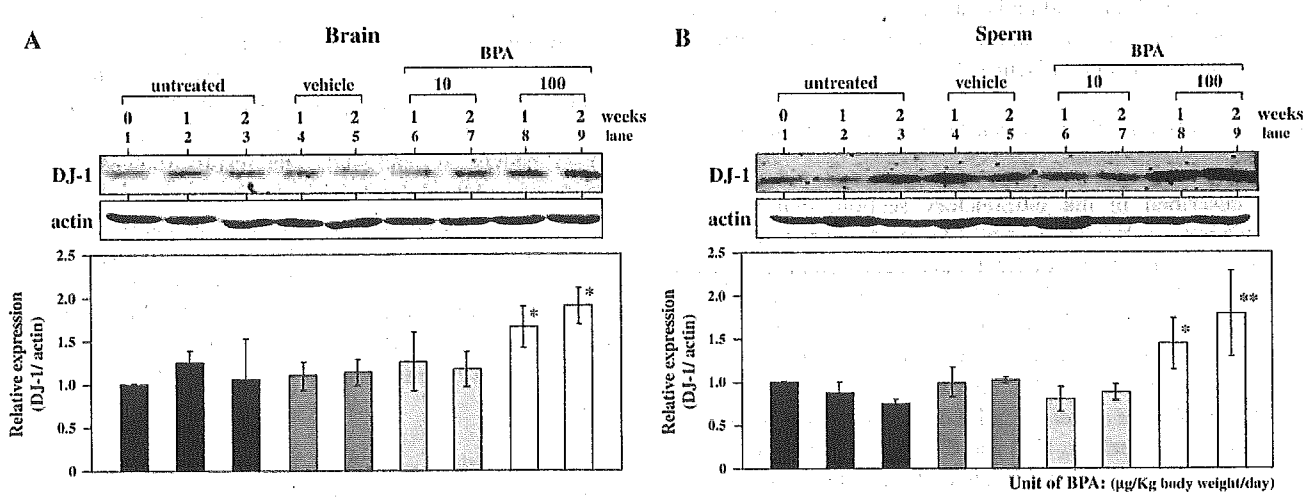


FIG. 1. Expression of DJ-1 in male mice after administration of BPA. Proteins extracted from the brain and sperm were analyzed by Western blotting with anti-DJ-1 and anti-actin antibodies. Intensities of bands were quantified as described in Materials and Methods. Means for each treatment group are represented in the bar graphs for the expression of DJ-1 relative to actin (error bars indicate standard deviation). (A) brain ($n = 4$). (B) sperm ($n = 4$). Asterisks indicate significant difference from the vehicle control; $*p < 0.05$; $**p < 0.01$.

