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Association of DJ-1 with chaperones and enhanced association and colocalization with mitochondrial Hsp70 by oxidative stress

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Abstract

DJ-1 is a novel oncogene and causative gene for familial form of the Parkinson's disease (PD). DJ-1 has been shown to play a role in anti-oxidative stress by eliminating reactive oxygen species (ROS). The onset of PD is thought to be caused by oxidative stress and mitochondrial injury, which leads to protein aggregation that results in neuronal cell death. However, the mechanism by which DJ-1 triggers the onset of PD is still not clear. In this study, we analyzed association and localization of DJ-1 and its mutants with various chaperones. The results showed that DJ-1 and its mutants were associated with Hsp70, CHIP and mtHsp70/Grp75, a mitochondria-resident Hsp70, and that L166P and M26I mutants found in PD patients were strongly associated with Hsp70 and CHIP compared to wild-type and other DJ-1 mutants. DJ-1 and its mutants were colocalized with Hsp70 and CHIP in cells. Furthermore, association and colocalization of wildtype DJ-1 with mtHsp70 in mitochondria were found to be enhanced by treatment of cells with H_2O_2. These results suggest that translocation of DJ-1 to mitochondria after oxidative stress is carried out in association with chaperones.

Keywords: *DJ-1*, chaperone, oxidative stress, Parkinson's disease

Introduction

DJ-1 was first identified by us as a novel oncogene that transforms mouse NIH3T3 cells in corporation with activated *ras* [1]. Elevated expression of DJ-1 has been reported in breast cancer, smoke-derived lung adenocarcinoma and prostate cancer [2–4]. DJ-1 was later found to be related to infertility of rats and mice and to participate in fertilization for sperm to penetrate into the zonae pellucida of eggs [5–9]. DJ-1 was found to be a positive regulator of the androgen receptor [10–12] and p53 [13] and to be a negative regulator of PTEN tumor suppressor [14] and pyrimidine tract-binding protein-associated splicing factor (PSF) [15]. Recently, DJ-1 has been shown to be responsible for onset of familial Parkinson's disease (PD), PARK7 [16] and 11 mutations in familial and sporadic forms of PD have been reported [17–19]. 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 [20–22].

PD involves an irreversible degeneration of the dopaminergic nigrostriatal pathway. Various lines of evidence also suggest that oxidative stresses contribute to the cascade leading to dopaminergic cell degeneration in PD [23–26], and expression of DJ-1 has been shown to be induced by oxidative stresses [27–30].

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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.

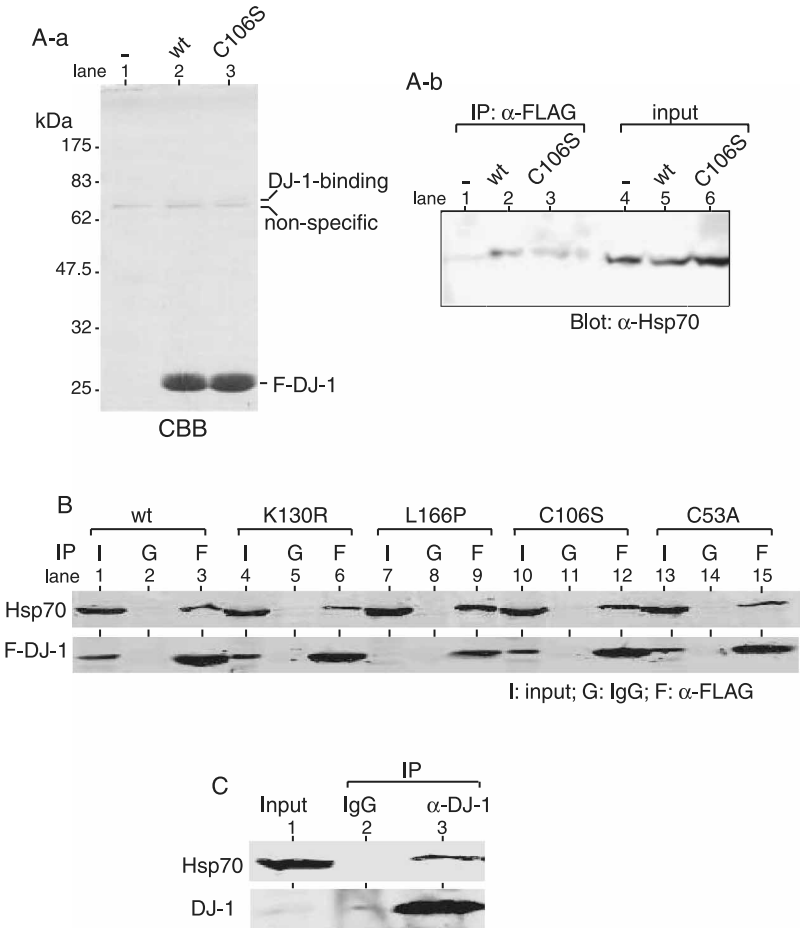

Figure 1. Association of DJ-1 with Hsp70. (A) Human 293T cells were transfected with pcDNA3-F-DJ-1 or pcDNA3-F-C106 by the calcium phosphate precipitation technique. Forty-eight hours after transfection, cell extracts were prepared and immunoprecipitated with an anti-FLAG antibody (M2, Sigma). The precipitates 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.

