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DJ-1 Modulates the p38 Mitogen-Activated Protein Kinase Pathway Through Physical Interaction With Apoptosis Signal-Regulating Kinase 1

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ABSTRACT

DJ-1 has been reported as a gene linked to early onset familial Parkinson's disease, and is functionally involved in transcriptional regulation and oxidative stress-induced cell death. To understand the role of DJ-1 in cellular stress, this study investigated DJ-1's effect on stress-activated protein kinase signaling and H_2O_2-induced activation of apoptosis signal-regulating kinase 1 (ASK1). According to the results, the overexpression of DJ-1 inhibited H_2O_2-induced activation of ASK1 as well as the activation of downstream kinases in the p38 mitogen-activated protein kinase (MAPK) signaling cascade. The results of both in vivo binding and kinase studies have revealed that ASK1 is the direct target of DJ-1, whereas it has shown no effect on either MKK3 or p38. DJ-1 blocked both the homo-oligomerization of ASK1 and inhibited ASK1 activity. Taken together, our data strongly suggest that DJ-1, by directly inhibiting ASK1, may act as a negative regulator in ASK1 signaling cascades. J. Cell. Biochem. 110: 229–237, 2010.

KEY WORDS: DJ-1; APOPTOSIS SIGNAL-REGULATING KINASE 1 (ASK1); p38 MITOGEN-ACTIVATED PROTEIN KINASE (MAPK); PROTEIN–PROTEIN INTERACTION

DJ-1 (SP22/CAP-1/RS/PARK7) has been identified as a novel oncogenic protein that acts co-operatively with H-Ras [Nagakubo et al., 1997]. DJ-1 proteins play diverse roles in oncogenesis, male fertility, controlling protein–RNA interactions, regulating inflammation, controlling hypoxic stress, and modulating androgen receptor transcription activity. Recently, DJ-1 was shown to be definitively causal in cases of familial Parkinson's disease (PD). Homozygous and heterozygous mutations of DJ-1 were identified in patients with familial or sporadic PD [Bonifati et al., 2003; Hague et al., 2003; Moore et al., 2003; Abou-Sleiman et al., 2004; Hedrich et al., 2004]. DJ-1 protein can be reversibly or irreversibly oxidized and this modification strikingly increases susceptibility to oxidative stress [Meulener et al., 2006]. Several lines of evidence implicate DJ-1's role in anti-oxidative stress reactions and that mutation of DJ-1 leads to cell death [Taira et al., 2004]. DJ-1 protects against oxidative stress and mitochondrial toxins in cell cultures and animal models. Thus far, several lines of evidence also appear to indicate that DJ-1 performs a cytoprotective function in cell death against oxidative stress by facilitating Akt/PKB signaling and suppressing apoptosis signal-regulating kinase 1 (ASK1) signaling via sequestration of Daxx. Most recently, we reported that DJ-1 modulates the c-Jun N-terminal kinase (JNK) signaling pathway through negative regulation of the functions of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (MEKK1) via physical interaction [Mo et al., 2008].

ASK1 is a member of the mitogen-activated protein kinase kinase kinase family and activates SEK1–JNK1 and MKK3/MKK6-p38 signaling cascades [Ichijo et al., 1997]. ASK1 is a pivotal component in apoptosis induced by various cytotoxic stresses, including tumor necrosis factor (TNF-α), hydrogen peroxide (H_2O_2), UV light, X-rays, heat shock, and growth factor- or serum withdrawal [Raingeaud et al., 1995; Xia et al., 1995; Kyriakis and Avruch, 1996; Verheij et al., 1996]. H_2O_2 increases the dimeric form of ASK1, and then ROS-mediated dimerization of ASK1 causes ASK1 activation and apoptosis [Gotoh and Cooper, 1998]. The ASK1 signaling pathway performs a critical role in 6-hydroxydopamine-induced apoptosis in human neuroblastoma cells, and the inhibition of ASK1 signaling

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using small molecules was shown to mitigate 6-hydroxydopamine-mediated neuronal death [Ouyang and Shen, 2006; Pan et al., 2007].