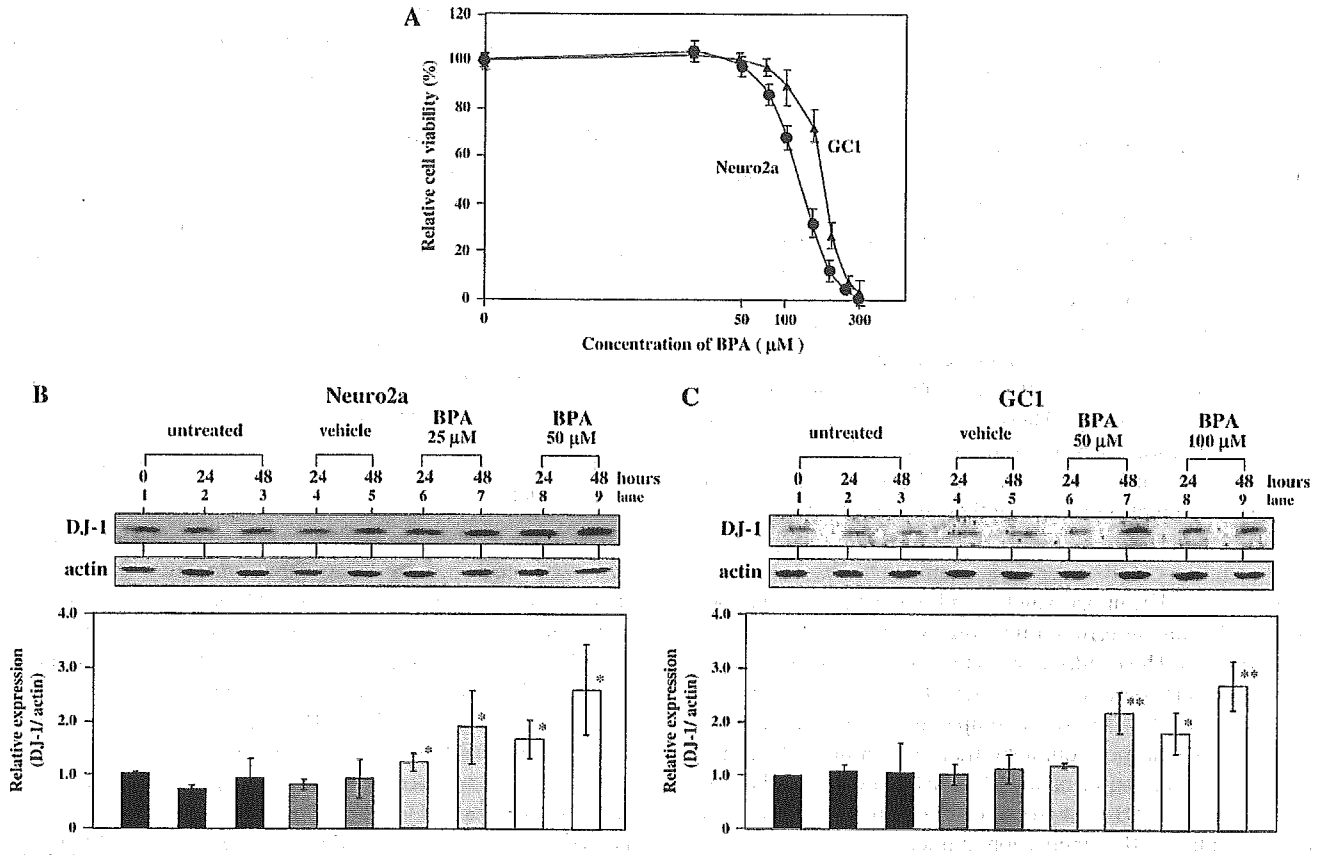


FIG. 2. Expression of DJ-1 in cultured cells after administration of BPA. Nueo2a and GC1 cells were administered various concentrations of BPA. (A) 48 h after administration, viability of cells was measured by an MTT assay. At 24 and 48 h after BPA administration, proteins extracted from cells were analyzed by Western blotting with anti-DJ-1 and anti-actin antibodies. Intensities of bands were quantified, as described in Materials and Methods, to measure relative expressions (DJ-1/actin) in Neuro2a (B) and GC1 cells (C). (B) Neuro2a ($n = 5$). (C) GC1 ($n = 5$). Asterisks indicate significant difference from the vehicle control; $*p < 0.05$; $**p < 0.01$.

GCI cells at 48 h after administration (Figs. 3C and 3D). The results indicate that BPA induces the production of various ROS, including hROS, in Neuro2A and GC1 cells.

pI Shift of DJ-1 in Neuro2a and GC1 Cells Induced by BPA Administration

As described in the introductory section, two types of modification, sumoylation and oxidation, occur on DJ-1. Various oxidized forms of DJ-1 have been found in *in vitro* cultured cells after oxidative stress (Kinumi *et al.*, 2004; Mitsumoto *et al.*, 2001; Mitsumoto and Nakagawa, 2001), and DJ-1 has been found to eliminate ROS by oxidizing DJ-1 itself *in vitro* and *in vivo* (Taira *et al.*, 2004b). We have further found that of three cysteines in DJ-1, C106 was first oxidized, and other two cysteines, C46 and C53, were then oxidized by using LC-MS and LC-MS/MS analyses (Kinumi *et al.*, 2004). pI shift of DJ-1, which indicates oxidation of DJ-1, is thought to be important for DJ-1 to exert its functions.

