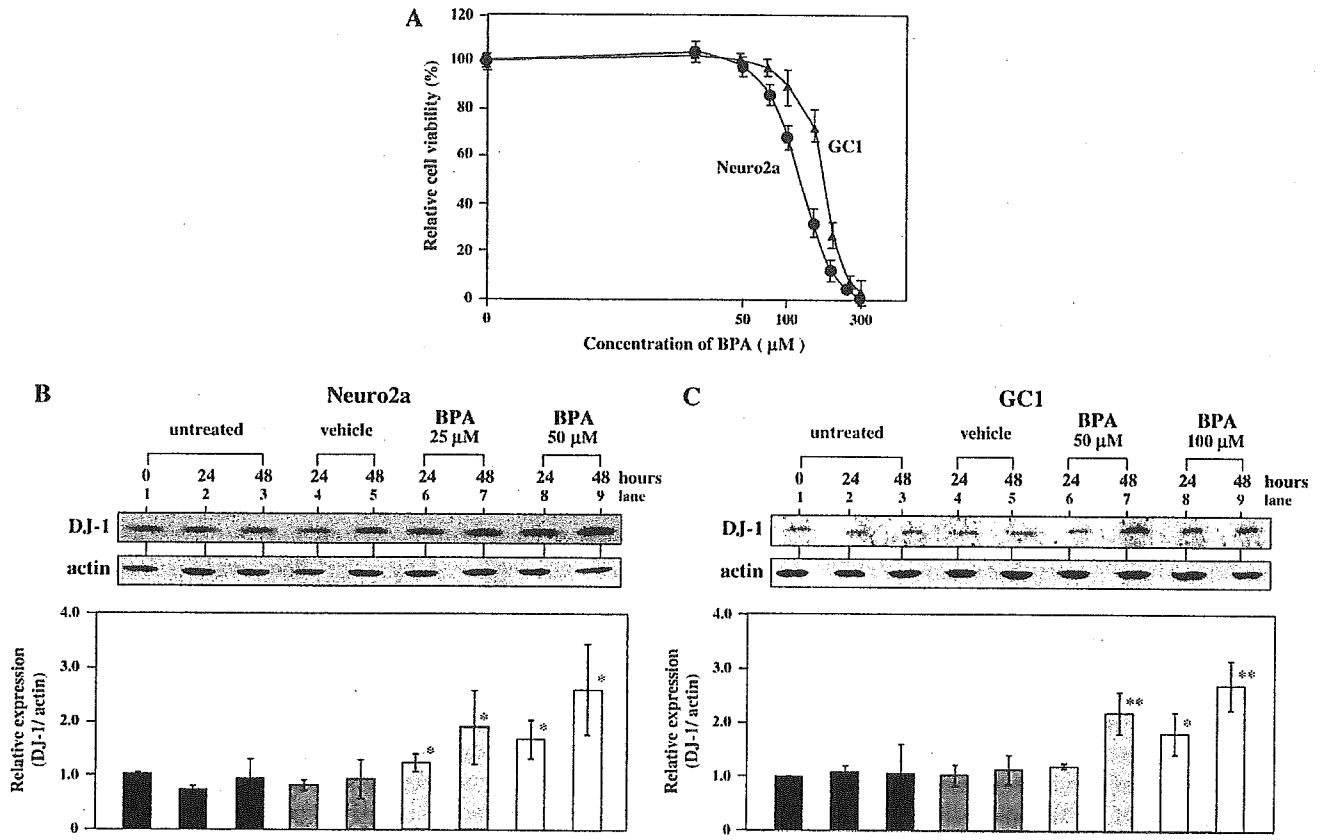


FIG. 2. Expression of DJ-1 in cultured cells after administration of BPA. Nueo2a and GC1 cells were administered various concentrations of BPA. (A) 48 h after administration, viability of cells was measured by an MTT assay. At 24 and 48 h after BPA administration, proteins extracted from cells were analyzed 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

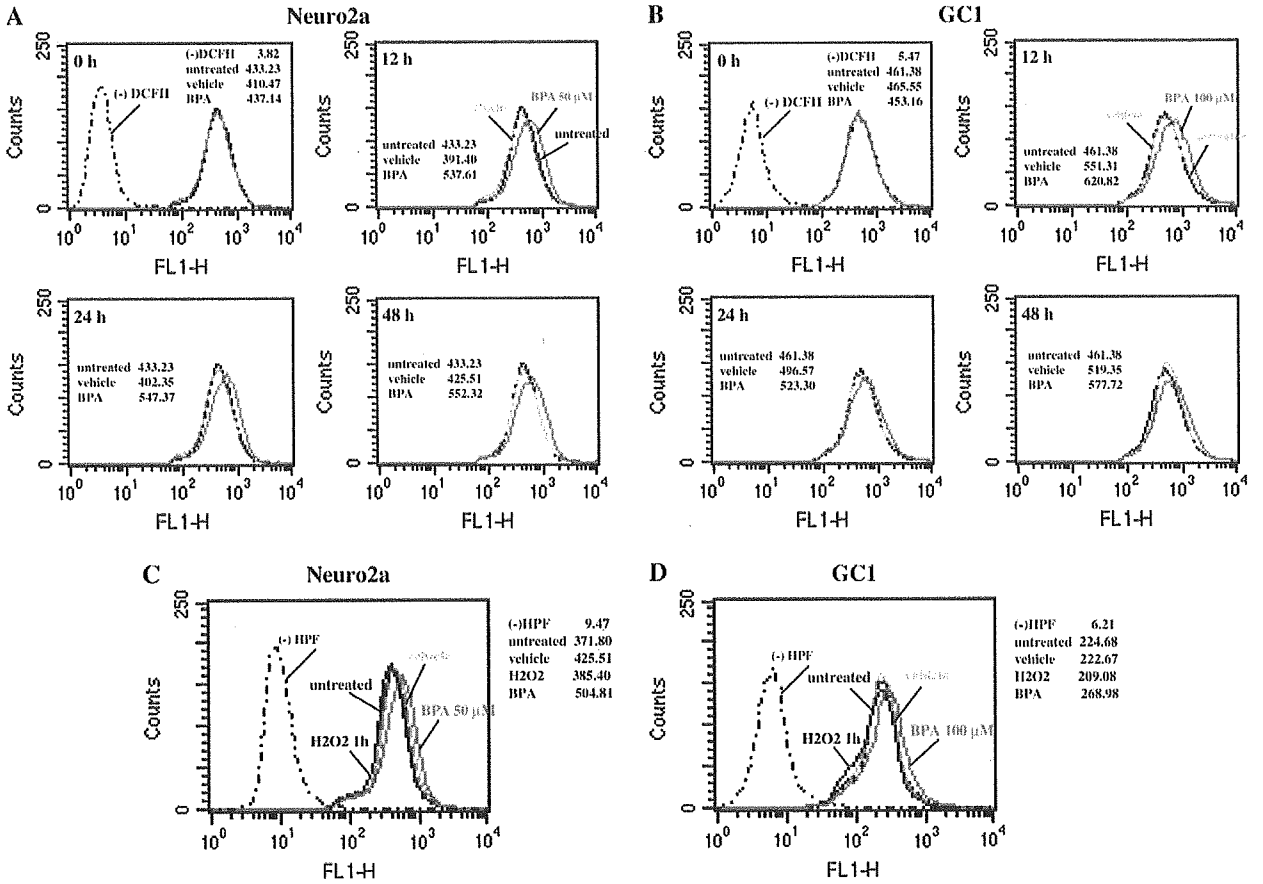


FIG. 3. Production of ROS in cultured cell induced by BPA administration. Neuro2a (A) and GC1 cells (B) were administered 50 and 100 μM BPA, respectively. At various times after administration, cells were treated with 10 mM DCFH-DA for 30 min, and levels of ROS were analyzed by flow cytometry. To detect 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

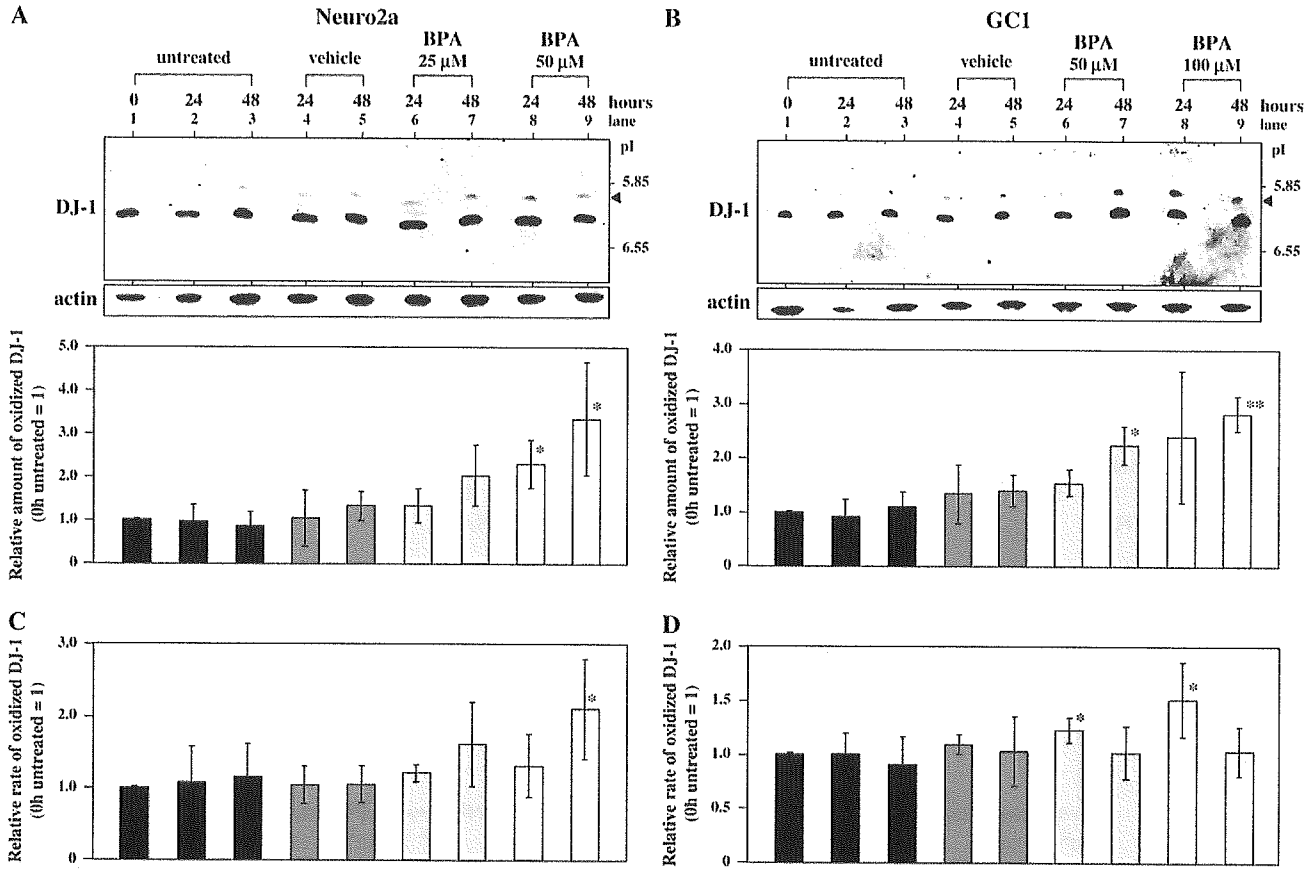
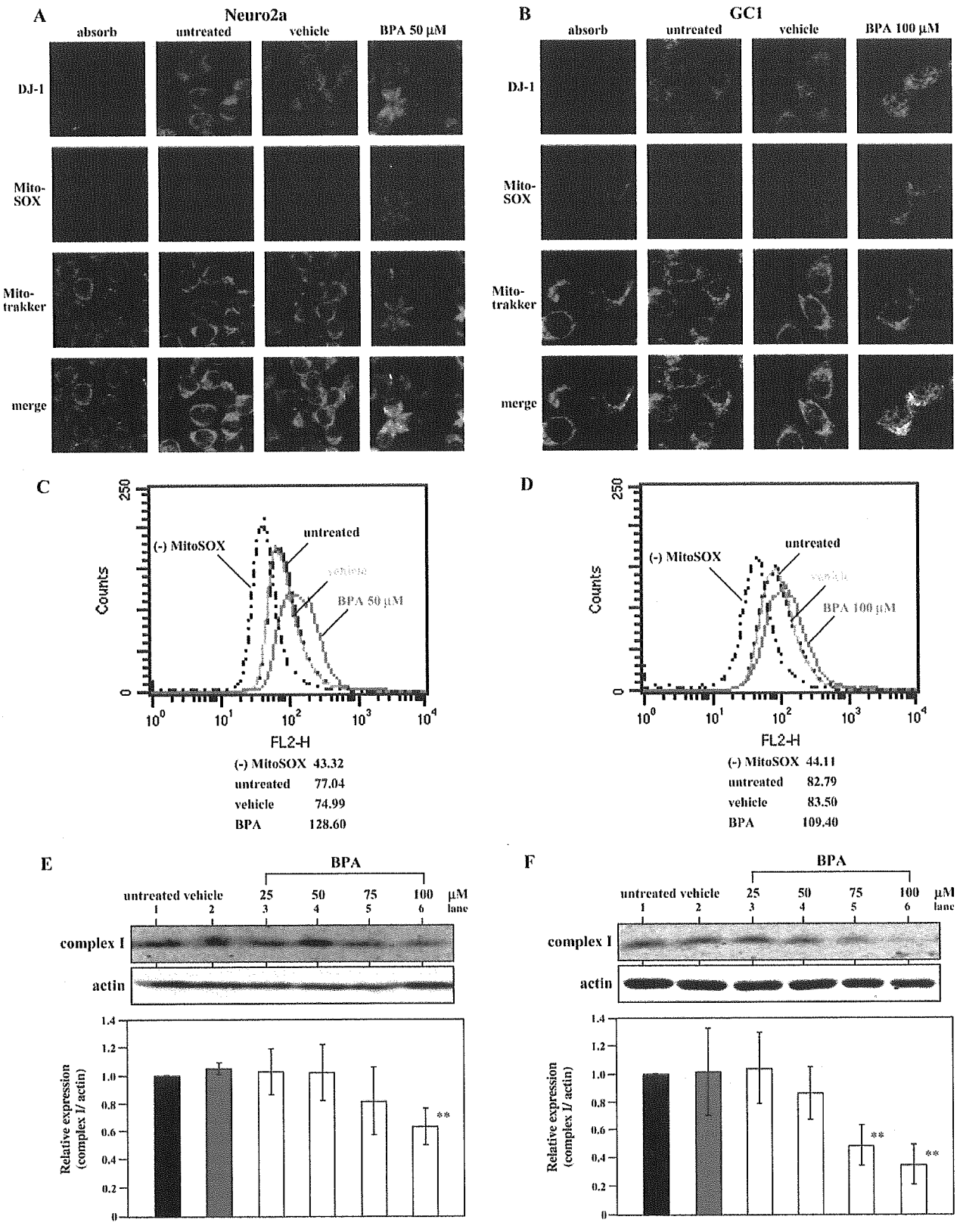