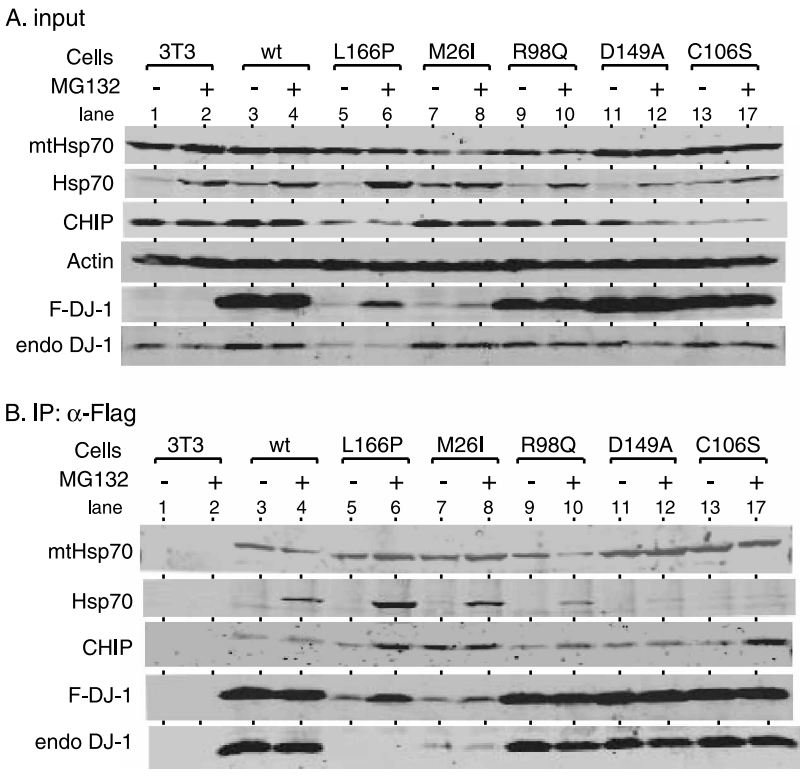

Figure 2. Association of wild-type and mutants of DJ-1 with chaperones in cells cultured in the presence of MG132. (A) Mouse NIH3T3 cells harboring FLAG-wild-type DJ-1 and its mutants were cultured in the presence of 10 μM MG132 for 12 h. Proteins extracted from cells were analyzed by Western blotting 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

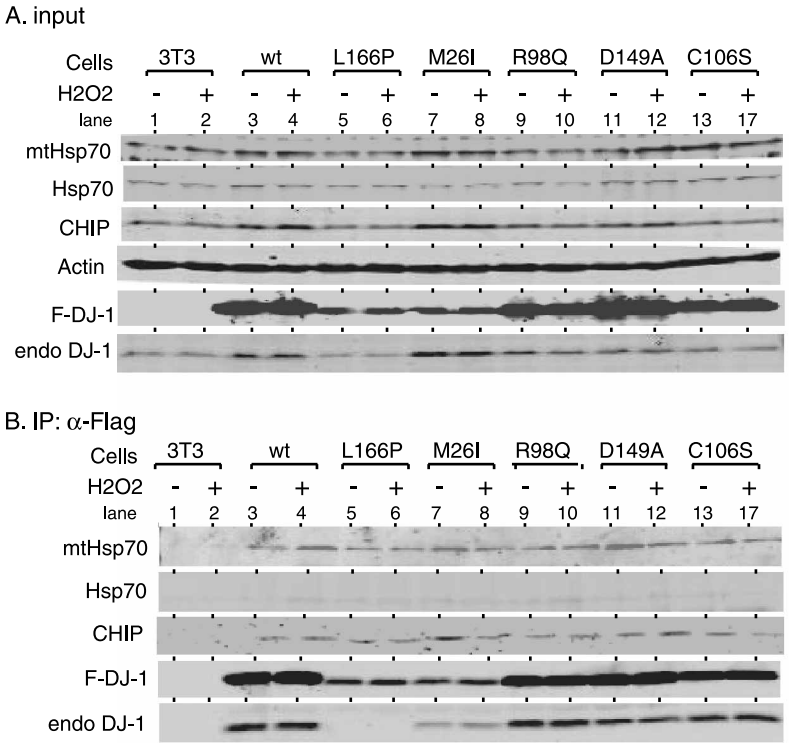

Figure 3. Association of wild-type and mutants of DJ-1 with chaperones in H_2O_2-treated cells. (A) Mouse NIH3T3 cells harboring FLAG-wild-type DJ-1 and its mutants were reacted with 100 μM of H_2O_2 for 4 h. Proteins extracted from cells were analyzed by Western blotting with anti-FLAG, anti-DJ-1, anti- 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.