Proteins were extracted from Neuro2a and GC1 cells at various times after administration of BPA, separated on isoelectric focusing gels, and analyzed by blotting with an anti-DJ-1 antibody (Figs. 4A and 4B, upper panels). The same aliquots of proteins on filters were also reacted with an anti-actin antibody to show loading controls, and intensities of bands were measured by an infrared imaging system (Figs. 4A and 4B in lower panels, 4C and 4D). The results showed that the amounts of oxidized DJ-1 in both Neuro2a and Gc1 cells increased with BPA administration in time- and dose-dependent manners (Figs. 4A and 4B). The rate of oxidized DJ-1/unoxidized DJ-1 was significantly increased at 48 h after administration of 50 μM BPA in Neuro2a cells and at 24 h after administration of 100 μM BPA in GC1 cells (Figs. 4C and 4D), indicating that BPA stimulates oxidization of DJ-1 in Neuro2a and GC1 cells.

Change in Localization of DJ-1 in Neuro2a and GC1 Cells Following BPA Administration

DJ-1 has been shown to be localized both in the cytoplasm and nucleus and to be translocated from the cytoplasm to nucleus upon mitogen stimulation (Nagakubo *et al.*, 1997) and

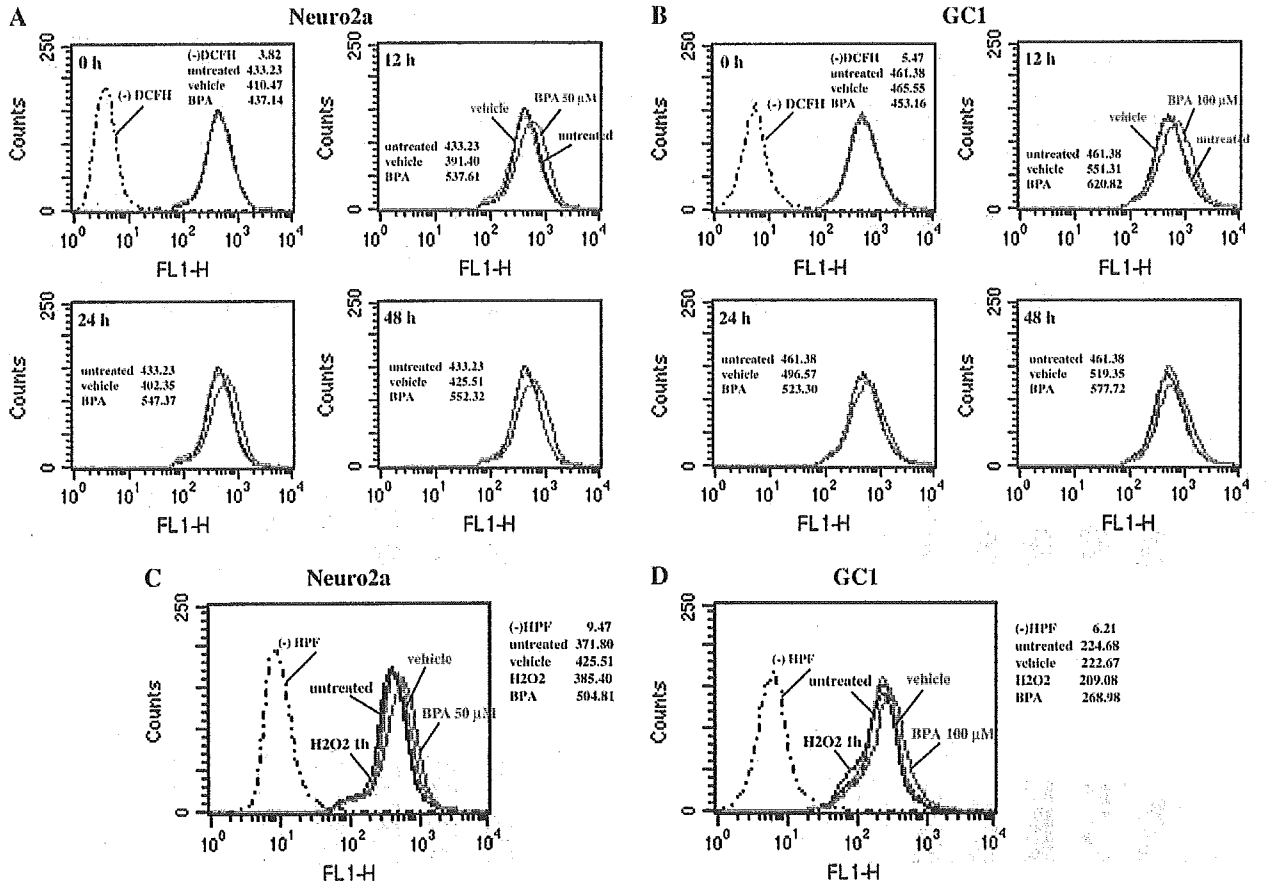


FIG. 3. Production of ROS in cultured cell induced by BPA administration. Neuro2a (A) and GC1 cells (B) were administered 50 and 100 μM BPA, respectively. At various times after administration, cells were treated with 10 mM DCFH-DA for 30 min, and levels of ROS were analyzed by flow cytometry. To detect hROS produced in cells, Neuro2a (C) and GC1 cells (D) were similarly administered BPA. At 48 h after administration, cells were treated with 10 mM HPF for 30 min, and levels of hROS were analyzed by flow cytometry. Cells were added with 100 mM H_2O_2 for 1 h, and their hROS levels were also analyzed. Amounts of fluorescences were quantified and were shown in an insert or beside of each figure. Experiments were carried out more than five times.