Nevertheless, despite the fact that many studies have been conducted regarding the subject, the physiological role of DJ-1 in cell death remains largely unknown. Here, we have demonstrated that DJ-1 modulates the ASK1 signaling pathway via negative regulation of the functions of ASK1, via physical interaction.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

Human embryonic kidney 293 (HEK293) and SH-SY5Y cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a humidified incubator with an atmosphere of 95% O_2 and 5% CO_2. For plasmid DNA transfection, the cells were plated at a density of 50–60% confluence, grown overnight, and transfected with appropriate expression vectors in the presence of indicated combinations of plasmid DNAs by using the calcium phosphate method or Lipofectamine-plus reagent. The HEK293 cells were stably transfected with pcDNA3 empty vector or pcDNA3 Flag-DJ-1, using Lipofectamin-Plus reagent. After 48 h of transfection, the cells were replated at a dilution of 1/20 and were maintained in complete medium containing 500 μg/ml of G418 (Invitrogen–GIBCO, USA) to select the neomycin-resistant cells [Mo et al., 2008].

IMMUNOBLOT ANALYSIS

After 48 h of transfection, the cultured HEK293 cells were harvested and lysed in RIPA buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM DTT, and 2 μg/ml each of leupeptin and aprotinin) for 30 min at 4°C. The cell lysates were subjected to 20 min of centrifugation at 12,000*g* at 4°C. The resultant soluble fraction was boiled in Laemmli buffer and subjected to SDS–PAGE. After gel electrophoresis, the separated proteins were transferred via electroblotting onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were then blocked with Tris-buffered saline solution (pH 7.4) containing 0.1% Tween 20 and 5% nonfat milk. The blotted proteins were then probed with anti-Myc antibody (9E10), anti-HA (12CA5) antibody, or anti-Flag M2 antibody (Sigma Chemical Co.), followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Inc.). The blots were developed using enhanced chemiluminescence (ECL) [Park et al., 2000].

IMMUNOCOMPLEX KINASE ASSAY

The cultured cells were harvested and lysed in buffer A, containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, 25 mM glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM sodium fluoride, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS for 30 min at 4°C. The cell lysates were then subjected to centrifugation at 12,000*g* for 20 min at 4°C. The soluble fraction was incubated for 3 h with appropriate antibodies against the indicated protein kinase at 4°C. The immunocomplexes were then coupled to protein G-agarose during an additional hour of incubation at 4°C, after which they were pelleted via centrifugation. The immunopellets were rinsed thrice with lysis buffer and then twice with 20 mM HEPES, at a pH of 7.4. The immunocomplex kinase assays were conducted via the incubation of the immunopellets for 30 min at 30°C with 2 μg of substrate protein in 20 μl of reaction buffer that contained 0.2 mM sodium orthovanadate, 10 mM $MgCl_2$, 2 μCi [^{32}P]ATP, 20 mM HEPES (pH 7.4). The phosphorylated substrates were separated by SDS–PAGE and quantified using a Fuji BAS 2500 phosphoImager. The GST fusion proteins to be used as substrates were expressed in *Escherichia coli* using pGEX-4T (Pharmacia) and purified using glutathione–sepharose, as described previously. The protein concentrations were determined by the Bradford method (Shimadzu) [Park et al., 2002].

REPORTER ASSAY

The cells were lysed in chemiluminescent lysis buffer (18.3% of 1 M K_2HPO_4, 1.7% of 1 M KH_2PO_4, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT)) and assayed for luciferase activity with a luciferase assay kit (Promega). The activity of the luciferase reporter protein in the transfected cells was normalized in reference to the β-galactosidase activity in the same cells.

DJ-1 KNOCKDOWN IN CELLS

The siRNA targeting DJ-1 was used as directed with the following target sequences: DJ-1: 5′-UGGAGACGGUCAUCCCUGUdTdT-3′ (upper strand) and 3′-dTdTACCUCUGCCAGUAGGGACA-5′ (lower strand). Sham control or DJ-1 siRNA was transfected into the HEK293 cells using Lipofectamine-Plus reagent (Invitrogen), in accordance with the manufacturer's instructions [Mo et al., 2008].