FIG. 4. pI shift of DJ-1 in cultured cells induced by BPA administration. Neuro2a (A) and GC1 cells (B) were administered BPA as described in Figures 3A and 3B. At 48 h after administration, proteins extracted from cells were separated on isoelectric focusing gels and analyzed by blotting with an anti-DJ-1 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.

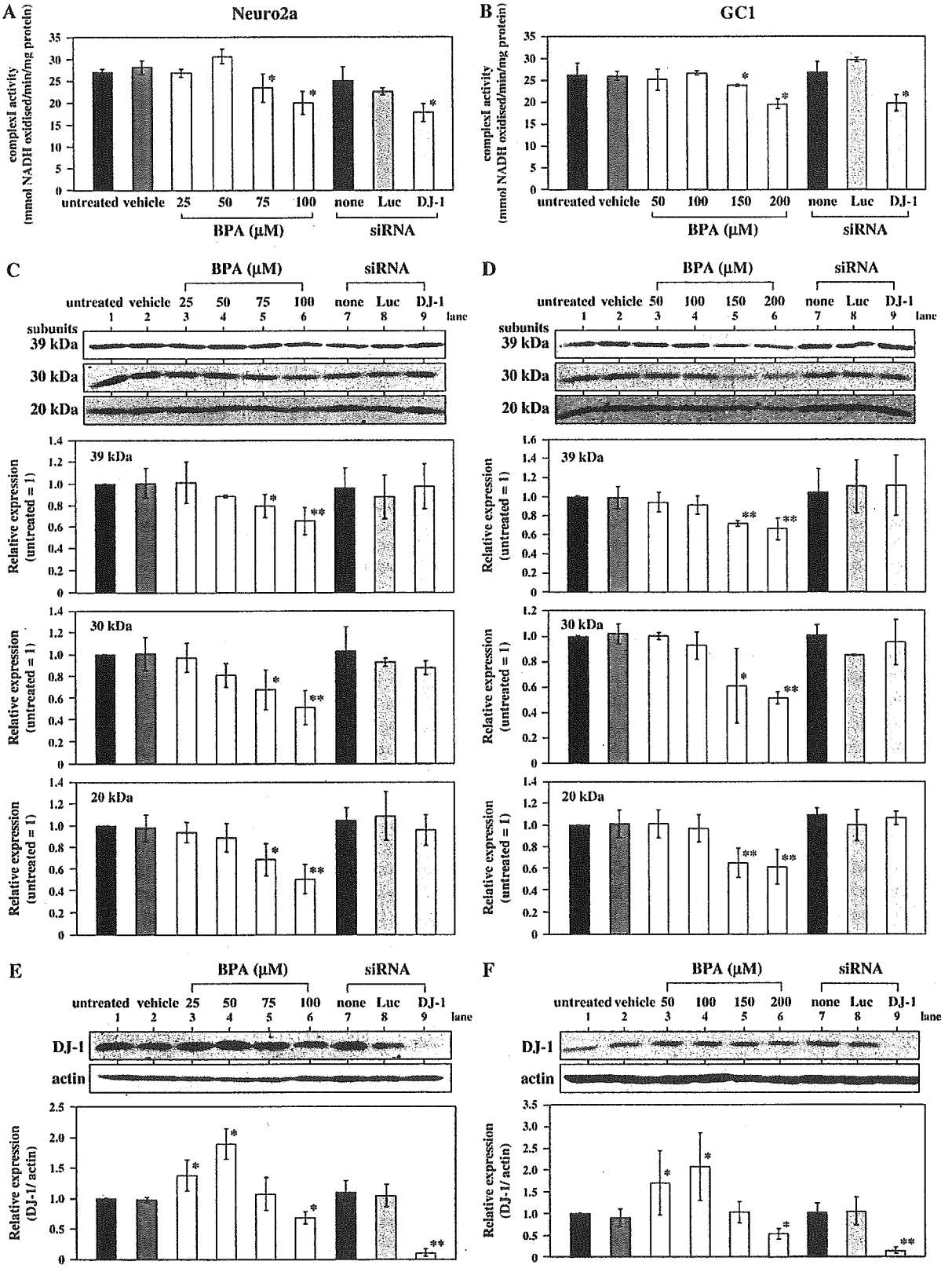
To further investigate the role of DJ-1 in BPA-induced cells death, Nero2a and GC1 cells were transfected with an expression vector for DJ-1, pcDNA3-F-DJ-1, using Lipofectamine 2000, and BPA was administered to cells 24 h after transfection. At 48 h after administration of BPA, cell viabilities were 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; $^*p < 0.05$; $^{**}p < 0.01$.


A
Neuro2a
complexI activity (mmol NADH oxidised/min/mg protein)
untreated vehicle 25 50 75 100 none Luc DJ-1
BPA (μM)
siRNA
B
GC1
complexI activity (mmol NADH oxidised/min/mg protein)
untreated vehicle 50 100 150 200 none Luc DJ-1
BPA (μM)
siRNA
C
D
BPA (μM)
siRNA
untreated vehicle 25 50 75 100 none Luc DJ-1
untreated vehicle 50 100 150 200 none Luc DJ-1
lane
subunits
39 kDa
30 kDa
20 kDa
Relative expression (untreated = 1)
E
F
DJ-1
actin
Relative expression (DJ-1/ actin)

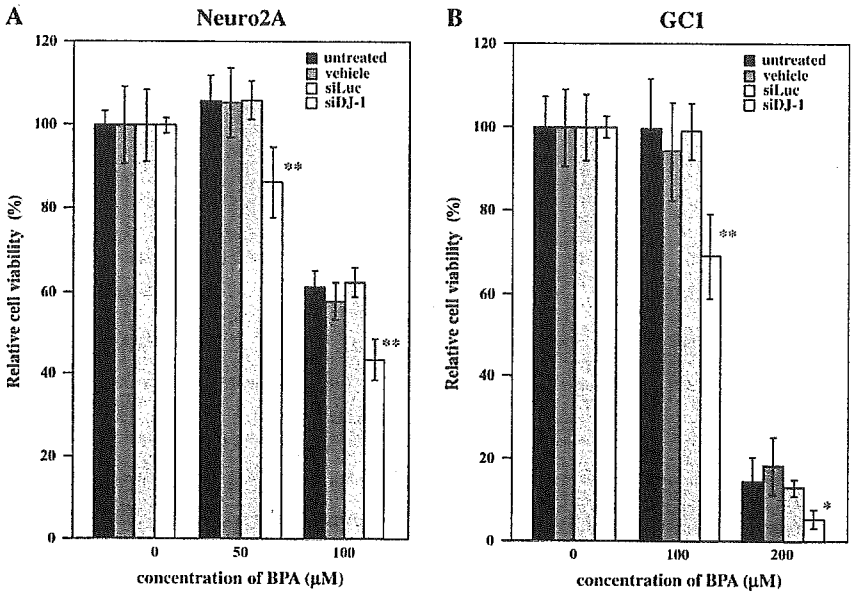


FIG. 7. Sensitization of BPA-induced cell death in DJ-1-knockdown cells. Neuro2a (A) and GC1 cells (B) were transfected with siRNA against *DJ-1* or *luciferase* by lipofectamine plus (Invirogen). At 24 h after transfection, cells were administered various concentrations of BPA. At 48 h after administration, viabilities of cells were measured by an MTT assay. (A) Neuro2a ($n = 5$). (B) GC1 ($n = 5$). Asterisks indicate significant difference from transfection of siRNA targeting the *luciferase* gene; $*p < 0.05$; $**p < 0.01$. Symbols of "untreated, vehicle, siLuc and siDJ-1" in figures indicate cells not transfected, transfected with lipofectamine plus alone, transfected with siRNA targeting the luciferase gene, and transfected with siRNA targeting the DJ-1 gene, respectively.

to restore the activity of complex 1. Since DJ-1 was located in the cytoplasm, nucleus, and mitochondria in various types of cells (Bandopadhyay *et al.*, 2004; Nagakubo *et al.*, 1997; Shendelman *et al.*, 2004; Shinbo *et al.*, 2005a, in press; Wagenfeld *et al.*, 2000; Zhang *et al.*, 2005), and some DJ-1 was translocated to mitochondria after oxidative stress (Blackinton *et al.*, 2005; Jin *et al.*, 2005; Li *et al.*, 2005), the localization of DJ-1 in mitochondria in the cells that had been administered BPA was thought to affect BPA-induced ROS production.