UV irradiation (Shinbo *et al.*, 2005a). Recent studies have also shown that some DJ-1 is also localized in mitochondria (Blackinton *et al.*, 2005; Canet-Aviles *et al.*, 2004; Jin *et al.*, 2005; Li *et al.*, 2005; Shinbo *et al.*, in press). We therefore examined the localization of DJ-1 after administration of BPA to cells. Neuro2a and GC1 cells that had been administered BPA were stained with MitoTrakker-Green, which stains mitochondria, and with MitoSOX-Red, which is a mitochondrial O_2^- indicator. MitoSOX-Red is permealized into cells and selectively targeted to mitochondria, in which MitoSOX-Red is oxidized by O_2^- but not by other ROS (see manufacturer's homepage, http://probes.invitrogen.com/lit/bioprobes47/bp47_5.pdf). Cells were also reacted with a rabbit anti-DJ-1 polyclonal antibody and then with a Cy5-conjugated anti-rabbit IgG and were visualized under a confocal laser microscope. These reactions give green, red, and blue colors, respectively, and these images were merged (Figs. 5A and 5B). DJ-1 was found to be localized in the cytoplasm and nucleus without BPA or with vehicle administration in Neuro2a and GC1 cells as reported in other cell types (Nagakubo *et al.*, 1997; Shinbo *et al.*, 2005a). After BPA administration, on the other hand, expression levels of DJ-1 were increased in both Neuro2a and GC1 cells concomitant with an increase in signals of O_2^--oxidized mitochondria (Figs. 5A and 5B). Intensities of the red fluorescence of MitoSOX-Red corresponding to levels of O_2^- in mitochondria were further measured by flow cytometry, and stimulation of O_2^- production by BPA administration in both Neuro2a and GC1 cells was confirmed (Figs. 5C and 5D).

It is interesting that the intensity of green fluorescence of MitoTracker-Green decreased in Neuro2a and GC1 cells that had been administered 50 μM BPA and 100 μM BPA for 48 h, suggesting that ROS compromised the mitochondria. To explore this possibility, expressions of a 20-kDa subunit of mitochondrial complex I in cells were examined by Western blotting with an anti-20-kDa subunit antibody, and intensities of bands were quantified. The results showed that levels of the 20-kDa subunit of mitochondrial complex 1 in both Neuro2a and GC1 cells decreased with BPA administration in a

