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Protein kinase A-induced phosphorylation at the Thr154 affects stability of DJ-1

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ABSTRACT

Introduction: Most cases of Parkinson's disease (PD) are sporadic, but genetic variations have been discovered in PD patients. PARK7/DJ-1 is a known cause of early-onset autosomal-recessive PD and is implicated in neuroprotection against oxidative stress. Although several post-translational modifications of DJ-1 have been proposed, phospho-modification of DJ-1 and its functional consequences have been less studied.

Methods: Putative phosphorylation sites of DJ-1 were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS analysis). Subsequently, phosphorylation site of DJ-1 was confirmed by *in vitro* kinase assay and cell-based pull-down assay. Impaired dimer formation of phospho-null mutant was measured using DSS crosslinking assay and immunoprecipitation assay. To evaluate physiological consequences of this event, protein stability of DJ-1 WT and DJ-1 phospho-null mutant were compared using cycloheximide chase assay and ubiquitination assay.

Results: Here, we showed that DJ-1 directly bound to the catalytic subunit of protein kinase A (PKAcα). We found that PKAcα is responsible for phosphorylation of DJ-1 at the T154 residue. Interestingly, dimerization of DJ-1 was not detected in a DJ-1 T154A mutant. Furthermore, stability of the DJ-1 T154A mutant was dramatically reduced compared with that of wild-type DJ-1. We found that DJ-1 T154A was prone to degradation by the ubiquitin proteasome system (UPS).

Conclusion: We identified a novel phosphorylation site of DJ-1. Furthermore, we determined protein kinase A that is responsible for this posttranslational modification. Finally, we demonstrated physiological consequences of this event focusing on dimerization and protein stability of DJ-1.

1. Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disease characterized by the selective loss of dopaminergic neurons. Most cases of PD are sporadic, but genetic variations have been discovered in PD patients. Among several identified PD-linked genes, DJ-1/PARK7 is causative of familial PD. DJ-1 is important for protecting cells against oxidative stress and functions as a molecular chaperone and transcriptional regulator [1]. DJ-1 is composed of highly conserved homodimers and is ubiquitously expressed in cells and tissues, including the brain. Intriguingly, several causative mutations of DJ-1 including L166P, L10P, and P158Delta destabilize the formation of DJ-1 homodimers [2]. For example, the pathogenic mutant DJ-1 L166P has a collapsed dimer structure that results in a loss of function [3]. Evidence demonstrates that its function as a sensor of anti-oxidant signaling is regulated by oxidative modifications especially at C106 [4]. DJ-1 is also modified by sumoylation and S-nitrosylation, which appear to affect the dimerization and activity of DJ-1 [5]. Regardless of these advances, the phospho-modification of DJ-1 and its functional consequences have been less studied.

Protein kinase A (PKA) is a member of a family of enzymes that is functionally dependent on the levels of cyclic AMP (cAMP). PKA is a tetrameric holoenzyme composed of two regulatory subunits bound to two catalytic subunits [6]. Binding of cAMP to a regulatory subunit destabilize the regulatory subunits and induces the release and subsequent activation of the catalytic subunits. Upon activation, the

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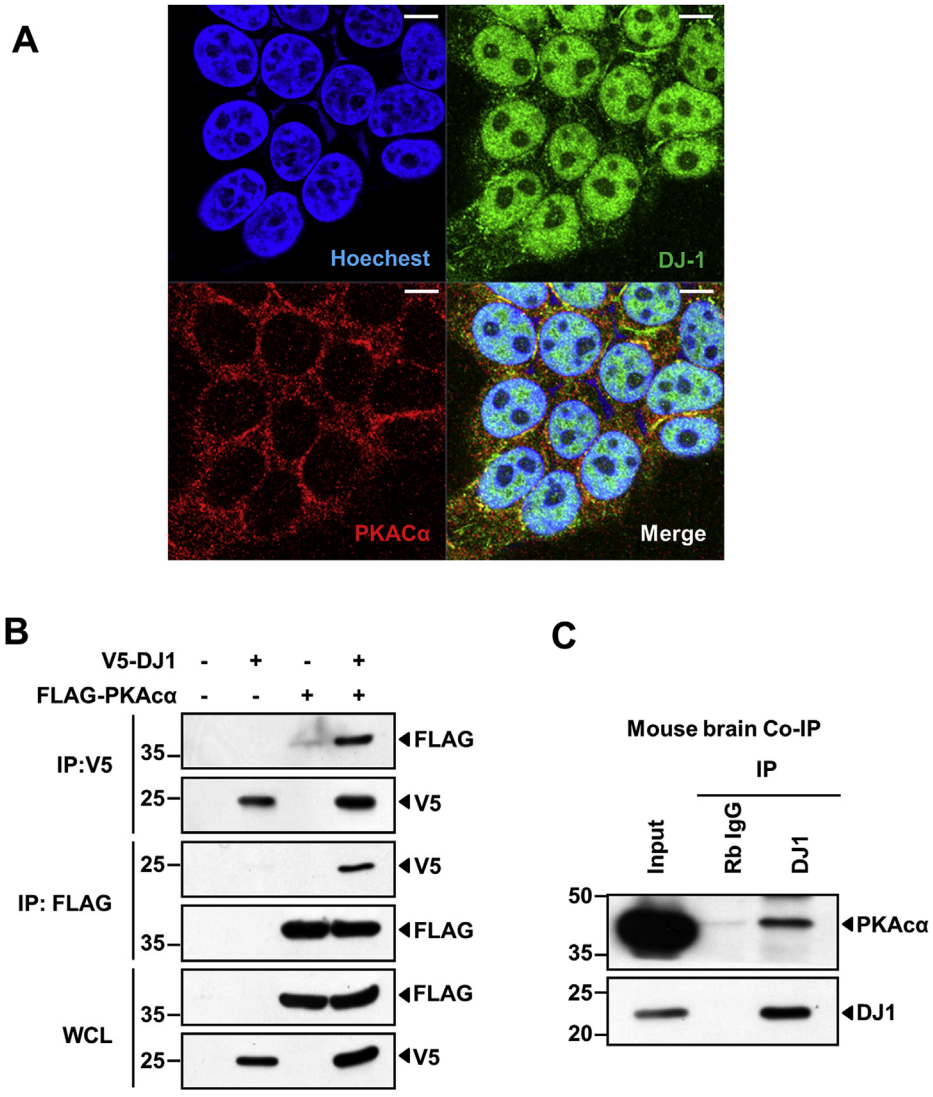