The activity of DJ-1 to sustain the activity of complex 1 was, however, observed at 50 and 100 μM BPA in Neuro2a and GC1 cells, respectively, and this activity was lost over these concentrations. Since, at high concentrations of BPA, cells began to die and the amount of DJ-1 also decreased, it is thought that the amount of DJ-1 is not sufficient to sustain the activity of complex 1. Alternatively, although DJ-1 contains three cysteines, and oxidation of a cysteine at amino acid number 106 (C106) is essential for DJ-1 to exert its anti-oxidative stress activity (Taira *et al.*, 2004b; Takahashi-Niki *et al.*, 2004), oxidation of all three cysteines by excess ROS, which is produced by high doses of BPA, for instance, may result in loss of its activity (Kinumi *et al.*, 2004; Taira *et al.*, 2004b).

Although DJ-1 is an abundant protein and approximately 5×10^5 molecules/cell of DJ-1 is present in cells, it is likely that, in addition to DJ-1, other ROS-scavenging proteins such as superoxide dismutase, glutathione peroxidase and catalase, and proteins belonging to other redox systems participate in protecting cells from BPA-induced cell injury. We have found that drastic changes in expressions of genes, including genes related to stress, apoptosis, oxidative stress, and neurotoxicity, occurred in DJ-1 knockdown and that expressions of some genes were regulated by DJ-1 (Nishinaga *et al.*, in press), suggesting that in addition to anti-oxidative activity of DJ-1, transcriptional function of DJ-1 toward genes related to stress functions is important.

In this study, levels of ROS production peaked at 12 h after BPA administration in GC1 cells and then decreased at 24 and

FIG. 6. Dysfunction of mitochondria in cultured cell induced by BPA administration. Neuro2a (A) and GC1 cells (B) were administered various concentrations of BPA. At 48 h after administration, mitochondria-rich fractions were prepared from cells, and complex 1 activities were measured using 70 μg proteins as described in Materials and Methods. Twenty μg of proteins were analyzed by Western blotting to detect 39-, 30-, and 20-kDa subunits of complex 1. Intensities of bands were quantified as described in Materials and Methods, and relative expression of each subunit to that of untreated cells (untreated) at 0 h is also shown (C, Neuro2a; D, GC1). DJ-1 expression in Neuro2a (E) and GC1 cells (F) that had been administered BPA was analyzed by Western blotting with an anti-DJ-1 antibody. Twenty nM siRNAs against *luciferase* or *DJ-1* were transfected into cells. At 72 h after transfection, complex 1 activity and expressions of subunits of complex 1 and DJ-1 were analyzed as described above. (A, C, and E) Neuro2a ($n = 6$). (B, D, and F) GC1 ($n = 7$). Asterisks indicate significant difference from the control and transfection of siRNA targeting the *luciferase* gene; $*p < 0.05$; $**p < 0.01$.

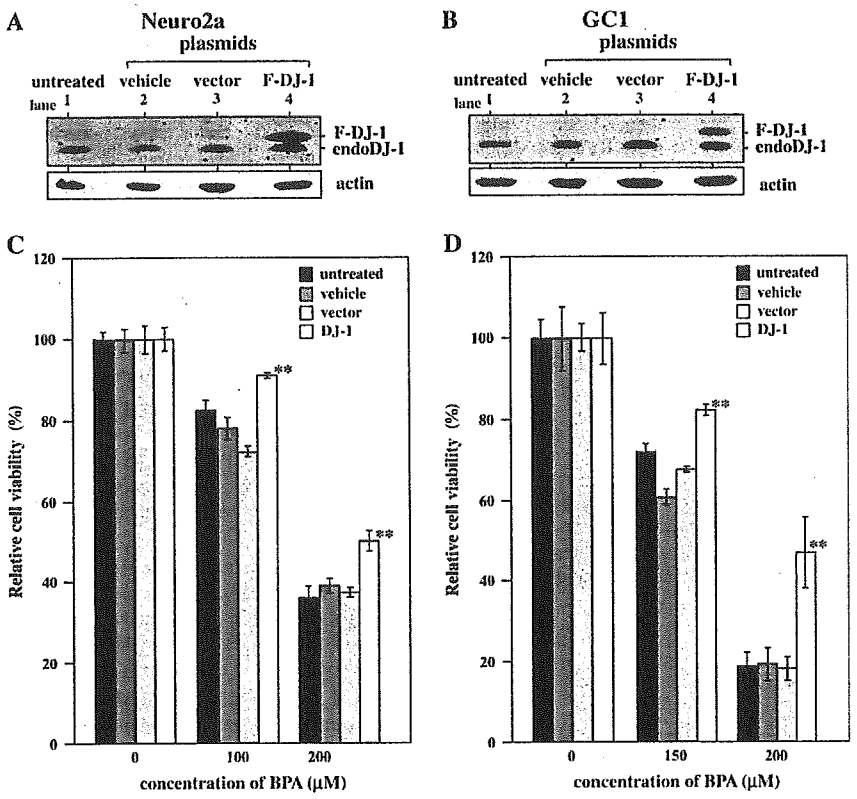


FIG. 8. Abrogation of BPA-induced cell death by DJ-1 in Neuro2a and GC1 cells. (A, B) Neuro2a and GC1 cells in 6-well plates were transfected with 650 and 400 ng of pcDNA3-F-DJ-1 or pcDNA3-F, respectively, using Lipofectamine 2000 (Invitrogen) according to the supplier's manual. At 72 h after transfection, proteins extracted from Neuro2a (A) and GC1 (B) cells were analyzed by Western blotting with anti-DJ-1 (ab4150, Abcam) and anti-actin (Chemicon) antibodies. Proteins were then reacted with an IRDye800-conjugated second antibody and visualized by an infrared imaging system (Odyssey, LI-COR). (C, D) Neuro2a and GC1 cells in 96-well plates were transfected with 16 and 10 ng of pcDNA3-F-DJ-1 or pcDNA3-F, respectively, using Lipofectamine 2000. At 24 h after transfection, Neuro2a (C) and GC1 (D) cells were administered various concentrations of BPA. At 48 h after administration, viabilities of cells were measured by an MTT assay. (C) Neuro2a ($n = 5$). (D) GC1 ($n = 5$). Asterisks indicate significant difference from transfection of pcDNA3, $*p < 0.05$; $**p < 0.01$. Symbols of "untreated, vehicle, vector and DJ-1" in figures indicate cells not transfected, transfected with lipofectamine plus alone, transfected with pcDNA3-F, and transfected with pcDNA3-F-DJ-1, respectively.

48 h. ROS production in Neuro2A cells, however, continued to occur during these periods. In the kidney, liver, and testis, an isoform of UDP-glucuronosyltransferase (UGTs) has been shown to metabolize BPA to BPA-glucuronide (Reinhechel *et al.*, 1995). Although the presence of UGTs in GC1 cells has not been examined, BPA produced at 12 h after BPA administration might be metabolized in GC1 cells.

The present study showed that BPA induced production of various ROS, including highly reactive hydroxy radicals, and ROS have been shown to attack mitochondrial complex I (Yokota *et al.*, 1999). Nakagawa and Toyama reported reduction of complex 1 activity after incubation of a mitochondria-rich fraction with BPA *in vitro,* but degradation of mitochondria and production of ROS were not examined (Nakagawa and Toyama, 2000). Since the present study showed that BPA induces ROS production and inhibits complex I activity by disrupting complex I, it would be interesting to examine whether degradation of complex 1 by BPA also occurs *in vitro* using an electron microscopy and whether this degradation is restored by DJ-1.

Environmental factors are thought to trigger the onset of PD. Several neurotoxins, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTT), rotenone, 6-hydroxydopamine (6-OHDA), and a metabolite of dopamine itself, have been reported to injure mitochondria, resulting in dopaminergic neuron death (Jenner, 2003). Recently, BPA has been reported to affect movement and to induce dopaminergic neuron death when BPA was administered to embryonal mice and into the substantia nigra of adult rats, respectively, though ROS levels were not examined (Kabuto *et al.*, 2004; Mizuo *et al.*, 2004; Suzuki *et al.*, 2003). BPA has been detected in several tissues and the blood serum of humans (Ikezuki *et al.*, 2002; Schonfelder *et al.*, 2002) and accumulates in adipose tissue and membranes, creating the potential for long-term exposure of humans to a low dose of BPA. Human exposure to PBA, and our resent results, suggest that BPA could be a risk factor for the

onset of familial and sporadic PD. Furthermore, there are many factors in the environment that cause oxidative stress to humans, including smoking, UV light, alcohol, and environmental contaminants. These factors, including BPA, might act by themselves or in combination as risk factors for diseases such as cancer, infertility, and neurodegenerative diseases.

Since the activity of mitochondrial complex I was significantly diminished by siRNA targeting DJ-1 in the cells of this study, it is possible that DJ-1 has a novel function to maintain complex I activity. In DJ-1-knockdown cells, complex 1 was still intact. Although the precise molecular basis of this reaction is not clear at present, it is important to clarify its mechanism.

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Proper SUMO-1 conjugation is essential to DJ-1 to exert its full activities

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Abstract

DJ-1 is a multifunctional protein that plays roles in transcriptional regulation and antioxidative stress, and loss of its function is thought to result in the onset of Parkinson's disease (PD). Here, we report that DJ-1 was sumoylated on a lysine residue at amino-acid number 130 (K130) by PIASxα or PIASy. The K130 mutation abrogated all of the functions of DJ-1, including *ras*-dependent transformation, cell growth promotion and anti-UV-induced apoptosis activities. Sumoylation of DJ-1 was increased after UV irradiation concomitant with a pI shift to an acidic point of DJ-1. Furthermore, L166P, a mutant DJ-1 found in PD patients, and K130RX, an artificial mutant containing four mutations in DJ-1, were improperly sumoylated, and they became insoluble, partly localized in the mitochondria and degraded by the proteasome system. Both L166P-expressing cells and DJ-1-knockdown cells were found to be highly susceptible to UV-induced cell apoptosis.
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Keywords: DJ-1; SUMO-1 conjugation; apoptosis; oxidative stress; cell transformation; cell growth

Abbreviations: AR, androgen receptor; PD, Parkinson's disease

Introduction

DJ-1 was first identified by our group as a novel candidate of the oncogene product that transformed mouse NIH3T3 cells in cooperation with activated *ras*.[1] The human *DJ-1* gene is mapped at chromosome 1p36.2–p36.3, where a hot spot of chromosome abnormalities has been reported in several tumors.[2] However, the mechanism by which cells are transformed has not been clarified. Another group has also identified RS, another name for human DJ-1, as a regulatory component of an RNA-binding protein complex.[3] Furthermore, CAP-1 or SP22, a rat homologue of human DJ-1, has been identified by other laboratories as a key protein related to infertility of male rats that were exposed to sperm toxicants such as ornidazole and epichlorohydrin. DJ-1 in the sperm and epididymis decreased in parallel with the subsequent infertility of the rats and was later found to be related to infertility.[4–9] It has also been shown that DJ-1 is a circulating tumor antigen in breast cancer, in which DJ-1 is secreted from cells to serum,[10] and that DJ-1 is overexpressed in smoker-derived lung adenocarcinoma.[11] DJ1 is therefore thought to play roles in both somatic cells and sperm.