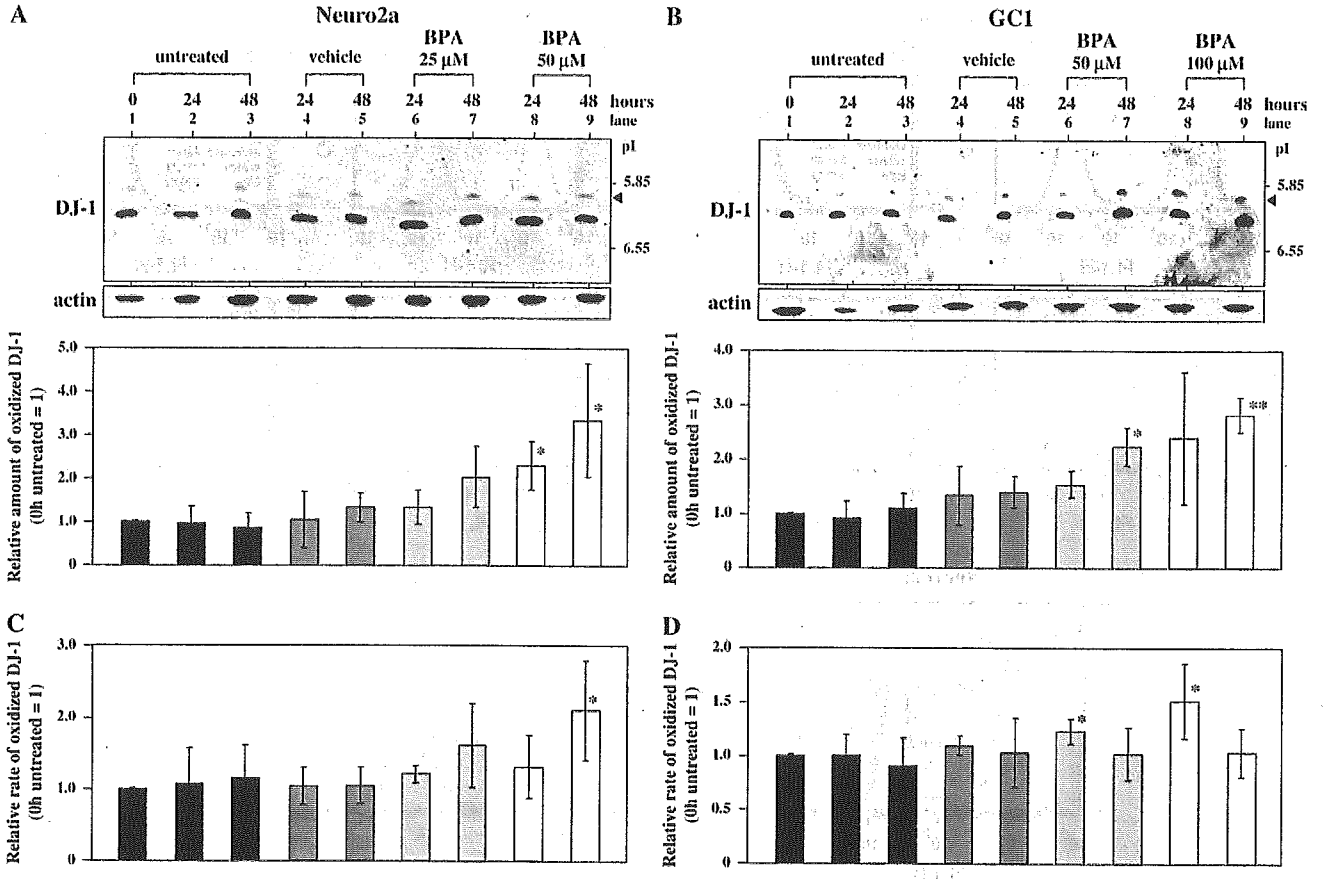
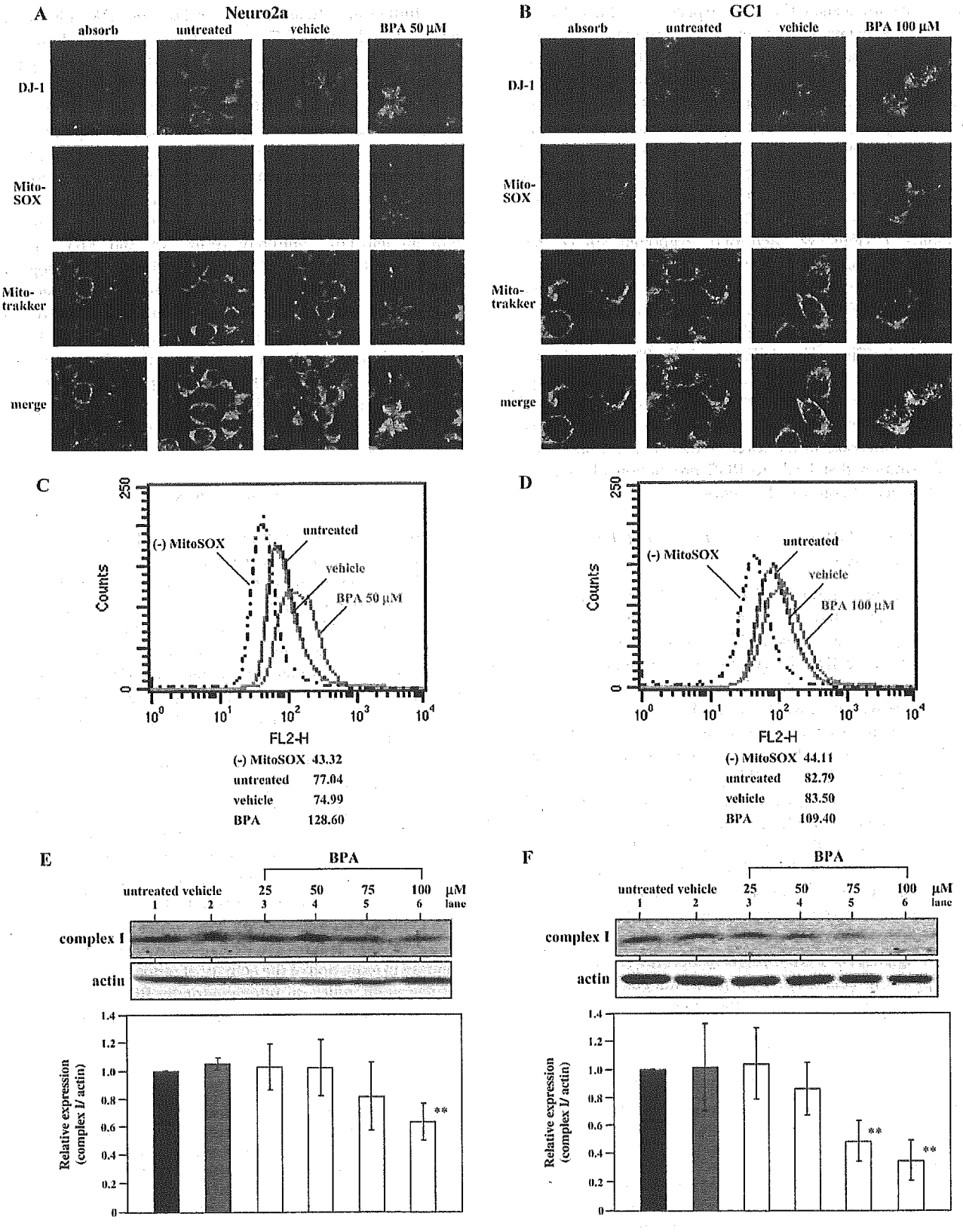