Fig. 1. DJ-1 binds to PKAcα. (A) Immunofluorescent co-localization of endogenous DJ-1 and PKAcα. HEK293 cells were fixed with 4% paraformaldehyde and subjected to immunofluorescence observation for detecting DJ-1 (green) and PKAcα (red). Nuclei were counterstained with Hoechst dye (blue). Merged images are shown in the right panels. Fluorescent images were obtained with an LSM700 confocal microscope. Scale bar: 10 μm. **(B)** Physical interaction between DJ-1 and PKAcα. Co-immunoprecipitation was performed using HEK293 cells that were transiently transfected with V5-tagged DJ-1 and/or FLAG-tagged PKAcα. At 48 h after transfection, cell lysates were collected for immunoprecipitation using *anti*-V5 antibody or anti-FLAG M2 affinity gel followed by immunoblot analyses using the indicated antibodies. **(C)** Co-immunoprecipitation of endogenous DJ-1 and PKAcα. Mouse whole brain tissues were homogenized and immunoprecipitated with *anti*-DJ-1 or control IgG antibody followed by immunoblot analyses using the indicated antibodies.

catalytic subunit alpha of PKA (PKAcα) phosphorylates serine and threonine residues of numerous target proteins and regulates their physiological functions. For example, PKAcα regulates neuronal development, synaptic plasticity, and neurotransmitter synthesis in the nervous system [7]. Notably, PKAcα-mediated signal transduction pathways also contribute to the etiology of several neurodegenerative diseases [8]. As PKAcα serves neuroprotective roles via regulating mitochondrial dynamics, trafficking, dendritic morphology, synaptic connectivity and neurotrophic support, PKAcα-mediated signaling is regarded as a potential therapeutic target in neurodegenerative diseases.

Here, we identified a novel posttranslational modification of DJ-1 by nano-LC-MS/MS. Among several identified phospho-modifications of DJ-1, PKAcα bound to and phosphorylated DJ-1 at T154 and this event may be critical for dimerization of DJ-1. Furthermore, T154 mutant was prone to degradation by UPS. Thus, our findings provide molecular evidence for a novel post-translational modification of DJ-1 and T154 residue may be critical for maintaining its functional role.

2. Materials and methods

2.1. Cell culture

HEK293 cells were cultivated at 37 °C in Dulbecco's Modified Eagle's Medium (GenDEPOT) supplemented with 10% heat-inactivated fetal bovine serum (GenDEPOT) in an atmosphere of 95% air and 5% CO_2. MN9D cells were cultured as previously described [9]. HEK293 cells were transfected using polyethylenimine (Sigma-Aldrich). Drug used included MG132 (Enzo Life Sciences Inc), H-89 (Millipore), forskolin (Sigma-Aldrich), cycloheximide (Sigma-Aldrich), or chloroquine (Sigma-Aldrich), and okadaic acid (Sigma-Aldrich).

2.2. Plasmids

FLAG- and V5-tagged human DJ-1 were generated by subcloning into pCI-neo mammalian expression vector (Promega) [9]. To generate His-tagged recombinant DJ-1 proteins, DJ-1 constructs were subcloned into pET-28a (+) vector (Millipore). For Ni-NTA pull-down assay, DJ-1 constructs were introduced into pcDNA3.1-V5/His mammalian expression vector (Invitrogen) [9]. FLAG-tagged PKAcα was generated by subcloning into FLAG-tagged pCI-neo vector. pRK5-FLAG-tagged Ub was provided by Dr. K. C. Chung (Yonsei University). To observe the cellular localization of DJ-1, GFP-tagged human DJ-1 was constructed by introducing DJ-1 PCR product into pAcGFP-C1 vector (Clontech). All DJ-1 mutants were generated using a site-directed mutagenesis kit (Agilent Technologies Inc.).