COIMMUNOPRECIPITATION ASSAYS

The cells were lysed in 1 ml of RIPA buffer for 30 min at 4°C. After centrifugation at 12,000*g* for 20 min, the supernatants were subjected to immunoprecipitation with appropriate antibodies coupled to protein A-agarose beads (Peptron). The resulting immunoprecipitates were washed thrice with phosphate-buffered solution (PBS, pH 7.4). Laemmli sample buffer was then added to the immunoprecipitated pellets; the pellets were heated at 95°C for 5 min and then analyzed by SDS–PAGE. The western blot was performed with the indicated antibodies [Park et al., 2001].

IMMUNOFLUORESCENCE STAINING

Assays were conducted as previously described with HEK293 cells plated at 1×10^5 cells per well onto cover slips (Fisher). A total of 0.5 μg of appropriate DNA per well was then transfected using Lipofectamine-plus reagent (Invitrogen). The transfected cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), and then permeabilized with 0.1% Triton-X 100 in PBS. Cells were blocked in 1% BSA in PBS. Mouse anti-FlagM2 antibody (Sigma) and anti-HA antibody (Novus Biologicals) were employed as the primary antibodies at a dilution of 1:100, washed thrice in PBS. Alexa-488 (Invitrogen) or Alexa-546 conjugated anti-mouse secondary antibody (1:100) was added, and then the DNA dye ToPro-3 was used for nuclear localization. The stained cells were evaluated for localization via confocal microscopy (Leica

TCS SPE). Each image is a single Z section at the same cellular level. The final images were obtained and analyzed using confocal microscopy with LAS AF software (Leica). Scale bars represent either 25 μm as indicated.

RESULTS

DJ-1 SUPPRESSES OXIDATIVE STRESS-INDUCED ASK1 ACTIVATION

To determine the possible role of DJ-1 in the regulation of the ASK1 signaling pathway, we subjected HEK293 cells to transient transfection with an expression plasmid encoding for Flag epitope-tagged DJ-1 (Flag-DJ-1) and then evaluated the H_2O_2-induced activation of ASK1 in control cells. As shown in Figure 1A, this effect was markedly inhibited in the DJ-1 transfected cells. We then assessed the H_2O_2-induced activation of ASK1 in HEK293 cells that had been stably transfected with an expression vector for Flag epitope-tagged human DJ-1 (HEK293-DJ-1 cells), or with the corresponding empty vector (HEK293-neo cells). The H_2O_2-induced activation of endogenous ASK1, which was apparent in the control HEK293-neo cells, was abolished in the HEK293-DJ-1 cells (Fig. 1B). The role of DJ-1 in the ASK1 signaling pathway was further evaluated by the knockdown of DJ-1 using short interference DJ-1. Unlike what was observed in the control short interference plasmid-transfected cells, the cells transfected with short interference DJ-1 harbored low expression levels of DJ-1 (Fig. 1C). Figure 1C also indicates that endogenous ASK1 enzyme activity, which was stimulated by H_2O_2, was promoted through the downregulation of DJ-1 expression. These data indicate that the level of DJ-1 expression results in the inhibition of the ASK1 signaling pathway.

DJ-1 INHIBITS DOWNSTREAM COMPONENTS OF THE ASK1 SIGNALING PATHWAY

We then attempted to determine whether DJ-1 influences signaling downstream of ASK1, including the MKK3 and p38 pathway. To determine whether DJ-1 is able to modulate MKK3 activity, we subjected HEK293 cells to transient transfection with an expression plasmid encoding HA epitope-tagged MKK3 (HA-MKK3), and then examined DJ-1's effect on the H_2O_2-induced activation of MKK3 in the transfected cells (Fig. 2A). While H_2O_2-induced MKK3 activation in the control HEK293 cells, this effect was markedly inhibited by DJ-1 co-expression. We next examined the effect of DJ-1 on p38 activity in HEK293 cells transfected with plasmid encoding HA epitope-tagged p38 (HA-p38). Exposing the cells to H_2O_2 increased