FIG. 4. pI shift of DJ-1 in cultured cells induced by BPA administration. Neuro2a (A) and GC1 cells (B) were administered BPA as described in Figures 3A and 3B. At 48 h after administration, proteins extracted from cells were separated on isoelectric focusing gels and analyzed by blotting with an anti-DJ-1 antibody. Aliquots of proteins were also analyzed by Western blotting with an anti-actin antibody. Intensities of bands were quantified as described in Materials and Methods, and relative expressions (DJ-1/actin) are shown (A, Neuro2a; B, GC1). Relative rates of the oxidized forms of DJ-1/unoxidized forms of DJ-1 to that in untreated cells (untreated) at 0 h are also shown (C, Neuro2a; D, GC1). Arrowheads indicate oxidized DJ-1. (A) Neuro2a ($n = 5$). (B) GC1 ($n = 5$). Asterisks indicate significant difference from the vehicle control; $^{*}p < 0.05$; $^{**}p < 0.01$.

dose-dependent manner (Figs. 5E and 5F). Moreover, the increased expression level of DJ-1 was found to be localized on the damaged mitochondria (see the merged figures, in which the color changed to white yellow). These results suggest that increased O_2^- produced by BPA compromises mitochondria and induces DJ-1 expression and that some of DJ-1 is localized in the injured spots.