We have shown that DJ-1 acts as a positive regulator for the androgen receptor (AR) by sequestering PIASxα or DJBP.[12–14] PIASxα, a member of the PIAS family of proteins, and DJBP, a novel DJ-1-binding protein, function as negative regulators for AR by preventing AR DNA-binding activity and by recruiting histone deacetylase complex, respectively.[12,13] We have also showed that DJ-1 bound to p53 to restore p53 transcription activity inhibited by Topors/p53BP3 and that transforming activity of DJ-1 was stimulated by Abstrakt, another DJ-1-binding protein.[15,16]

Deletion and point (L166P) mutations of *DJ-1* have recently been shown to be responsible for the onset of familial Parkinson's disease (PD), PARK7,[17] and other homozygous and heterozygous mutations of *DJ-1* have been identified in patients with familial or sporadic PD.[18–20] PD involves an irreversible degeneration of the dopaminergic nigrostriatal pathway. Genes responsible for rare familial early-onset PD, including α-synuclein,[21] Parkin[22] and UCH-L1,[23] have been identified, and they are thought to play a role in ubiquitin–proteasome dysfunction in PD. L166P DJ-1 has been reported to be degraded, in part, by the ubiquitin–proteasome system.[24–28] DJ-1 was 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.[29–31] Various lines of evidence also suggest that oxidative stresses contribute to the cascade leading to dopaminergic cell degeneration in PD,[32–35] and expression of DJ-1 was shown to be induced by oxidative stresses.[36–39] A pI shift of DJ-1 towards a more acidic isoform has been observed in PD patients.[29] We previously reported that DJ-1 plays a role in the antioxidative 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 L166P, lead to cell death.[40,41] Recently, PINK1, a causative gene product of *PARK6*, has also been suggested to play a role in an antioxidative stress response,[42] but precise roles of PINK1 are not clarified.

SUMO-1 conjugation to lysine residues of proteins is catalyzed in three steps by E1, E2 and E3 enzymes and modulates interactions, localization, activity and stability of proteins to be sumoylated (see recent reviews and references therein).[43,44] E3 SUMO-1 ligases promote attachment of the SUMO modifier to target proteins, and some SUMO-1 ligases have been identified. PIAS family proteins, which include

PIAS1, PIAS3, PIASxα, PIASxββ and PIASy, were initially identified as negative regulators of STAT-transcription factors and later found to function as transcriptional coregulators in various other cellular pathways, including Wnt signaling, the p53 pathway and steroid hormone signaling. PIAS proteins were then found to act as E3 SUMO-1 ligases that stimulate SUMO-1 conjugation to proteins, including p53,[45–47] AR,[47] Jun[46] and Lef-1.[48] In most cases, the PIAS/SUMO pathway appears to be an important mechanism of transcriptional regulation.

In this study, we found that mutation of lysine at amino-acid number 130 of DJ-1 abrogated all of the functions of DJ-1 and that improperly sumoylation to L166P mutant DJ-1 makes DJ-1 insoluble, which may trigger the onset of PD.

Results

SUMO-1 conjugation of DJ-1 in cells

We have reported that DJ-1 binds to PIASxα and regulates the AR transcription activity.[12] In addition to PIASxα, both Ubc9 and SUMO-1 were identified as DJ-1-binding proteins by a yeast two-hybrid screening. Ubc9 is an E2 enzyme in the SUMO-1 conjugation system. These findings indicate the possibility of sumoylation of DJ-1. To test this possibility, human H1299 cells were lysed in SDS-containing buffer, boiled, and immunoprecipitated with an anti-DJ-1 antibody or nonspecific IgG. Precipitates were analyzed by Western blotting with an anti-DJ-1 antibody or an anti-SUMO-1 antibody (Figure 1). In addition to a 26-kDa band corresponding to DJ-1, a 44 kDa band appeared in the immunoprecipitate with the anti-DJ-1 antibody but not with IgG (Figure 1, lanes 2 and 1, respectively). Since SUMO-1 conjugation to proteins is known to give an addition of approximately 20 kDa to proteins in SDS-polyacrylamide gel and since the 44-kDa band reacted with the anti-SUMO-1 antibody, the results clearly showed that DJ-1 was sumoylated *in vivo* in H1299 cells (Figure 1, lane 4).

Then EST clones for cDNAs encoding DJ-1 homologs of chicken, fish (Atlantic salmon) and *Drosophila* were obtained, and monkey DJ-1 cDNA was constructed by RT-PCR using total RNA from monkey COSI cells as a template. A search for amino-acid sequences for DJ-1 homologs of *Xenopus*, nematode and *Drosophila* were carried out in the NIH-NCI Database. Interestingly, two homologs of DJ-1 were found to be present both in nematode and *Drosophila*. Amino-acid sequences of DJ-1s were found to be highly conserved among species, and the identities of the sequences of monkey, mouse, rat, chicken, *Xenopus*, fish and *Drosophila* to that of human DJ-1 are 98, 91, 91, 89, 78, 80, 52 (nematode 1), 44 (nematode 2), 52 (*Drosophila* 1) and 58% (*Drosophila* 2), respectively (Figure 2a). Of 16 lysines, candidates for the sumoylated amino acid in human DJ-1, the lysine at amino-acid number 130 was found to be conserved among all of the species (Figure 2a). We therefore converted all of the lysines of human DJ-1 to arginine and tested the SUMO-1 conjugation to these mutants of DJ1s in H1299 cells into which FLAG-DJ-1, HA-Ubc9 and T7-SUMO-1 were cotransfected. At 48 h after transfection, the cells were lysed in SDS-containing buffer, boiled, and immunoprecipitated with an anti-FLAG antibody. Precipitates were analyzed by Western blotting with the anti-FLAG antibody (Figure 2b). The results showed that wild-type DJ-1 and all of the arginine-substituted mutants except for K130R were sumoylated, indicating that K130 is a major sumoylation site of DJ-1 (Figure 2, lane 12). It was noted that both the K99R mutant and its sumoylated form of DJ-1 run slowly in gel, suggesting that this mutant possesses a conformation different from that of wild-type DJ-1 (Figure 2, lane 10).

We previously reported that DJ-1 binds to both PIASxα and PIASy,[12] both of which are known to function as an E3 SUMO-1 ligase. We therefore tested whether PIASxα and PIASy are E3 SUMO-1 ligases for DJ-1. H1299 cells were cotransfected with FLAG-DJ-1, HA-Ubc9, T7-SUMO-1 and various amounts of HA-PIASxα or -PIASy and SUMO-1 conjugation to DJ-1 was examined. Expressions of HA-PIASxα and -PIASy were first confirmed by blotting the precipitates with an anti-HA antibody (Figure 3a, lanes 4–9, 11 and 12 in lower part). The results showed that while the HA-tag alone did not stimulate sumoylation of DJ-1, both HA-PIASxα and -PIASy stimulated

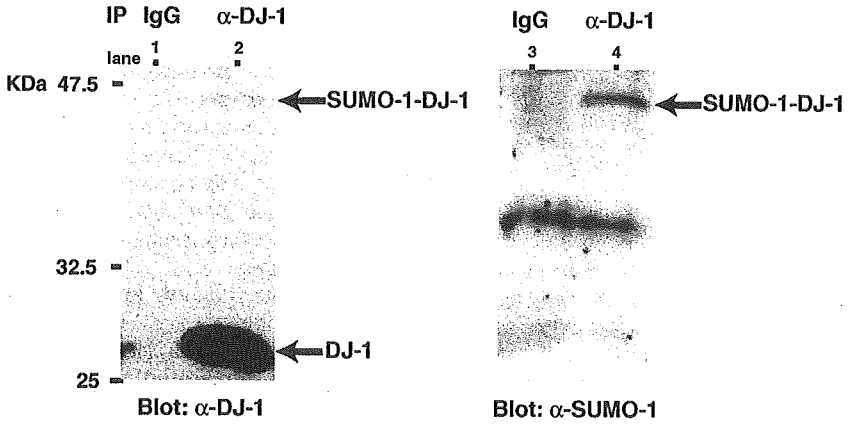

Figure 1 SUMO-1 conjugation to DJ-1 *in vivo*. Proteins were extracted from H1299 cells, immunoprecipitated with an anti-DJ-1 polyclonal antibody, and blotted with an anti-DJ-1 monoclonal antibody or anti-SUMO-1 polyclonal antibody as described in Materials and Methods

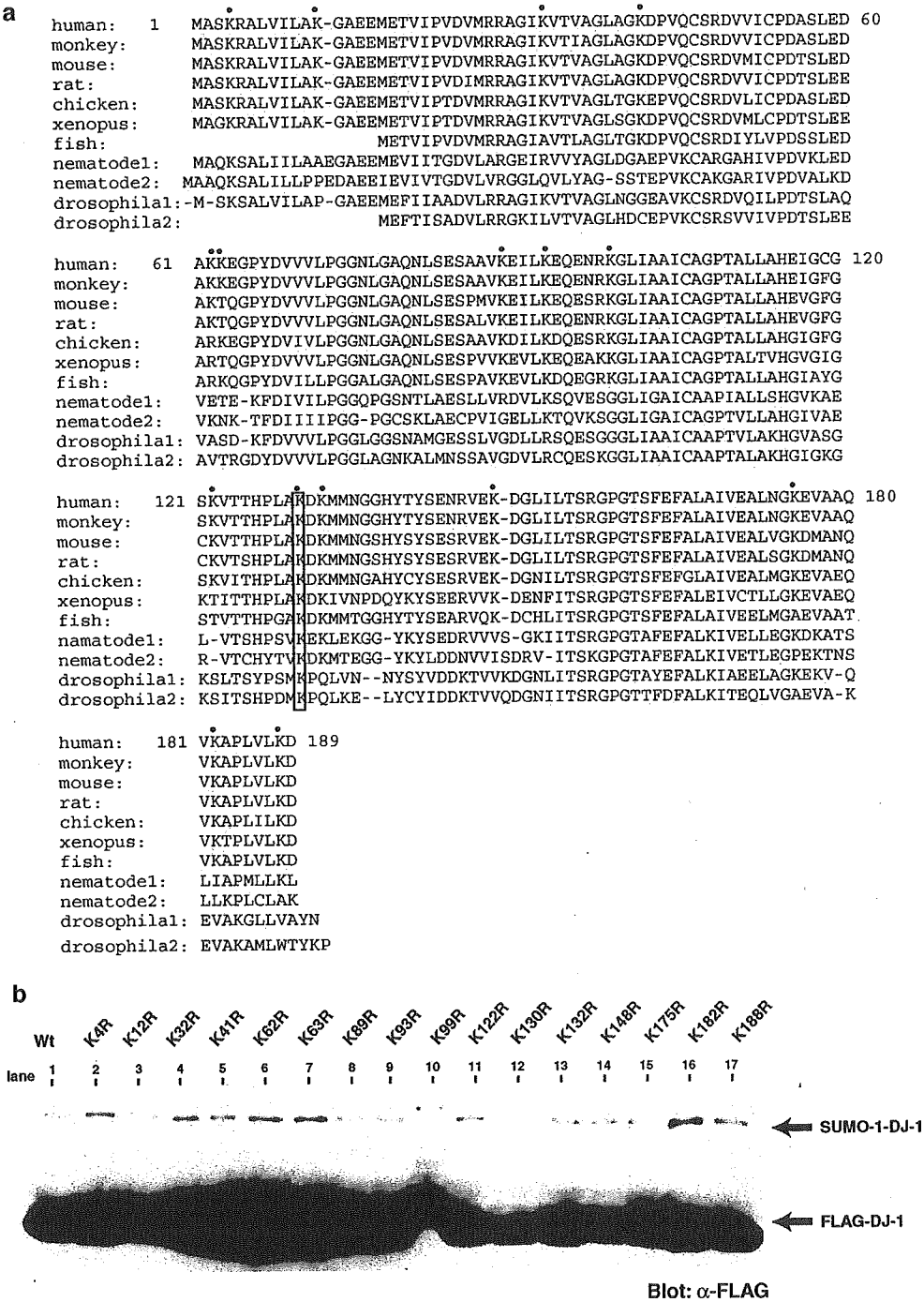

Figure 2 Alignment of deduced amino-acid sequences of DJ-1s from human to *Drosophila* and identification of the sumoylation site on DJ-1. (**a**) Accession numbers for amino-acid sequences are D61380 (human), AB073863(monkey), AB015652 (mouse), AJ007291 and AF157511 (rat), AB076264 (chicken), AF394958 (*Xenopus*), AB079598 (Atlantic salmon), AAB37889 (nematode 1), AAK18904 (nematode 2), AB079599 (*Drosophila* 1) and AAF58316 (*Drosophila* 2). The identical amino acids are drawn in gray, and the lysines are indicated by dots. Lysines corresponding to lysine at amino-acid number 130 of human DJ-1 are boxed. (**b**) H1299 cells were transfected with FLAG-DJ-1 and lysine-substituted mutants ofDJ-1. At 48 h after transfection, proteins extracted from transfected cells were immunoprecipitated with an anti-FLAG antibody and blotted with the anti-FLAG antibody as described in Materials and Methods