2.3. In-gel digestion and MS analysis

General procedure was basically followed as described previously

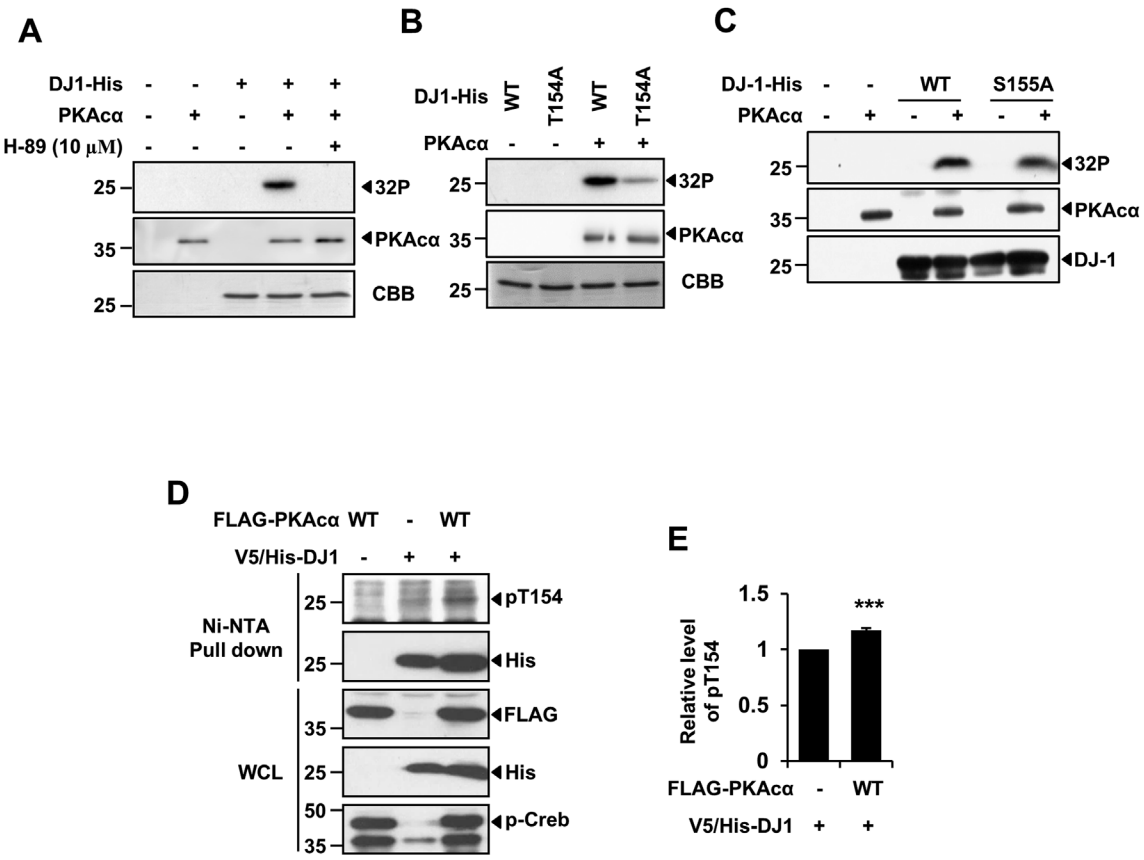

Fig. 2. PKA phosphorylates DJ-1 at T154. (A, B) *In vitro* kinase assay for measuring phosphorylation of DJ-1. Purified WT DJ-1 and DJ-1 T154A mutant protein (1 μg) was incubated with or without 0.1 μg PKAcα in the presence of [γ-^{32}P] ATP. H-89 was used as a PKAcα inhibitor. Reaction mixtures were resolved by SDS-PAGE and subjected to autoradiography. Coomassie Brilliant Blue (CBB) staining for DJ-1 protein was used as a loading control. Immunoblot analyses using anti-PKAcα antibody were separately performed to measure the expression levels of PKAcα. **(C)** To rule out the possibility of phosphorylation of DJ-1 at Ser155, purified WT DJ-1 and DJ-1 S155A were incubated with or without PKAcα in the presence of [γ-32P] ATP. Reaction mixtures were resolved by SDS-PAGE and subjected to autoradiography. **(D)** Cell-based assay for phosphorylation of DJ-1. HEK293 cells were transiently transfected with V5/His-tagged DJ-1 in combination with FLAG-tagged WT PKAcα. At 48 h after transfection, cell lysates were pulled down using 50 μl Ni-NTA-agarose beads and immunoblotted with *anti*-pT154 antibody or the indicated antibodies. PKAcα activity was confirmed by immunoblot analyses using *anti*-p-Creb antibody. **(E)** After normalization to the intensity of the pull-down His-tag signal, the relative intensity of pT154 was expressed as a fold change relative to control (value = 1). Bars represent the mean ± SD of three independent experiments. $^{***}p < 0.001$; n.s., not significant.

[10] and described in detail in Supplementary Materials and Methods. Briefly, purified DJ-1 protein from MN9D cells was separated by 7.5% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue (CBB) G-250. The DJ-1 protein band was directly cut out of gels, destained with 50% acetonitrile in 50 mM ammonium bicarbonate, and dried in a speed vacuum concentrator. DJ-1 protein was analyzed with a QTRAP 5500 hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems) connected to an Easy-nLC II system (Thermo Fisher Scientific).

2.4. Production of recombinant DJ-1 protein and in vitro kinase assay

His-tagged WT DJ-1 and its mutants were expressed in BL21 bacteria after induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich) at 37 °C for 4 h. Recombinat DJ-1 protein was obtained from bacterial pellet and used for *in vitro* kinase assay basically as described previously in our laboratory [11].

2.5. Preparation of anti-pT154 DJ-1 antibody

To generate rabbit polyclonal DJ-1 Thr154 phospho-specific antibody, we designed the peptide DGLIL(pT)SRGPG corresponding to sequences surrounding the Thr^{154} residue on mouse DJ-1. The antibody was generated by Abmart.