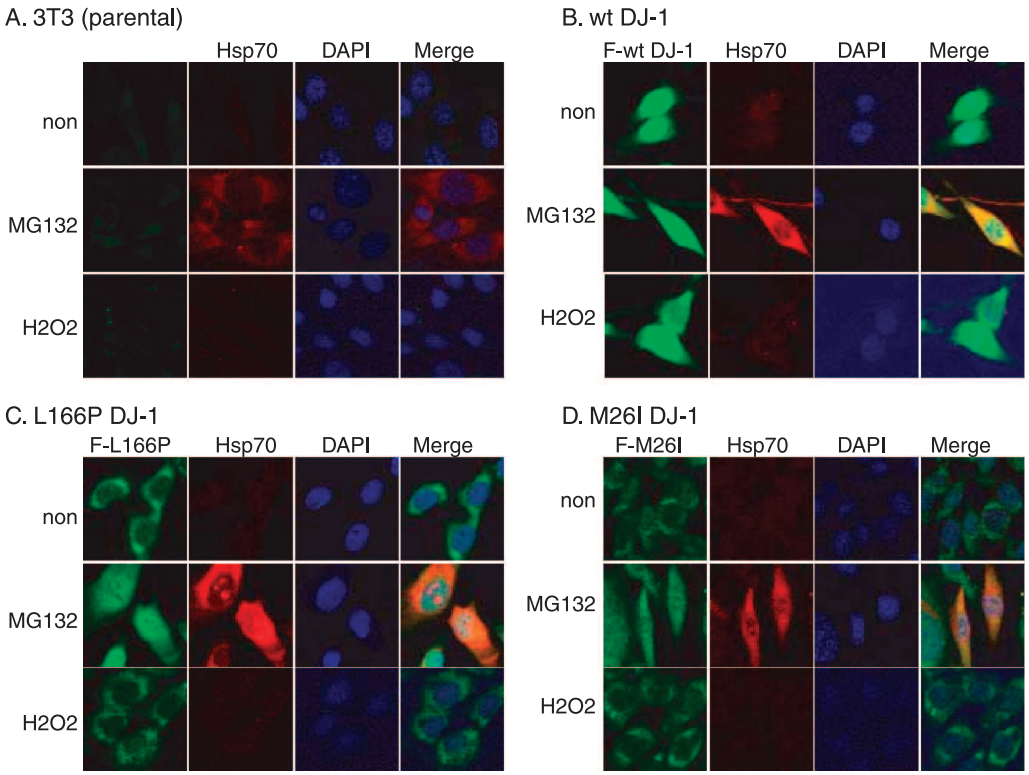

Figure 4. Colocalization of Hsp70 with DJ-1 and its mutants. Mouse NIH3T3 cells harboring FLAG-wild-type DJ-1 and its mutants were reacted with 10 μM MG132 for 12 h or with 100 μM of H_2O_2 for 4 h. The cells were then fixed with paraformaldehyde and reacted with a combination of a rabbit anti-FLAG polyclonal 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