sumoylation of wild-type DJ-1 but not K130R in a dose-dependent manner (Figure 3a), indicating that both PIASxα and PIASy work as E3 SUMO-1 ligases for DJ-1. To examine the effect of PIASxα on sumoylation to endogenous DJ-1, H1299 cells were cotransfected with PIASxα-HA, T7-SUMO-1 and Ubc9-HA, and SUMO-1 conjugation to DJ-1 was

examined. As in the case of the ectopic expression system shown in Figure 3a, endogenous DJ-1 was sumoylated by PIASxα in a dose-dependent manner (Figure 3b, lanes 2–5), and similar results were obtained when PIASy was cotransfected into H1299 cells (data not shown). These results clearly indicate that both PIASxα and PIASy are E3 SUMO-1 ligases for DJ-1.

Abrogation of DJ-1 activities by K130 mutation

To know the importance of K130 for DJ-1 activities, transformation activities of several lysine-substitution mutants of DJ-1 were tested in rat 3Y1 cells by cotransfection of DJ-1 mutants with activated *ras* (Figure 4a). As previously reported in mouse NIH3T3 cells,[1] wild-type DJ-1 gave a number of transformed foci only in the case of cotransfection with activated *ras*. While DJ-1 mutants of both K62, 63R, in which lysines at #62 and 63 were changed to arginine, and K93R still gave numbers of foci similar to that given by wild-type DJ-1, DJ-1 mutants of K130R and K130RX, in which three other amino acids, S57R, E96G and H126Y, had been changed,[12] as well as K130 gave smaller numbers of foci than that without activated *ras*. These results clearly indicate that K130 in DJ-1 is of functional importance for the transforming activity of DJ-1.

Many proteins possessing transforming activity often have cell growth-stimulating activity. To test this activity of DJ-1, rat 3Y1 cells were transfected with the expression vectors for wild-type and mutant forms of DJ-1 that were cloned in pcDNA3 containing a neomycin-resistant gene, and they were cultured in a medium containing G418. G418-resistant colonies were then counted 14 days after transfection (Figure 4b). Transfection with c-Myc as a positive control gave a large number of colonies, while transfection with the vector alone or no-DNA control (none) gave few or no colonies. Wild-type or non-K130 mutants of DJ-1, K62, 63R and K93R, gave similar numbers of colonies to those of c-Myc. K130R and K130RX mutants of DJ-1, on the other hand, abolished the colony-forming activity to the level of the vector alone. These results indicate that DJ-1 possesses cell growth-promoting activity that is dependent upon K130.

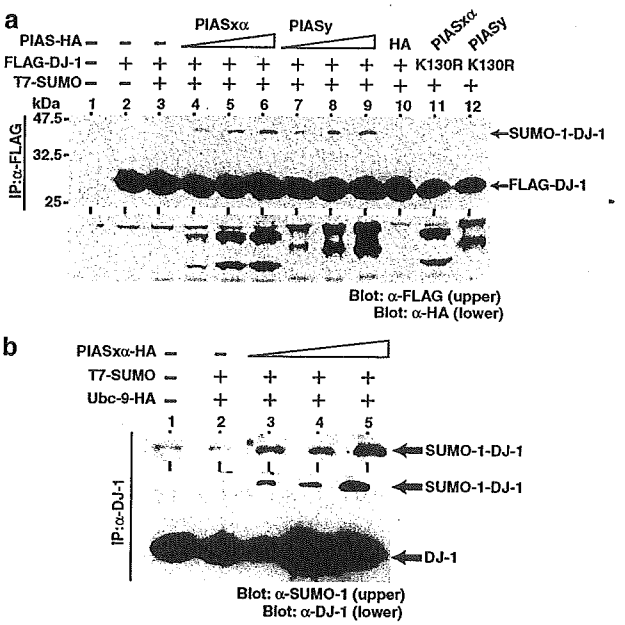

Figure 3 Stimulation of SUMO-1 conjugation to DJ-1 by PIASxα and PIASy. (a) H1299 cells were cotransfected with FLAG-DJ-1, T7-SUMO-1, Ubc9-HA and various amounts of PIASxα-HA or PIASy. At 48 h after transfection, proteins extracted from transfected cells were immunoprecipitated with an anti-FLAG antibody and blotted with the anti-FLAG antibody or an anti-HA antibody as described in Materials and Methods. The amounts of expression vectors for PIASxα-HA and PIASy were 2.5, 5.0 and 7.5 μg in lanes 4 and 7, 5 and 8, and 6 and 9, respectively. (b) H1299 cells were cotransfected with T7-SUMO-1, Ubc9-HA and various amounts of PIASxα-HA. At 48 h after transfection, proteins extracted from transfected cells were immunoprecipitated with an anti-DJ-1 antibody and analyzed by Western blotting with the anti-DJ-1 antibody or an anti-SUMO-1 antibody as described in Materials and Methods. The amounts of expression vectors for PIASxα-HA were 2.5, 5.0 and 7.5 μg in lanes 3, 4 and 5, respectively

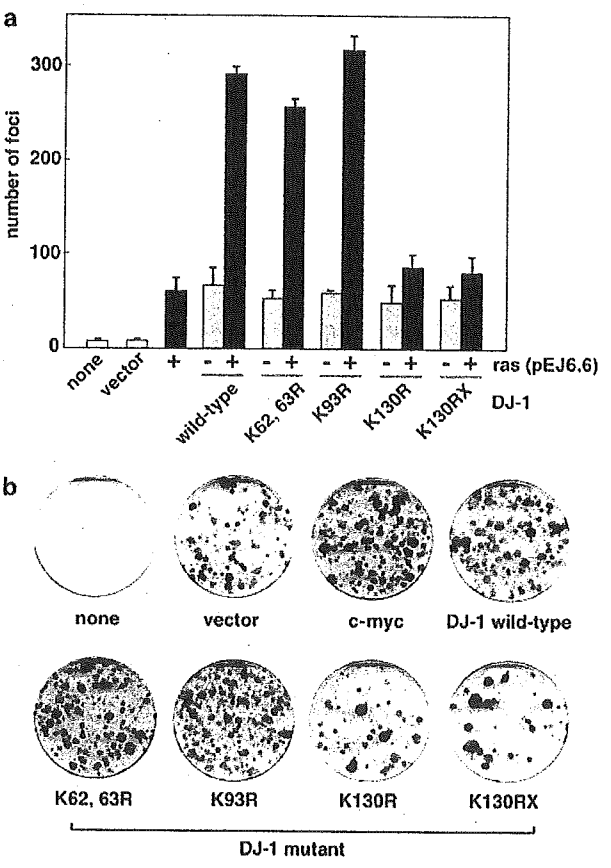

Figure 4 Effects of lysine mutations of DJ-1 on the transforming and cell growth promoting activities of DJ-1. (a) Rat 3Y1 cells were transfected with expression vectors for activated wild-type DJ-1or mutants of DJ-1 with or without activated H-*ras*. In total, 14 days after transfection, the transformed cell foci were counted. (b) Rat 3Y1 cells were transfected with neomycin-resistant gene-containing expressionvectors for wild-type DJ-1 or mutants of DJ-1, and they were cultured in the presence of G418. At 14 days after transfection, the G418-resistant cell colonies were counted. Pictures of the colonies are shown

Induction of DJ-1 expression and stimulation of SUMO-1 conjugation to DJ-1 by UV irradiation

Expression of DJ-1 was induced in various cells that had been subjected to treatment with paraquat, endotoxin or iron, which yielded ROS.[36–39] We also showed that expression of DJ-1 was induced in human neuroblastoma SH-SY5Y cells treated with hydrogen peroxide.[41] Since ultraviolet (UV) irradiation is known also to induce ROS, we first examined whether DJ-1 expression is induced after 20 J/m^2 UV irradiation by using p53-positive and p53-negative cell lines, ME180 and H1299 cells, respectively, in which p53 is a positive control as an induced protein against UV irradiation (Figure 5A). In ME180 cells, while expression of p53 was induced at 2 h after UV irradiation, expression of DJ-1 was induced at 15 min after UV irradiation and the level peaked at 30–45 min and then gradually decreased up to 3 h after UV irradiation (Figure 5A-a). In H1299 cells, induction of DJ-1 expression started at 15 min after UV irradiation and the level peaked at 30–45 min and then gradually decreased until 6 h after UV irradiation (Figure 5A-b). These results suggest that expression of DJ-1 is induced by UV irradiation in a p53-independent manner.

Shifts of the isoelectric point of DJ-1 in cells caused by oxidative stresses have been reported.[36–41] H1299 cells were irradiated with 20 and 50 J/m^2 UV, and proteins prepared from cells were separated on isoelectric focusing gel over the pI range of 5–8 (Figure 5B). After blotting with an anti-DJ-1 antibody, pI of DJ-1 was found to be shifted to a more acidic point, from 6.3 to 5.2, at 1 h after UV irradiation (Figure 5B). SUMO-1 conjugation to DJ-1 was also found to be stimulated at 25 min, peak at 30 min and then gradually decreased after UV irradiation (Figure 5C). These results suggest that DJ-1 is first sumoylated and then oxidized after UV irradiation.

ME180 cells after UV irradiation were stained with a rabbit anti-DJ-1 polyclonal antibody, reacted with an FITC-conjugated anti-rabbit IgG, and visualized under a confocal laser microscope (Figure 5D). Nuclei and the mitochondria in the cytoplasms were also stained with DAPI and MitoTracker, respectively. Endogenous DJ-1 was found to be localized mainly in the cytoplasm and sparsely in the nucleus without UV irradiation (Figure 5D, time 0 h). After UV irradiation, almost all the DJ-1 were translocated to the nucleus, which was shown in the merged figures as light blue color (Figure 5D, time 0.25–0.75 h). At 1 h after UV irradiation, some DJ-1 were relocalized in the cytoplasm and almost all the DJ-1 were located in the cytoplasm at 24 h after UV irradiation. These results indicate that a portion of DJ-1 is shuttled in between the cytoplasm and nucleus of UV-irradiated H1299 cells.