Dysfunction of Mitochondrial Complex 1 in Cells Induced by BPA Administration

Dysfunction of mitochondria is thought to be responsible for the onset of neurodegenerative disorders, including PD, and DJ-1 is a causative gene of familial PD. Dysfunction of mitochondrial complex 1 has been found in PD patients, and rats or mice that had been administered drugs that injure mitochondrial complex 1 have been reported to show PD-like phenotypes, including dopaminergic neuronal cell death (see recent reviews, Corti *et al.*, 2005; Shen and Cookson, 2004; Tretter *et al.*, 2004). Moreover, since we have found that BPA induced production of ROS in the mitochondria of Neuro2a and GC1 cells and injured the mitochondria, activity and expression levels of subunits of mitochondorial complex 1 were examined. Neuro2a and GC1 cells were administered various concentrations of BPA. At 48 h after administration, mitochondria-rich fractions were prepared, and their activities and expression levels of subunits of complex 1, including subunits of 39, 30, and 20 kDa, were analyzed by Western blotting with anti-complex 1 subunit antibodies (Fig. 6). The expression of DJ-1 in mitochondrial-rich fractions was also examined. While the expression levels of all of the subunits of mitochondrial complex 1 decreased with BPA administration in a dose-dependent manner, complex I activity level was found to increase in Neuro2a cells or to hardly change in GC1 cells at low concentrations of BPA and then to significantly decrease in a dose-dependent manner at high concentrations of BPA (decrease at more than 50 and 100 μM


A
Neuro2a
absorb
untreated
vehicle
BPA 50 μM
DJ-1
Mito-SOX
Mito-trakker
merge
B
GC1
absorb
untreated
vehicle
BPA 100 μM
DJ-1
Mito-SOX
Mito-trakker
merge
C
(-) MitoSOX
untreated
vehicle
BPA 50 μM
Counts
FL2-H
(-) MitoSOX 43.32
untreated 77.04
vehicle 74.99
BPA 128.60
D
(-) MitoSOX
untreated
vehicle
BPA 100 μM
Counts
FL2-H
(-) MitoSOX 44.11
untreated 82.79
vehicle 83.50
BPA 109.40
E
BPA
untreated vehicle 25 50 75 100 μM
1 2 3 4 5 6 lane
complex I
actin
Relative expression (complex I/ actin)
F
BPA
untreated vehicle 25 50 75 100 μM
1 2 3 4 5 6 lane
complex I
actin
Relative expression (complex I/ actin)

BPA in Neuro2a and GC1 cells, respectively) (Figs. 2, 6A–6D). It is notable that patterns of complex 1 activities, initial increase and then decrease, paralleled those of DJ-1 expression in cells (Figs. 6E and 6F). To assess the relationship between DJ-1 expression and complex 1 activity, siRNA targeting the DJ-1 or *luciferase* gene, which is a nonspecific control, was transfected into Neuro2a and GC1 cells to knock down expression of the respective gene, and activities of complex 1 were measured at 3 days after transfection. Introduction of these siRNAs into cells was confirmed not to affect the expressions of subunits of complex 1 (Figs. 6C and 6D). Although siRNA against luciferase affected neither the expression of DJ-1 nor the activity of complex 1, siRNA against DJ-1 reduced both the expression level of DJ-1 and activity level of complex 1 to 10% and 70% of those without siRNA in Neuro2a and GC1 cells, respectively (Figs. 6E and 6F, lanes 7–9). These results suggest that BPA abrogates complex I activity by disrupting complex 1 and that DJ-1-knockdown decreases complex I activity without decrease in complex I expression. The results also suggest that the initial increase in the level of DJ-1 expression upon BPA administration that leads to ROS production plays a role in maintenance of complex 1 activity.