2.6. Immunoblot and antibodies

All mice were handled in accordance with the guideline for animal care and use of the Yonsei University. All experimental procedures were approved (2017-10-647-01 and 2018-01-689-01). General immunoblot assay is followed as described [11]. For immunoprecipitation, cell lysates (1–2 mg) were pre-incubated with protein A agarose beads (Millipore) for pre-clearing and further incubated with 1–2 μg of the indicated antibodies or 20 μl anti-FLAG M2 affinity gel beads (Sigma-Aldrich) overnight at 4 °C. Proteins were eluted from the beads were subsequently processed for immunoblot analyses. Antibodies used included horseradish peroxidase (HRP)-conjugated anti-FLAG antibody (A8592, Sigma-Aldrich), HRP-conjugated *anti*-V5 antibody (R961, Invitrogen), *anti*-V5 antibody (R960, Invitrogen), anti-His antibody (H15, Santa Cruz Biotechnology), anti-HA antibody (F7, Santa Cruz Biotechnology), *anti*-phospho-Creb antibody (87G3, Cell Signaling Technology), *anti*-DJ-1 antibody (ab18257, Abcam), *anti*-PKACα (610981,BD Transduction Laboratories), *anti*-Ub antibody (P4D1, Santa Cruz Biotechnology), *anti*-LC3B antibody (2775, Cell Signaling Technology), and *anti*-GAPDH antibody (Mab374, Millipore). Specific bands were detected by enhanced chemiluminescence (PerkinElmer). Band intensity was measured using ImageJ software (NIH).