Improper sumoylation and insoluble form of L166P mutant of DJ-1 found in PD patients

Homozygous point mutations of the *DJ-1* gene have been found in Italian kindred of PD patients,[17] and its gene product L166P mutant form of DJ-1 has been reported to be unstable and to be degraded, in part, by the ubiquitin–proteasome system.[24–28,49] We therefore examined the sumoylation status of L166P DJ-1 (Figure 6). H1299 cells were transfected with FLAG-tagged wild type and three mutants of DJ-1 together with SUMO-1 and Ubc9, and protein extracts from transfected cells were immunoprecipitated with an anti-FLAG antibody. Precipitates were then separated on two gels and analyzed by Western blotting with the anti-FLAG antibody or an anti-SUMO-1 antibody (Figure 6A). Sumoylation of wild-type DJ-1 but not K130R DJ-1 was first confirmed (Figure 6A-a, lanes 1, 2, 6 and 7). Characteristics of the band of 62 kDa observed above the DJ-1-SUMO-1 bands are not clear at present. It is interesting that L166P and K130RX showed ladder bands over the range of SUMO-1-DJ-1 in a blot with the anti-FLAG antibody and that these bands also reacted with the anti-SUMO-1 antibody (Figure 6A-a, lanes 8, 9, 3 and 4), indicating that L166P and K130RX mutants of DJ-1 are conjugated with multi- or poly-SUMO-1, or alternatively that these mutant proteins became misfolded, resulting in the formation of the abnormal aggregates. Since a lysine residue at amino-acid number 130 (K130) in K130RX DJ-1 was changed to arginine, it is possible that sumoylation occurs on lysine residue(s) other than K130 in K130RX DJ-1 and that this is also true for sumoylation of L166P DJ-1. To examine this possibility, K130 in L166P DJ-1 was changed to arginine and sumoylation assay of this mutant (K130R, L166P DJ-1) was carried out. This mutant showed ladder bands as did K130RX and L166P (Figure 6A-a, lanes 5 and 10), indicating that sumoylation occurred some lysine residues, including L130, in L166P DJ-1. This improper sumoylation of L166P and K130RX mutants of DJ-1 was also observed in an *in vitro* reconstituted system using recombinant proteins comprised of SAE1/SAE2 complex, Ubc9 and SUMO-1 (Figure 6A-b, lanes 3 and 4), suggesting that the structure of L166P and K130RX mutants of DJ-1 possess easier accessibility to be sumoylated than does wild-type DJ-1 and was changed to be improperly sumoylated.

We then examined the solubility of wild-type DJ-1 and mutants of DJ-1 in cells. Human 293T cells were transfected with FLAG-tagged wild-type DJ-1 and four mutants of DJ-1, L166P, K130R, K130RX and C106S. An amino acid at C106 is a putative active center for protease activity of DJ-1, and a C106S mutant had no protease and antioxidative stress activities.[28,41,50–52] At 48 h after transfection, the soluble proteins were extracted with 0.1% NP-40 and insoluble proteins were further dissolved in 2.0% SDS, and these proteins were analyzed by Western blotting with an anti-FLAG antibody (Figure 6B). While wild-type DJ-1, C106S DJ-1 and K130R DJ-1 were in soluble forms, both L166P and K130RX were found to be in insoluble forms. Since insoluble forms of DJ-1 have been observed in patients with degenerative neuronal diseases, it is interesting that improperly sumoylated DJ-1 becomes insoluble (see Discussion).

We then examined the cellular localization of these mutants of DJ-1. H1299 cells were transfected with FLAG-tagged wild-type DJ-1 and four mutants of DJ-1. At 48 h after transfection, cells were stained with an ant-FLAG antibody and visualized with an FITC-conjugated anti-mouse antibody (Figure 7). Since L166P DJ-1 has been reported to be localized, in part, in the mitochondria[17] and we have reported that DJ-1 also played a role in antiendoplasmic reticulum (ER) stress-induced cell death,[40] cells were also reacted with MitoTracker and an anti-GRP78 to see the mitochondria and ER, respectively (Figures 7a and data not shown, respectively).

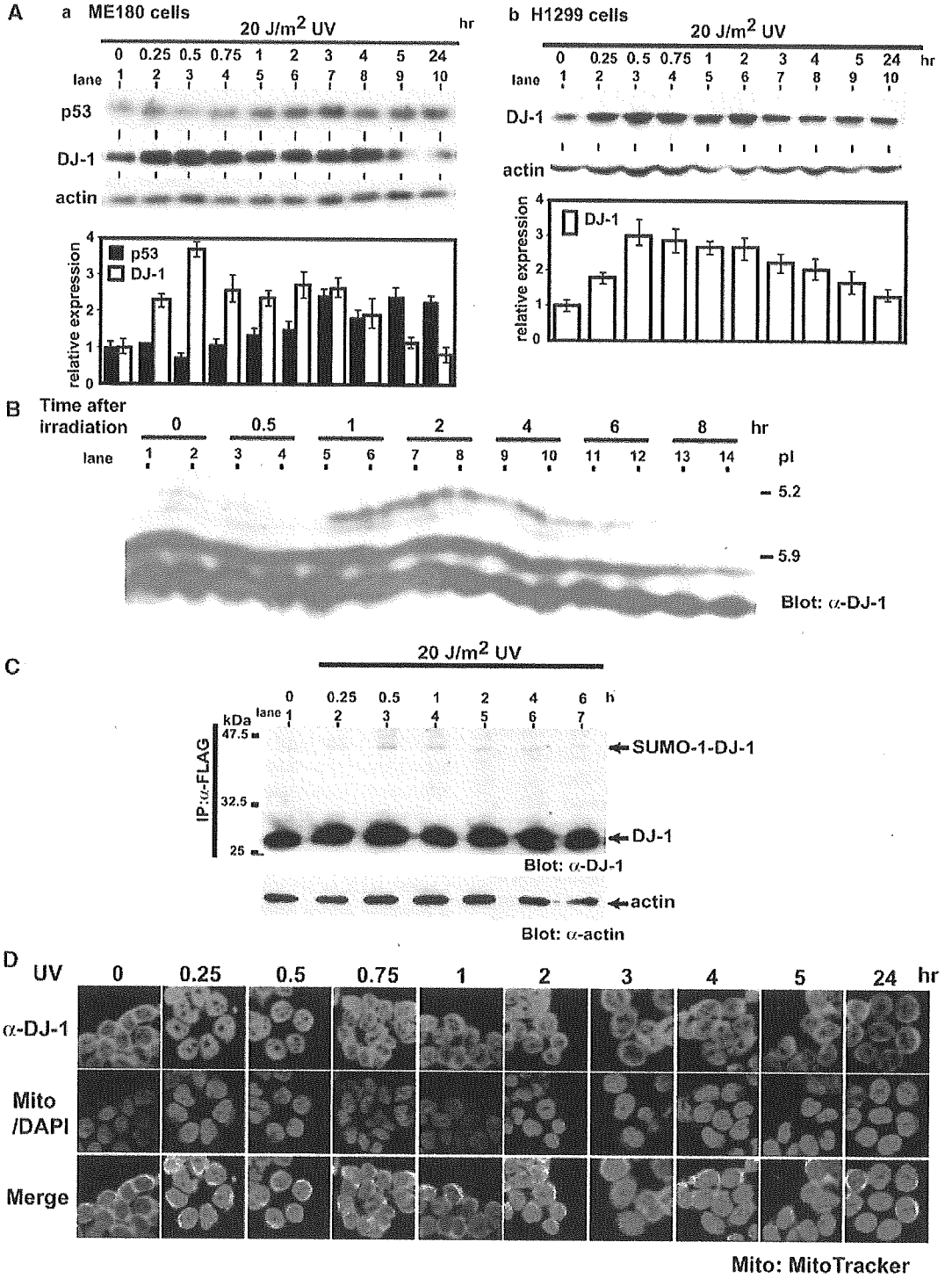

Figure 5 Induction of expression, pI shift and stimulation of sumoylation of DJ-1. (**A**) ME180 cells (a) and H1299 cells (b) were irradiated with 20 J/m^2 of UV, and at various times after irradiation, proteins extracted from cells were blotted with an anti-p53 (FL-393, Santa Cruz), anti-DJ-1 polyclonal and anti-actin antibodies (upper panel). Bands that reacted with the antibody were quantitated using a densitometer (lower panel). (**B**) H1299 cells were irradiated with 20 and 50 J/m^2 of UV, and proteins in the extracts were then analyzed by isoelectric focusing phoresis gel as described in Materials and Methods. (**C**) H1299 cells were irradiated with 20 J/m^2 of UV. At various times after irradiation, proteins extracted from cells were immunoprecipitated with an anti-DJ-1 polyclonal antibody and blotted with an anti-DJ-1 monoclonal antibody or anti-actin antibody as described in Materials and Methods. (**D**) ME180 cells were irradiated with 20 J/m^2 of UV. At various times after irradiation, cells were stained with an anti-DJ-1 polyclonal antibody. The cells were then reacted with an FITC-conjugated anti-rabbit IgG and observed under a confocal laser fluorescent microscope. The cells were also stained with DAPI and MitoTracker-red (Molecular probe)