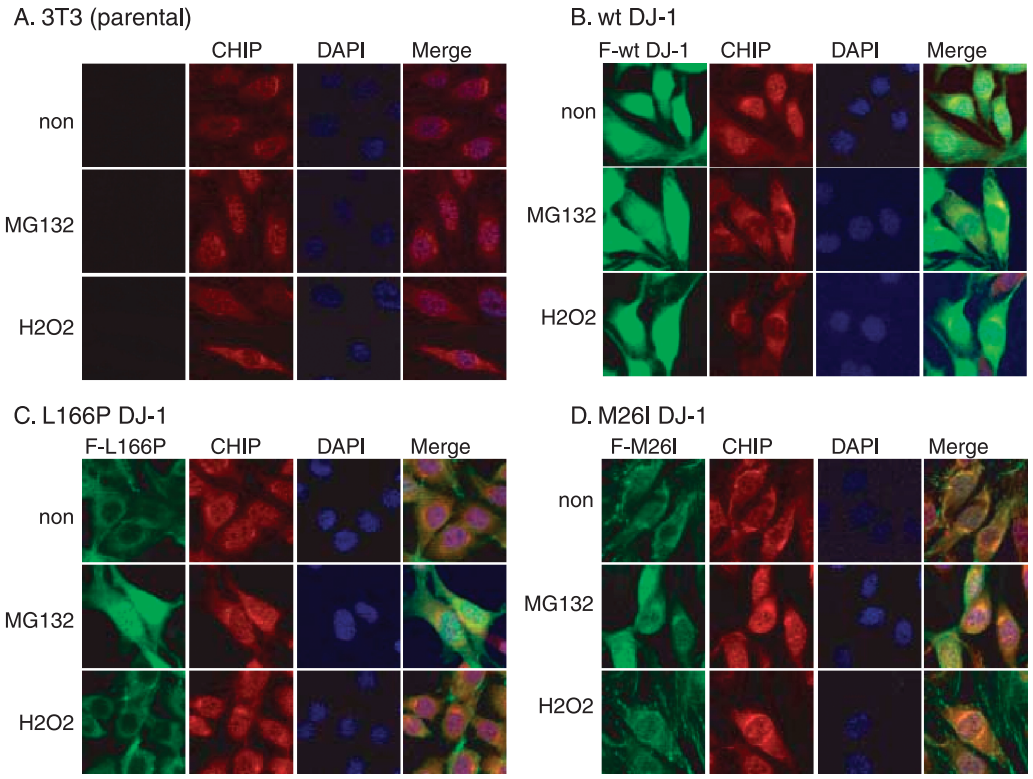

Figure 5. Colocalization of CHIP with DJ-1 and its mutants. Mouse NIH3T3 cells harboring FLAG-wild-type DJ-1 and its mutants were reacted with 10 μM MG132 for 12 h or with 100 μM of H_2O_2 for 4 h. The cells were then fixed with paraformaldehyde and reacted with a combination of a mouse anti-FLAG monoclonal 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

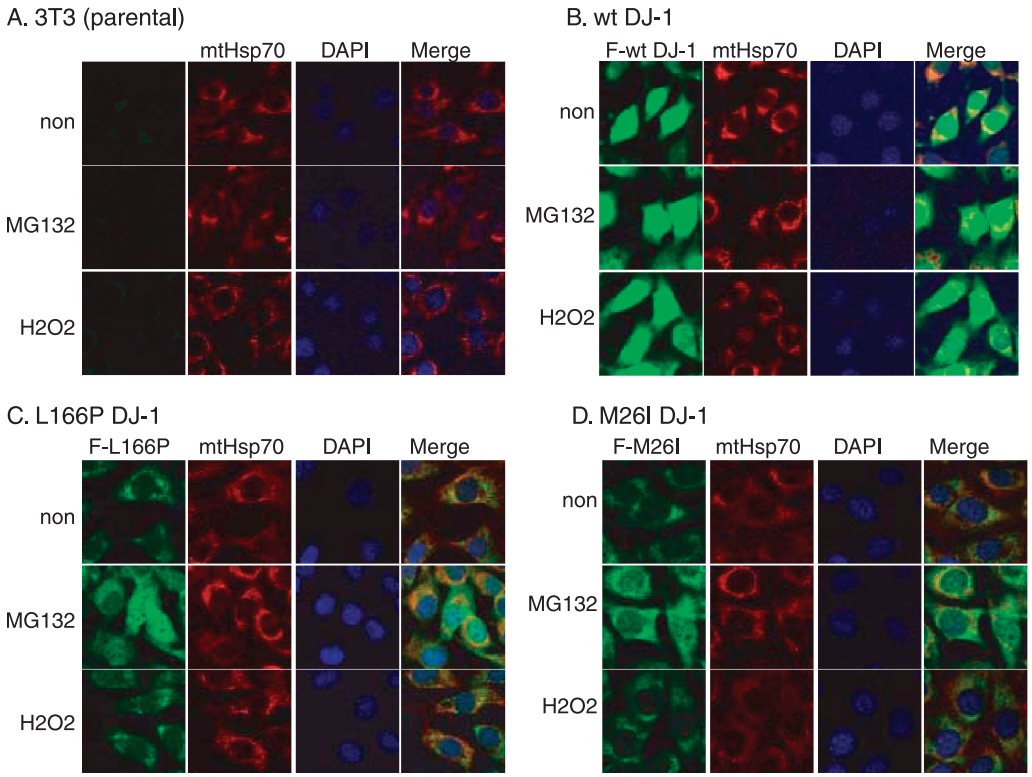

Figure 6. Colocalization of mtHsp70 with DJ-1 and its mutants. Mouse NIH3T3 cell lines harboring FLAG-wild-type DJ-1 and its mutants were reacted with 10 μM MG132 for 12 h or with 100 μM of H_2O_2 for 4 h. Cells were then fixed with paraformaldehyde and reacted with a combination of a rabbit anti-FLAG 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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