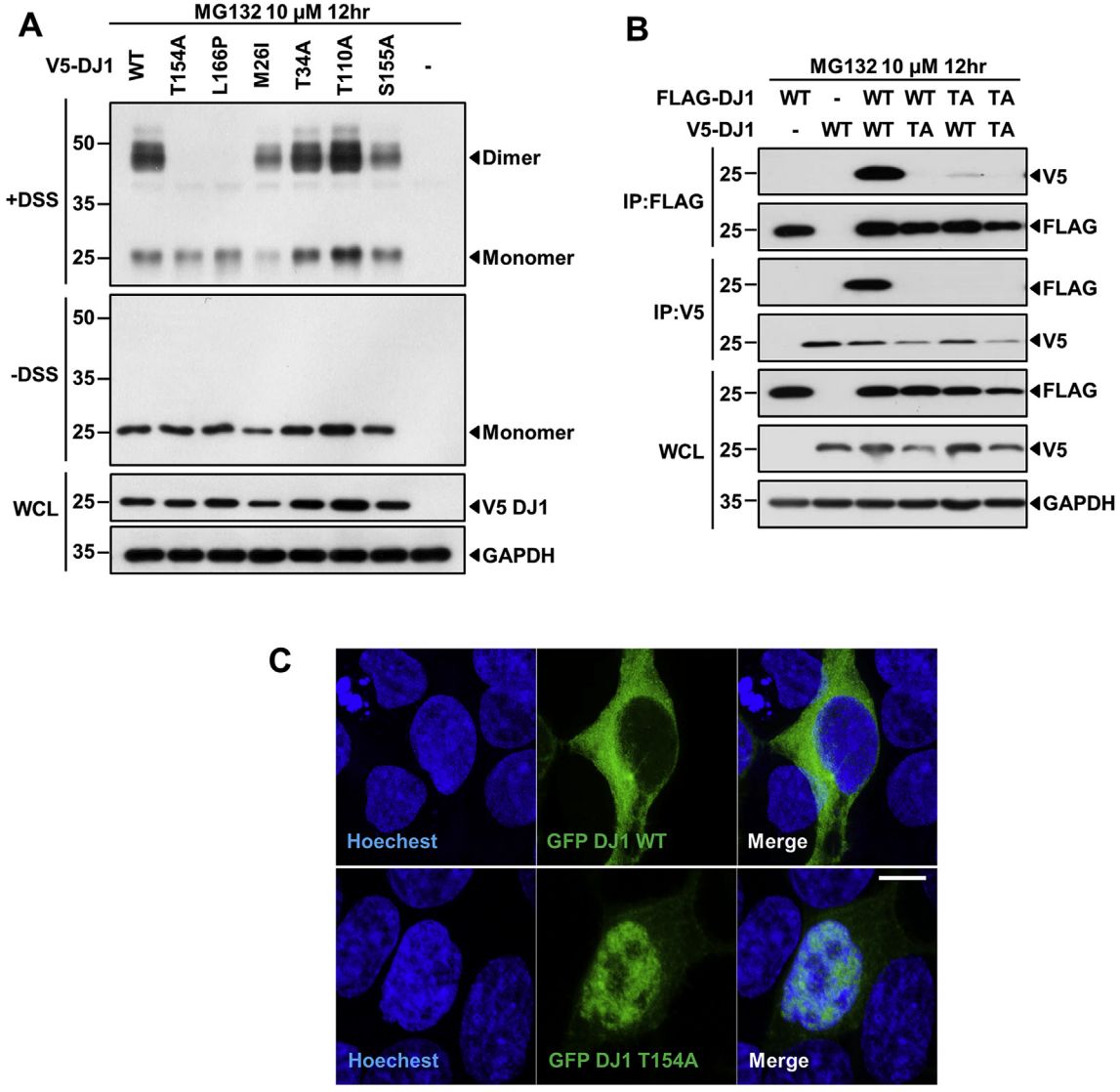

Fig. 3. T154 site of DJ-1 affects dimer formation and cellular localization. (A) Impaired dimerization of DJ-1 T154A mutant. HEK293 cells were transfected with V5-tagged WT DJ-1 or one of several DJ-1 mutants including DJ-1 T154A. To inhibit proteolytic degradation, transfected cells were incubated with 10 μM MG132 for 12 h. Cell lysates were incubated with disuccinimidyl suberate (DSS) for cross-linking reaction. Reaction mixtures were resolved by SDS-PAGE and subjected to immunoblot analyses using *anti*-V5 antibody. **(B)** Analyses of dimerization of WT DJ-1 and DJ-1 T154A by co-immunoprecipitation. The indicated combinations of WT DJ-1 or DJ-1 T154A (TA) were transiently overexpressed in HEK293 cells and further cultivated for 12 h in the presence of 10 μM MG132. Cell lysates were subjected to immunoprecipitation with anti-FLAG or *anti*-V5 antibody. Co-immunoprecipitated DJ-1 was probed with the indicated HRP-conjugated antibodies. **(C)** Immunocytochemical localization of WT DJ-1 and DJ-1 T154A. HEK293 cells were transiently transfected with GFP-tagged WT DJ-1 or DJ-1 T154A. At 48 h after transfection, fluorescent images of cellular DJ-1 (green) were obtained with an LSM700 confocal microscope. Nuclei were counterstained with Hoechst dye (blue). Scale bar: 10 μm.

2.7. Ni-NTA-based pull-down assay

To detect phosphorylation of DJ-1 at Thr^{154} at a cellular level, HEK293 cells were transfected with V5/His-tagged WT DJ-1 in combination with FLAG-tagged PKACα. Cell lysates (2 mg) were incubated at 4 °C overnight with 50 μl Ni-NTA-agarose beads (Invitrogen) on a shaking rotator. After centrifugation at 3000 g for 2 min, precipitates were washed three times with lysis buffer containing 20 mM imidazole. Proteins were separated on SDS-PAGE followed by immunoblot analyses using an antibody that specifically detect DJ-1 pT154 site.

2.8. Fluorescence microscopy

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Science) at RT for 15 min and permeabilized with 0.25% Triton X-100 in PBS at RT for 15 min. Cells were then processed for immunofluorescent staining by incubating at 4 °C overnight with *anti*-DJ-1 antibody (ab18257) and PKACα (610981) followed by incubation at RT for 1 h with Alexa 488-conjugated goat anti-rabbit IgG (A11001, Invitrogen) and Alexa 568-conjugated goat anti-mouse IgG (A11004, Invitrogen). Nuclei were counter-stained with 2 μg/ml Hoechst 33258 (H3570, Invitrogen). Fluorescence images were photographed under a confocal microscope (LSM 700, Carl Zeiss).

2.9. Cross-linking assay

HEK293 cells transiently transfected with the indicated DJ-1 mutants were washed with ice-cold PBS and incubated in a lysis buffer containing 1% Triton-X 100 in PBS. Cross-linking assay was performed according to the manufacturer's instructions. Briefly, cell lysates (20 μg) were incubated with 5 mM DSS (21655, Thermo Fisher Scientific) for 30 min at RT. The reaction was quenched by incubation with 50 mM