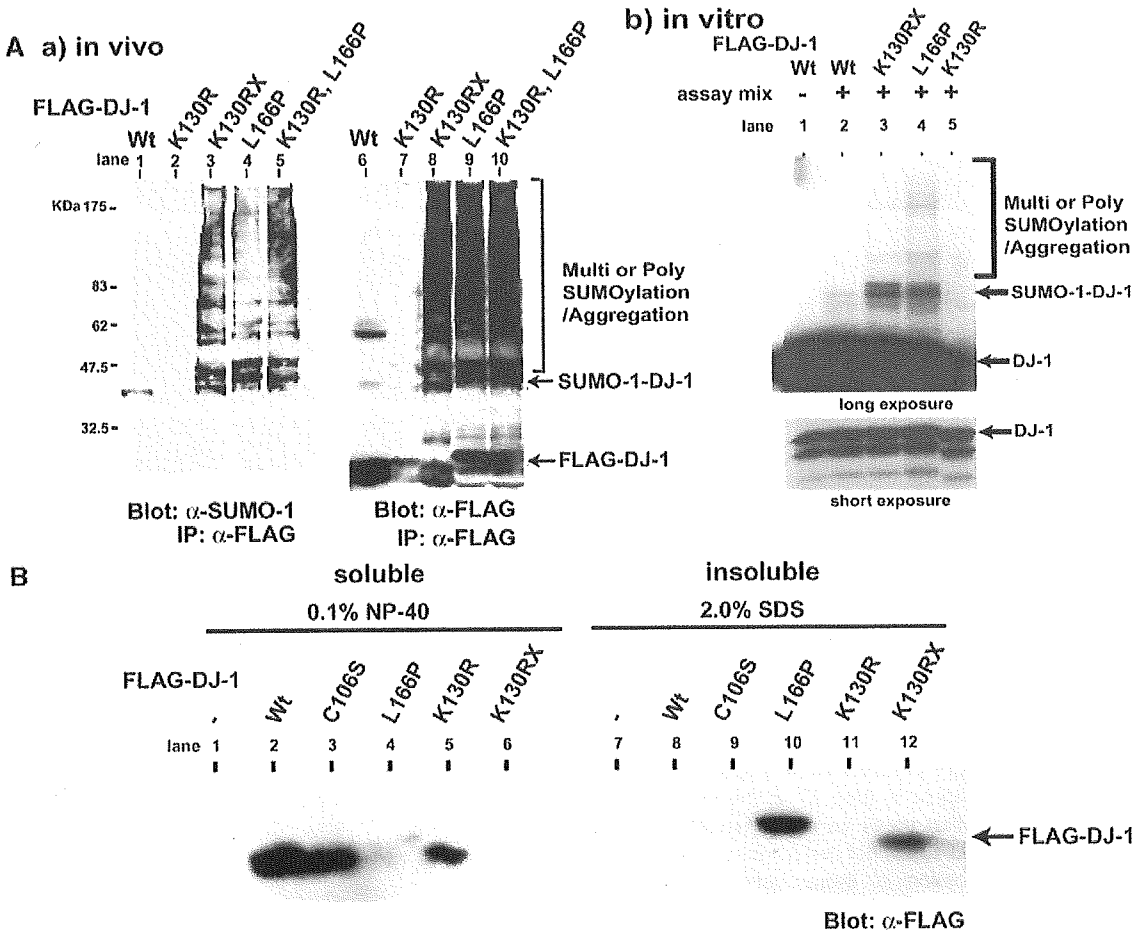

Figure 6 Improper sumoylation of L166P mutant of DJ-1. (**A** (a)) H1299 cells were cotransfected with FLAG-DJ-1 or mutants, T7-SUMO-1 andUbc9-HA. At 48 h after transfection, proteins extracted from transfected cells were immunoprecipitated with an anti-FLAG antibody and analyzed by Western blotting with the anti-FLAG antibody or an anti-SUMO-1 antibody as described in Materials and Methods. (b) *In vitro* sumoylation of DJ-1 and its mutant were carried out using the reaction mixture containing ^{35}S-DJ-1 and its mutated DJ-1 and the recombinant proteins of E1 enzyme, Ubc9 and SUMO-1 as described in Materials and Methods. Labeled proteins in the mixture were separated on polyacrylamide gel and visualized by fluorography. (**B**) 293T cells were transfected with FLAG-DJ-1 or its mutants. At 48 h after transfection, soluble proteins was first extracted from transfected cells with 0.1% SDS-containing buffer and insoluble proteins were then dissolved with 2% SDS. These were analyzed by Western blotting with an anti-FLAG antibody

Nuclei were also stained with DAPI. Wild-type DJ-1 and K130R DJ-1 were localized both in the cytoplasm and nucleus, and some portion of DJ-1 were also localized in the mitochondria as shown by merged figures in which the green and red have become yellow (Figure 7a, k and l). K130RX, L166P DJ-1 and a double mutant (K130R, L166P) were, on the other hand, found to be predominantly localized in the cytoplasm and almost all of them were localized in the mitochondria (Figure 7a, m, n and o). Neither wild-type DJ-1 nor any of the four mutants of DJ-1, on the other hand, was found to be localized in the ER in cells without ER stress (data not shown). Wild-type DJ-1 and the four mutants of DJ-1 were also not found to be localized in the Golgi apparatus (data not shown).

Since L166P DJ-1 has been reported to be degraded, in part, by the ubiquitin–proteasome system[24–28,49] and DJ-1 possess cysteine protease activity,[28] we then examined the stability of mutants of DJ-1 against degradation by proteasome and cysteine protease using their specific inhibitors, MG132 and E64, respectively. Proteins were extracted from NIH3T3 cells transfected with FLAG-tagged wild-type DJ-1 and mutants of DJ-1 in the presence or absence of MG132 or E64 and blotted with an anti-FLAG antibody or an antiactin antibody (Figure 8). The results first showed that the amounts of L166P and K130RX DJ-1 were smaller than those of wild-type DJ1 and K130R DJ-1 in the absence of MG132 (Figure 8, lanes 1, 4, 7, 10, 13 and 16) and that E64 had no effect on the stability of wild-type DJ-1 or any of the mutants of DJ-1 (Figure 8, lanes 3, 6, 9, 12, 15 and 18). Although wild-type DJ-1 and K130R DJ-1 were not affected by MG132, the amounts of L166P and K130RX increased partially in the presence of MG132 (Figure 8, lanes 14 and 17 in the middle part), suggesting that K130RX and L166P DJ-1 were degraded, in part, by the proteasome system. It was noted that

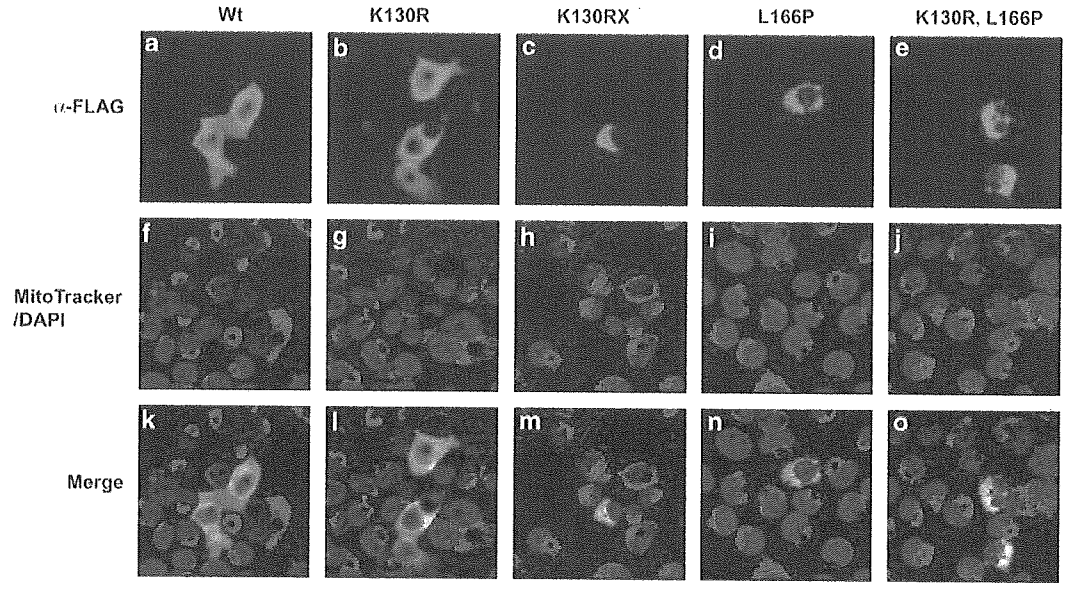

Figure 7 Localization of DJ-1 and its mutants in cells. H1299 cells were transfected with FLAG-wild-type (Wt) or its mutants. At 48 h after transfection, cells were fixed, reacted with a combination of an anti-FLAG antibody (**a–e**), DAPI and MitoTracker (**f–j**), and visualized with a fluorescein isothiocyanate-conjugated anti-mouse antibody (**a–e**) and self-fluorescences of DAPI and MitoTracker (**f–j**). The two figures have been merged (**k–o**)

slow-migrating bands of K130RX DJ-1 appeared in the presence of MG132 (Figure 8, lane 17 in the upper part). Although it is possible that these bands correspond to the ubiquitinated K130RX DJ-1, it is not certain at present due to the inability of identification of these bands against an antiubiquitin antibody (data not shown). These results suggest that mutants of DJ-1 localized in the mitochondria are improperly sumoylated, insoluble and unstable.

Loss of antiapoptosis activity of DJ-1 mutants

We then examined the effects of K130R and L166P mutations of DJ-1 on UV-induced apoptosis. Mouse NIH3T3-Wt, -K130R and -L166P cells, which are stable cell lines expressing wild-type, K130R and L166P DJ-1 as described previously,[41] and NIH3T3-D2 cells, which are stable DJ-1-knockdown cells expressing siRNA toward DJ-1,[49] were irradiated with two doses of UV. At 22 h after irradiation, DNA from cells was stained with propidium iodide and the distribution of cells in the cell cycle was analyzed by flow cytometry (Figure 9). DJ-1 expression in D2 cells decreased to about 10% of that of parental NIH3T3 cells (Figure 9a) and the characteristics of D2 cells has been described.[49] Apoptotic cells that appeared at the position below the G1/G0 phase were counted using the 'ModiFit LT' program (Figure 9b). As in the case of hydrogen peroxide-induced cell death,[41] cells harboring exogenously added wild-type DJ-1 were much more resistant to UV than were parental nontransfected cells (host). Of the cell lines harboring mutants, on the other hand, the cell line harboring L166P was found to be most sensitive to UV and the K130R line followed in terms of sensitivity to UV. DJ-1-knockdown cells were found to have similar sensitivity to that of L166P cells. These results clearly indicate that DJ-1 plays a role in protection against UV-induced apoptosis and that mutations or knockdown of DJ-1 lead to cell death.

Discussion

SUMO-1 conjugation to DJ-1

In this study, we first found that a lysine at amino-acid number 130 of DJ-1 (K130) was sumoylated by PIASxα and PIASy in cells and that this sumoylation of DJ-1 was essential for DJ-1 to exert its full activities, including *ras*-dependent transforming and cell growth-stimulating activities. PIAS family proteins, which include PIAS1, PIAS3, PIASxα, PIASx$\beta\beta$ and PIASy, are known to function as E3 SUMO-1 ligases and the proteins to be sumoylated by each PIAS family protein were identified.[43,44] In most case, transcription factors have been identified as target proteins for PIAS family proteins and DJ-1 is also a transcription factor as a coactivator to regulate expressions of genes for AR, p53 and PTEN.[12,13,15,53,54] Since identifications of almost all the proteins that had been sumoylated were performed by overexpression system using expression vectors for SUMO-1 and Ubc9, the level of sumoylation of DJ-1, which was observed without overexpression of SUMO-1 or Ubc9, is thought to be high.

DJ-1 expression and its sumoylation were induced by UV irradiation and DJ-1 was translocated from the cytoplasm to nucleus. Since induction of DJ-1 expression after irradiation of UV occurred both in p53 (−) and p53 (+) cells and this occurred faster than did induction of p53 expression in p53 (+) cells, induction of DJ-1 expression by UV irradiation is p53-independent. UV irradiation is known to induce ROS and

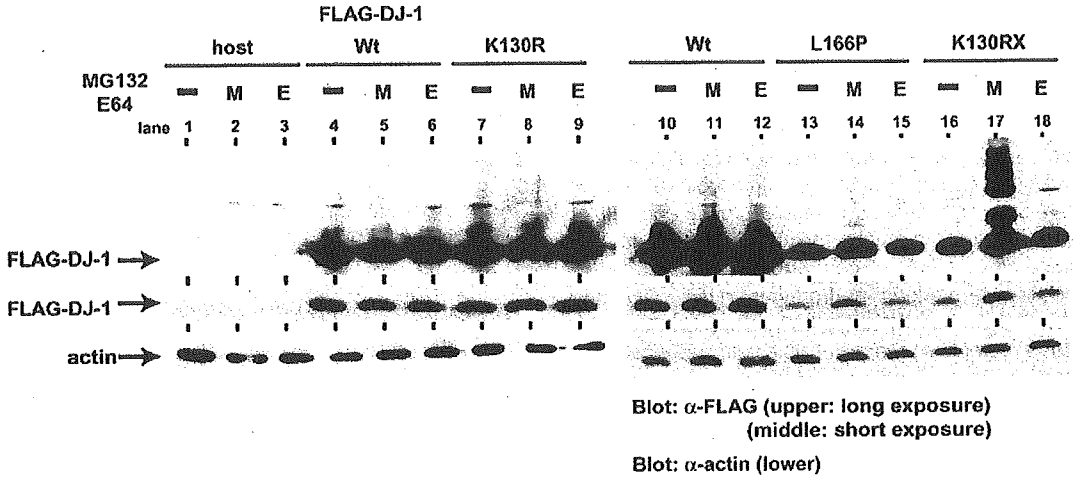

Figure 8 Stability of DJ-1 and its mutants. Mouse NIH3T3 cell lines harboring FLAG-wild-type DJ-1 and its mutants were cultured in the presence of 10 mM MG132 or 8 μg/ml of E64 for 12 h. Proteins extracted from cells were analyzed by Western blotting with anti-FLAG and antiactin antibodies. Host indicates parental NIH3T3 cells