Abrogation of BPA-Induced Cell Death by DJ-1 in Neuro2a and GC1 Cells

To investigate the role of DJ-1 in BPA-induced cell injury, siRNA against DJ-1 was transfected into Neuro2a and GC1 cells using Lipofectamine 2000 to reduce the expression levels of DJ-1 in the cells. siRNA targeting the *luciferase* gene was used as a negative control, and transfection using Lipofectamine 2000 alone without siRNA was used as a vehicle control. BPA was then administered into cells at 24 h after transfection of siRNA. At 48 h after administration of BPA, cell viabilities were examined by an MTT assay (Fig. 7). The concentrations of BPA used are those that induce cell death as shown in Figure 2A, 50 and 100 μM into Neuro2a cells and 100 and 200 μM of BPA. We first confirmed that the expression levels of DJ-1 in transfected cells were reduced to 10% of those in nontransfected cells as described in Figures 6E and 6F, and that cell death did not occur after transfection of siRNA into cells without BPA administration (data not shown). Neuro2a and GC1 cells transfected with siRNA against DJ-1 were found to become significantly more susceptible to cell death than those transfected with siRNA against *luciferase* or with a buffer alone.

To further investigate the role of DJ-1 in BPA-induced cells death, Nero2a and GC1 cells were transfected with an expression vector for DJ-1, pcDNA3-F-DJ-1, using Lipofectamine 2000, and BPA was administered to cells 24 h after transfection. At 48 h after administration of BPA, cell viabilities were examined by an MTT assay (Fig. 7). Transfection with vector alone, pcDNA3-F, and that without plasmid DNA were used as negative (vector) and vehicle controls. Expression of transfected FLAG-tagged DJ-1 in transfected Neuro2a and GC1 cells was confirmed by Western blotting with an anti-DJ-1 antibody (Figs. 8A and 8B). In these blottings, relatively equal expression levels of endogenous DJ-1 in Neuro2a and GC1 cells were observed. In contrast to the case of transfection with siRNA into cells, Neuro2a and GC1 cells transfected with DJ-1 were found to become significantly more resistant to cell death than those transfected with a vector or with a buffer alone (Figs. 8C and 8D). These results suggest that DJ-1 is one of the proteins that prevent BPA-induced cell death in Neuro2a and GC1 cells.

DISCUSSION

Oxidative stresses caused by excess ROS production in mitochondria and microsomes are known to damage nucleic acid, lipid, and protein, resulting in the onset of various diseases, including cancer, infertility, and neurodegenerative diseases such as PD. Chemicals with structures similar to that of BPA have been reported to accumulate in adipose tissues and to be partitioned preferentially into membranes containing interior hydrophobic protein rather than the polar hydrophilic part (Law *et al.*, 1986; Nunez *et al.*, 2001). Since the mitochondria membrane is composed of this type of protein, BPA is thought to accumulate in the mitochondrial membrane, resulting in an uncoupling of the oxidative phosphorylation, thereby inhibiting complex I activity. The molecular mechanism of ROS production by BPA, however, remains unclear.

In this study, we examined the expression and oxidative levels of DJ-1 after administration of BPA to mice and cultured cells, and we found that expression of DJ-1 was induced by BPA-induced ROS production concomitant with oxidation of DJ-1. We also found that BPA compromised mitochondria, resulting in reduction of the activity of mitochondrial complex 1 and that upon injury of mitochondria by BPA, an elevated expression level DJ-1 was observed on the injured mitochondria

FIG. 5. Localization of DJ-1 after administration of BPA in cultured cells. Neuro2a (A) and GC1 cells (B) were administered 50 and 100 μM BPA. At 48 h after administration, cells were reacted with 500 nM MitoTracker-Green for 40 min, 5 mM MitoSOX-Red for 10 min, and an anti-DJ-1 antibody followed by reaction with an Cy5-conjugated anti-rabbit IgG, and then visualized under a confocal laser microscope. The images were merged (merge). Levels of superoxide (O_2^-) in mitochondria of Neuro2a (C) and GC1 cells (D) were also measured by flow cytometry, and the amounts of fluorescences quantified were shown under each figure. These experiments were carried out more than five times. Proteins extracted from Neuro2a (E) and GC1 cells (F) at 48 h after administration were analyzed by Western blotting with an anti-20-kDa subunit of a complex I monoclonal antibody and anti-actin antibody. (E) Neuro2a ($n = 5$). (F) GC1 ($n = 5$). Asterisks indicate significant difference from the vehicle control; $^*p < 0.05$; $^{**}p < 0.01$.