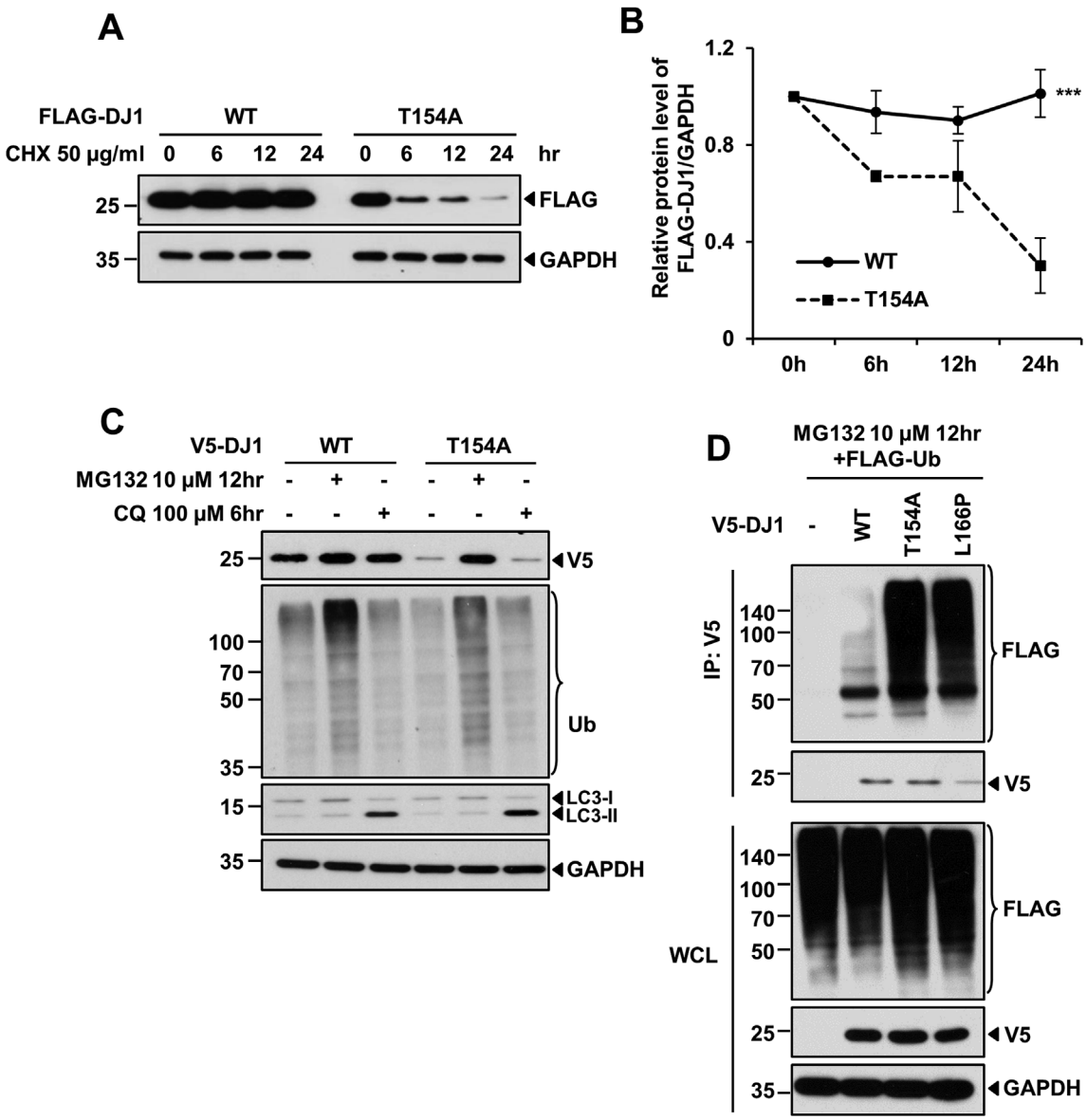

Fig. 4. DJ-1 bearing T154A is prone to proteasome-mediated degradation. (A) Decreased stability of DJ-1 T154A. At 24 h after transfection with the indicated constructs, HEK293 cells were incubated with 50 μg/ml cycloheximide (CHX) for the indicated time periods. Cell lysates obtained in each condition were subjected to immunoblot analyses using anti-FLAG antibody. **(B)** After the intensity of DJ-1 was normalized to that of GAPDH at the indicated time points, relative protein levels compared with those at time 0 were evaluated. The below graph shows the mean ± SD of a representative experiment performed in triplicate. **(C)** Proteasome-mediated degradation of DJ-1 T154A. HEK293 cells were transfected with V5-tagged WT DJ-1 or DJ-1 T154A. HEK293 cells were treated with 10 μM MG132 for 12 h or 100 μM chloroquine (CQ) for 6 h. Cell lysates were subjected to immunoblot analyses using the indicated antibodies. **(D)** Ubiquitination of WT DJ-1 and several DJ-1 mutants. At 48 h after transfection with the indicated combinations of constructs, HEK293 cells were further maintained for 12 h in the presence of 10 μM MG132. Cell lysates were subjected to immunoprecipitation analyses using *anti*-V5 antibody. The extent of ubiquitination of DJ-1 was detected using HRP-conjugated anti-FLAG antibody.

Tris-HCl (pH 7.5) in PBS for 15 min at RT.

2.10. siRNA-mediated silencing of PKAcα

Using Lipofectamine 2000 (Invitrogen), MN9D cells were transfected for 72 h with 100 nM siRNAs specific for silencing mouse PKAcα (numbers 1412481, 1412482, 1412383; Bioneer Corp) or a non-targeting sequence (SN-1001, Bioneer Corp.) according to the manufacturer's instructions.

2.11. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of three independent experiments. To determine the significance of differences between groups, two-tailed Student's t-tests or one-way analysis of variance followed by Tukey's post-hoc tests were performed using Prism 6 software (GraphPad Inc). The statistical significance of differences is indicated as follows: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.

3. Results

3.1. Interaction with and phosphorylation of DJ-1 by PKAcα

To investigate the existence of phosphorylated DJ-1 in MN9D cells, we performed immunoprecipitation with DJ-1 followed by nano-LC-

MS/MS. Three threonine phosphorylation sites on DJ-1 were identified by the Mascot search engine (Supplementary Figs. 1A–C). Modified peptides identified from tryptic digestions represented the detected phosphorylation sequences (Supplementary Fig. 1D), suggesting T34, T110, and T154 are phosphorylated. Previously, DJ-1 was described as a putative substrate of PKACα and potentially phosphorylated at T154 or S155 [12]. To precisely demonstrate PKACα-mediated phosphorylation of DJ-1, we examined the interaction between DJ-1 and PKACα. Confocal microscopy revealed co-localization of endogenous DJ-1 and PKACα in the cytosol (Fig. 1A). Co-immunoprecipitation assay indicated interaction between DJ-1 and PKACα in HEK293 cells (Fig. 1B). We confirmed their binding in whole brain lysates (Fig. 1C). By incubating purified His-tagged DJ-1 with PKACα, we found that DJ-1 was phosphorylated by PKACα, and this event was blocked in the presence of H-89, a PKACα inhibitor (Fig. 2A). Next, based on a previous report suggesting the possibility of PKACα-mediated phosphorylation of DJ-1 at T154 or S155 [12], we attempted to determine which residue of DJ-1 was phosphorylated by performing *in vitro* kinase assay. The extent of phosphorylation was decreased in a DJ-1 T154A mutant compared with wild type (WT) DJ-1 (Fig. 2B). By contrast, the extent of phosphorylation was not discernibly altered in a DJ-1 S155A mutant (Fig. 2C), suggesting that PKACα is responsible for phosphorylation of the T154 residue of DJ-1. In addition, the extent of phosphorylation of T34A and T110A was not altered by PKACα (Supplementary Fig. 2), indicating that these two residues are not likely phosphorylated by PKACα. Considering that there was still a considerable level of phosphorylation left in T154A mutant, however, we did not completely rule out the possibility that other unidentified residues of DJ-1 could be additionally phosphorylated by PKACα. To further confirm PKACα-mediated phosphorylation of DJ-1 at T154 at a cellular level, Ni-NTA-agarose pulldown assay was performed and followed by immunoblotting with a rabbit polyclonal antibody that specifically detects DJ-1 bearing pT154. A band with the expected size of DJ-1 was detected in cells expressing both FLAG-tagged WT PKACα and His/V5-tagged WT DJ-1 (Fig. 2D and E). Additionally, Ni-NTA pulldown assay using cells transfected with V5-His-tagged DJ-1 WT or T154A mutant indicated that this antibody specifically recognizes pT154 form of DJ-1 (Supplementary Fig. 3). As determined by expression levels of p-Creb in HEK293 cells, we confirmed that transient transfection of PKACα alone can lead to PKA activation and is equivalent to forskolin (PKA activator; Supplementary Figs. 4A and B).