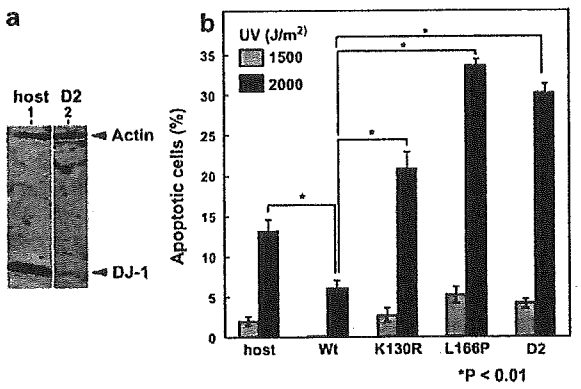

Figure 9 Abrogation of antiapoptosis activity of DJ-1 by mutations. (a) Proteins extracted from NIH3T3 cells (host) and DJ-1-knockdown cells (D2) were analyzed by Western blotting with anti-mouse DJ-1 and antiactin antibodies. The proteins that reacted with primary antibodies were visualized with IRDye800-conjugated or Alexa Fluor680-conjugated secondary antibodies using an infrared imaging system (Odyssey, LI-COR). (b) Mouse NIH3T3 cell lines harboring FLAG-wild-type DJ-1 and its mutants were irradiated with 100 and 2000 J/m^2 UV. At 22 h after irradiation, DNA in cells was stained with propidium iodide and the distribution of cells in the cell cycle was analyzed by flow cytometry. Distribution of cells in the cell cycle by analysis of flow cytometry was quantitated by using a 'ModiFit LT' program. Results ($n=3$) were statistically processed using Student's *t*-test. Significance: $^*P<0.01$ *versus* apoptotic cells in wt-DJ-1 cells

to give the oxidative stress to cells, and DJ-1 expression has been reported to be induced by several chemicals that had also been known to induce ROS.[36–39] UV irradiation of cells also shifted the pI of DJ-1 to acidic points, as in the case of direct addition of hydrogen peroxide, one of the ROS, to human SH-SY5Y and mouse NIH3T3 cells, in which ROS were scavenged by DJ-1, as described previously.[41] The amount of pI-shifted DJ-1, however, was again reduced 4 h after UV irradiation concomitant with a reduction of the amount of the sumoylated form of DJ-1 and relocalization of DJ-1 in the cytoplasm, suggesting that self-oxidation of DJ-1 following SUMO-1 conjugation to DJ-1 occurs depending on the amounts of ROS in cells and that the sumoylated form of DJ-1 is an active form to eliminate ROS. High-dose of UV irradiation of cells induces apoptosis, and an NIH3T3 cell line expressing exogenously added wild-type DJ-1 became resistant to UV-induced apoptosis compared to the parental cells. This antiapoptotic activity of DJ-1 was abrogated by the mutation into an amino acid K130, a sumoylation site of DJ-1, as well as those of *ras*-dependent transforming and cell growth-stimulating activities, indicating that SUMO-1 conjugation to K130 of DJ-1 is essential for DJ-1 to function.

Improper SUMO-1 conjugation to L166P DJ-1, a mutant of DJ-1 found in PD patients

In a latter part of this paper, we described the properties of L166P DJ-1, a mutant of DJ-1 found in PD patients,[17] and K130RX DJ-1, an artificial mutant possessing four mutations including K130R, in terms of sumoylation and loss of function. Both L166LP and K130RX were improperly sumoylated *in vivo* compared to wild-type DJ-1, to which one SUMO-1 molecule was conjugated. It is possible that this improper SUMO-1 conjugation to L166P and K130RX occurs on multiple lysine residues (multisumoylation) or on a specific lysine residue with poly-SUMO-1 (polysumoylation). It is also possible that L166P and K130RX are misfolded, resulting in sumoylation of multiple lysine residues (multisumoylation) and then abnormal aggregation. Although polysumoylation, which had been thought not to occur, has recently been reported,[55,56] it is not clear whether there was excess sumoylation of DJ-1 mutants in either case. Since K130RX lost lysine at 130, a lysine(s) other than K130 was sumoylated. Furthermore, both L166LP and K130RX were also improperly sumoylated in an *in vitro* reconstituted system using recombinant proteins. The crystal structure of DJ-1 has been determined by five groups, including us, and it has been shown that L166 is located in the α-helix near the C-terminal region of DJ-1 and that L166P mutation might disrupt dimer formation.[57–61] Structure of K130RX was also thought to be

drastically changed by the computer-based analysis. The protective activity of DJ-1 against hydrogen peroxide-induced cell death has been reported to be abrogated by L166P mutation.[41,49] It is therefore thought that the structures of these mutants themselves changed to allow for improper sumoylation or abnormal aggregation. Both L166P and K130RX DJ-1 were found to become insoluble. Recently, insoluble forms of DJ-1 have been reported to be colocalized with tau or α-synuclein in fibrillar inclusions in patients with Pick's disease and multiple system atrophy, suggesting that DJ-1 is related to various neurodegenerative diseases, including PD.[30,31] It would therefore be interesting to examine whether such insoluble forms of DJ-1 in patients with neurodegerative diseases are sumoylated.

Localization of K130R DJ-1 in the cytoplasm and nucleus was found to be the same as that of wild-type DJ-1, suggesting that SUMO-1 conjugation is not sufficient to determine the localization of DJ-1 in cells. Some of wild-type DJ-1 and K130R DJ-1 were also found to be localized in the mitochondria. Both L166P and K130RX DJ-1 were then found to be localized in the mitochondria. Since the mitochondrion is a major ROS-production organelle and since low activity levels of the complex 1 that reside in mitochondria have been found in PD patients (see review and references therein),[62] it is interesting that improperly sumoylated DJ-1 was localized in the mitochondria. Since DJ-1 has no mitochondria-import/export sequences, wild-type DJ1 and mutants of DJ-1, especially L166P and L130RX, may associate with other protein(s) that are targeted in mitochondria. To determine the importance of different localizations of DJ-1 in cells, localization of DJ-1 in specific organelles such as mitochondria in patients with various neurodegenerative diseases, including PD, must be investigated.

K130RX is unstable and degraded, in part, by the ubiquitin–proteasome system, like L166P as described previously.[24–28,49] In the presence of the proteasome inhibitor MG132, a ladder above the position of the K130RX monomer was observed in polyacrylamide gel. This is likely to be a ubiquitinated form of K130RX DJ-1, and further study is needed to confirm this. Wild-type DJ-1 and its mutants were not affected by the presence of E64, an inhibitor of cysteine protease, indicating that DJ-1 is not regulated by its protease activity.

We established DJ-1-knockdown cells (D2 cells) in which siRNA targeting to the mouse DJ-1 gene was expressed under the control of the U6 promoter,[49] and we found that D2 cells were highly susceptible to UV-induced apoptosis, indicating that DJ-1 plays a role in antiapoptotic activity. A cell line that harbors exogenously added L166P DJ-1 had sensitivity against UV-induced apoptosis similar to that of D2 cells. It is therefore thought that L166P DJ-1 acts in a dominant-negative fashion at least in this cell line, in which wild-type DJ-1 is present. In PD patients harboring L166P mutation of the *DJ-1* gene, loss of function of DJ-1 triggers onset of disease.

It has been reported that improper sumoylation was related to the onset of polyglutamine diseases, Alzheimer's disease and Huntington's disease.[63–65] Together with the results of those studies, the results of this study suggest that proper sumoylation is essential for DJ-1 to exert its full activities and that improper sumoylation makes DJ-1 insoluble, mislocalized and unstable, which trigger the onset of PD.

Materials and Methods

Cells

Human H1299, ME180, 293T, HeLa and rat 3Y1 cells were cultured in Dulbecco's modified Eagle's medium with 10% calf serum.

Construction of lysine mutants of DJ-1

Nucleotide sequences of the oligonucleotide used for PCR primers were as follows: DJ-1-K62R (+), 5′-CTGAAGATGCAAGAAAAGAG-3′; DJ-1-K62R (−), 5′-CATATGGTCCCTCTTTTCTTGC-3′; DJ-1-K62, 63R (+), 5′-CTGAAGATGCAAGAAGAGAG-3′; DJ-1-K62, 63R (−), 5′-CATATGG TCCCTCTCTTCTTGC-3′; DJ-1-K63R (+), 5′-CTGAAGATGCAAAAA GAGAG-3′; DJ-1-K63R (−), 5′-CATATGGTCCCTCTCTTTTTGC-3′; DJ-1-K130R (+), 5′-CAACACACCCTCTTGCTAGAGAC-3′; DJ-1-K130R (−), 5′-CATCATTTTGTCTCTAGCAAG-3′; T7, 5′-CCCTATAGTGAGTCG TATTA-3′; Sp6, 5′-TTTAGGTGACACTATAGAAT-3′. The first PCR was carried out on pcDNA3-FLAG-DJ-1 as a template either with DJ-1-K (+) and T7 or with DJ-1K (−) and Sp6 as primers. The products were mixed and used as templates for the second PCR with T7 and Sp6 as primers. The resultant PCR product was digested with *Hin*dIII and *Xho*I and inserted into the respective site of pcDNA3.

SUMO-1 conjugation to DJ-1 *in vivo*

Proteins were extracted from human H1299 cells with a buffer containing 0.1% NP-40, 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl and by sonication. Proteins were then immunoprecipitated with an anti-DJ-1 polyclonal antibody-conjugated agarose,[1] and the precipitates were dissolved with a sample buffer containing 30 mM Tris-HCl (pH 6.8), 6% SDS, 0.006% bromophenol blue, 12% 2-mercaptoethanol and 50% glycerol, boiled for 15 min, and blotted with an anti-DJ-1 monoclonal antibody (3E8, specificity described by Yoshida *et al.*[9]) or an anti-SUMO-1 polyclonal antibody (FL-101, Santa Cruz). H1299 cells were transfected with 5 μg of FLAG-DJ-1 or its mutants, 2 μg of Ubc9-HA and 2 μg of T7-SUMO-1 by the calcium phosphate precipitation method. At 48 h after transfection, the proteins extracted from cells were immunoprecipitated with an anti-FLAG monoclonal antibody (M2, Roche), blotted with an anti-T7 monoclonal antibody (Novagen) or the anti-FLAG monoclonal antibody, and visualized by ECL (Amersham BioScience).

SUMO-1 conjugation to DJ-1 *in vitro*

Recombinant proteins of human SUMO-1, E1 enzyme (SAE1 and SAE2) and Ubc9 were purchased from Alexis Biochemicals. ^{35}S-FLAG-DJ-1 and its mutant were synthesized *in vitro* using a TnT-Coupled reticulocyte lysate system (Promega). Reaction mixture containing 100 ng of E1 enzyme, 900 ng of Ubc9, 10 μg of SUMO-1 and ^{35}S-FLAG-DJ-1 and its mutant was incubated at 30°C for 2 h, mixed with the sample buffer, and boiled for 5 min. The proteins were then separated on SDS-containing polyacrylamide gel and visualized by fluorography.

Focus forming and growth assays

Rat 3Y1 cells cultured in a 10-cm dish were transfected with 1 μg each of pEJ6.6, pCMV-F-DJ-1 or pCMV-F-DJ-1-mutant by the calcium phosphate