3.2. T154 site of DJ-1 is important for dimer formation

DJ-1 retains the ability to form homodimers and serves chaperone or antioxidant activities in living cells [13]. However, an abnormal monomer of human DJ-1 is found in DJ-1 L166P and is related to neurodegeneration [13,14]. To examine whether the DJ-1 T154 residue is involved in dimer formation, the extent of DJ-1 dimer was compared between HEK293 cells transfected with WT DJ-1 or DJ-1 T154A. Due to the protein instability of monomeric DJ-1, transfected cells were treated with MG132 to block proteasome-mediated degradation [15]. Cell lysates were then subjected to cross-linking assay using a membrane permeable cross-linking reagent disuccinimidyl suberate (DSS). A higher level of dimer formation of WT DJ-1 was detected in the presence of DSS. No discernible level of dimer was detected in cells overexpressing DJ-1 T154A (Fig. 3A). In HEK293 cells transfected with DJ-1 L166P, we did not find the dimeric form of DJ-1. However, a loss of dimer formation was not detected in HEK293 cells transfected with DJ-1 M26I, another disease-related mutant. Intriguingly, the extent of DJ-1 dimerization was not affected in other phospho-null mutants. To compare binding efficacy between WT DJ-1 and DJ-1 T154A (TA), HEK293 cells were transfected with the indicated combinations. A higher level of DJ-1 binding was detected in HEK293 cells doubly transfected with WT DJ-1 (Fig. 3B). By contrast, the extent of DJ-1 binding was dramatically decreased in HEK293 cells transfected with WT DJ-1 and DJ-1 T154A or with FLAG- and V5-tagged DJ-1 T154A. These data indicated that distortion of T154 is limited to DJ-1 dimerization. The distinct cellular localization of WT DJ-1 and T154A DJ-1 was detected (Fig. 3C). Consistent with previous reports [4,16,17], DJ-1 was predominantly localized in the cytosol, whereas DJ-1 T154A was primarily detected in the nucleus. Taken together, our data indicate that the Thr^{154} residue of DJ-1 contributes to homodimeric binding and appropriate cellular localization.

3.3. DJ-1 T154A is prone to proteasome degradation

We compared the stability of WT DJ-1 and DJ-1 T154A in HEK293 cells. Cycloheximide chase assay indicated that the stability of T154A DJ-1 was less than that of WT DJ-1 (Fig. 4A and B). In eukaryotic cells, two major pathways—UPS and lysosomal proteolysis—mediate protein degradation [18]. To distinguish these two systems, HEK293 cells transfected with WT DJ-1 or DJ-1 T154A were treated with MG132 or chloroquine (CQ), a lysosomotropic agent. As pattern of ubiquitination and the appearance of microtubule-associated protein light chain 3 (LC3)-II indicates, these two drugs were effective in inhibiting UPS- or autophagy-mediated protein degradation, respectively (Fig. 4C). Under these conditions, levels of WT DJ-1 remained the same regardless of drug treatment. However, the decreased level of T154A was restored in HEK293 cells treated with MG132 but not with CQ, indicating that the degradation of DJ-1 depends on the UPS. To compare the extent of ubiquitination between WT DJ-1 and representative DJ-1 mutants, HEK293 cells were transfected with FLAG-tagged ubiquitin in combination with V5-tagged WT DJ-1, DJ-1 T154A, or DJ-1 L166P. Levels of ubiquitinated DJ-1 were dramatically augmented in cells expressing DJ-1 T154A or DJ-1 L166P compared with those in WT DJ-1 (Fig. 4D).

4. Discussion

The *DJ-1* gene was first identified as a novel oncogene [19]. A large deletion and various missense mutations were demonstrated to be associated with autosomal recessive early-onset parkinsonism [20]. Among the multiple functions of DJ-1 [5], DJ-1 has extensively been shown to play a role in protecting cells from oxidative stress-induced cell death [1]. For example, DJ-1, as a sensor of anti-oxidant signaling, is regulated by oxidative modification at the C106 residue [4]. Notably, proper oxidative modification at the C106 residue is important to maintain DJ-1 activity and stability. However, highly oxidized forms of DJ-1 are accumulated and destabilized in patients with sporadic PD [21]. Other modifications such as sumoylation and S-nitrosylation may also affect the neuroprotective activity of DJ-1 [5]. Lin et al. reported several post-translationally modification of DJ-1 in whole blood samples of patients with PD and Alzheimer's disease [22], raising the possibility that post-translational modifications of DJ-1 dynamically regulate its protein activity, subcellular localization, and stability.

Phosphorylation has traditionally been viewed as a key mechanism governing the signaling network [23]. Indeed, various neurodegenerative diseases, including PD and Alzheimer's disease, share common mechanisms of regulating the phosphorylation-to-dephosphorylation ratio of specific disease-related proteins relevant to the pathogenesis of disease progression [24]. Although phospho-modifications of DJ-1 have been less studied, S/T/Y residue of DJ-1 was found to be phosphorylated in a p53-dependent manner [25]. Using MS-based screening, DJ-1 Tyr67 appears to be phosphorylated by an unknown kinase [26,27]. However, it remains unclear as to which specific kinase is responsible for the phosphorylation of a specific site of DJ-1 and the resulting effect on its functions. Here, we identified three novel phosphorylated threonine residues on DJ-1: Thr^{34}, Thr^{110}, and Thr^{154}. Based on previous reports that PKACα contributes to the etiology of several neurodegenerative diseases and that the level of PKACα was reduced during neurodegeneration [7,8], we focused on determining whether PKACα is

responsible for the phosphorylation of these newly identified threonine sites of DJ-1. Assays using purified DJ-1 with PKACα indicated that PKACα-dependent phosphorylation of DJ-1 occurs at Thr154. This was confirmed by using antibody that specifically recognizes pT154. Molecular modeling studies predict that DJ-1 phosphorylation on Thr154 may induce substantial conformational distortion in this region, which, in turn, might be transferred to the dimeric interface (Supplementary Fig. 5). Consequently, this conformational change in the dimeric interface could promote dimerization of DJ-1. Moreover, we provided evidence that PKACα-dependent phosphorylation is an important step toward enhanced DJ-1 dimerization. Specifically, we detected that DJ-1 dimerization appeared to be increased by the phosphorylation status of DJ-1 (Supplementary Fig. 6). First, pharmacological assays after treatment with okadaic acid or forskolin indicated that dimerization of DJ-1 was increased (Supplementary Figs. 6A–D). When PKACα was down-regulated by siRNA-mediated silencing, the extent of DJ-1 dimerization was accordingly reduced (Supplementary Figs. 6E and F). Co-immunoprecipitation assays also indicated that physical interaction of DJ-1 was increased by PKACα over-expression and this enhancement was returned to a basal level in presence of H-89 (Supplementary Fig. 6G). Furthermore, no discernible level of dimer was detected in cells over-expressing DJ-1 T154A mutant. Intriguingly, compared with cells overexpressing WT DJ-1, levels of dimeric DJ-1 remained relatively unchanged in other phospho-null mutants including DJ-1 T34A, T110A, and S155A, raising the possibility that phosphorylation of specific residues of DJ-1 contributes to enhanced DJ-1 dimerization. In this study, we demonstrated that PKACα-mediated formation of dimeric DJ-1 is important for maintaining the physiological level of DJ-1 by permitting its escape from cellular degradation systems. This argument is supported by our findings that DJ-1 T154A was relatively unstable and that DJ-1 T154A was prone to UPS-mediated degradation. Intriguingly, we examined whether endogenous levels of DJ-1 are affected in PKA-regulating experimental paradigms: PKA knockdown cell lines and pharmacological PKA inhibitor/activator treatment. The results from these independent studies showed that PKA activity does not affect endogenous levels of total DJ-1, indicating that PKA activation is limited to enhancing DJ-1 dimerization and subsequent stability (Supplementary Fig. 7).

DJ-1 is expressed in almost all cells including neurons and glial cells [19,20] and is localized in the cytosol, mitochondria, and nucleus. In addition to DJ-1 dimerization and stability, the subcellular localization of DJ-1 is also important for determining its neuroprotective functions [9,16]. For example, nuclear-localized DJ-1 interacts with p53 or ASK1 and subsequently alleviates pro-apoptotic molecules expression [28,29]. When DJ-1 is localized in the mitochondria, it regulates mitochondrial quality control and neuroprotective effect [16]. DJ-1 L166P is also reported to be mainly localized in the nucleus and mitochondria [30], and to promote cell death induced by Bax and Daxx. In a previous study, we demonstrated that DJ-1 translocates into the nucleus upon oxidative stress and upregulates pro-apoptotic proteins [9]. In the present study, we showed that DJ-1 T154A was mainly localized in the nucleus, whereas WT DJ-1 was predominantly detected in the cytosol. Although further investigation is needed, Thr154 residue of DJ-1 which promotes its protein stability via dimerization and proper subcellular localization may comprise a critical loop that maintains its neuroprotective roles. Finally, it remains to be determined whether the biological significance of our findings can be recapitulated in more pathophysiologically relevant animal brain models and in human. Similarly, it would be intriguing to investigate the potential combinatorial effects of phosphorylation and other post-translational modifications on DJ-1 dimerization and its stability.

Conflicts of interest

None.

Author contributions

YJO, YUK, and SJK designed the research, analyzed the data, and wrote the manuscript. YUK, SJK, and JHL conducted most of the experiments. MYS and KSP performed LC-MS/MS and analyzed the data. HSC and JBP performed the molecular modeling of DJ-1 p154T.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parkreldis.2019.07.029.

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