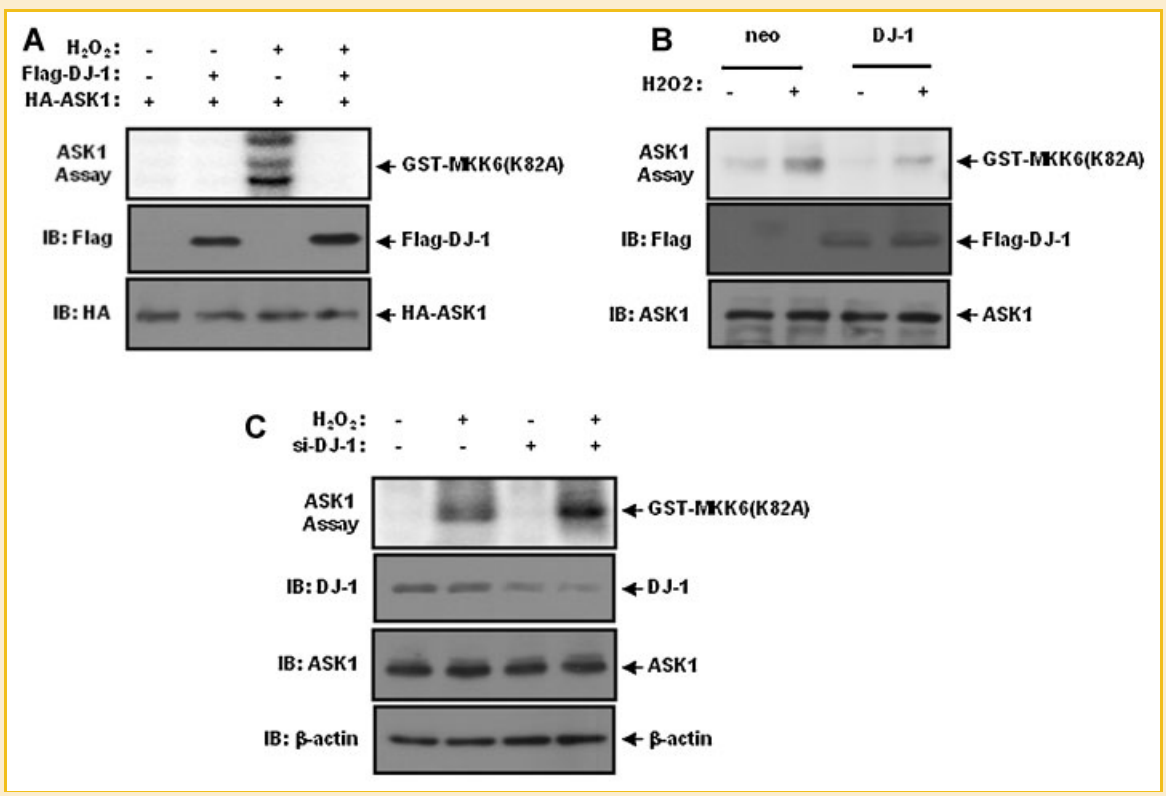

Fig. 1. DJ-1 suppresses oxidative stress-induced ASK1 activation. A: HEK293 cells were transiently transfected with plasmids expressing pcDNA3-HA-ASK1 or pCMV-Flag-DJ-1 as indicated. After 48 h of transfection, the cells were treated with 2 mM H_2O_2 for 20 min at 37°C, and then assayed for ASK1 activity via immunocomplex kinase assays. B: HEK293-neo or HEK293-DJ-1 cells were treated with 2 mM H_2O_2 for 20 min at 37°C, and then assayed for ASK1 activity via an immunocomplex kinase assay. C: HEK293 cells were transfected with siRNA targeting DJ-1 (pSUPER-siDJ-1) or control siRNA (pSUPER-siCon). After 48 h of transfection, the cells were treated with 2 mM H_2O_2 for 20 min at 37°C, and assayed for ASK1 activity via an immunocomplex kinase assay. DJ-1 expression was analyzed by immunoblotting using anti-DJ-1 monoclonal antibody. A–C: The cell lysates were also subjected to immunoblotting analysis with the indicated antibodies. β-actin expression was analyzed by immunoblotting using an anti-β-actin monoclonal antibody as a loading control. The immunocomplex ASK1 kinase assays were conducted using GST-MKK6(K82A) as the substrate. IB, immunoblot.

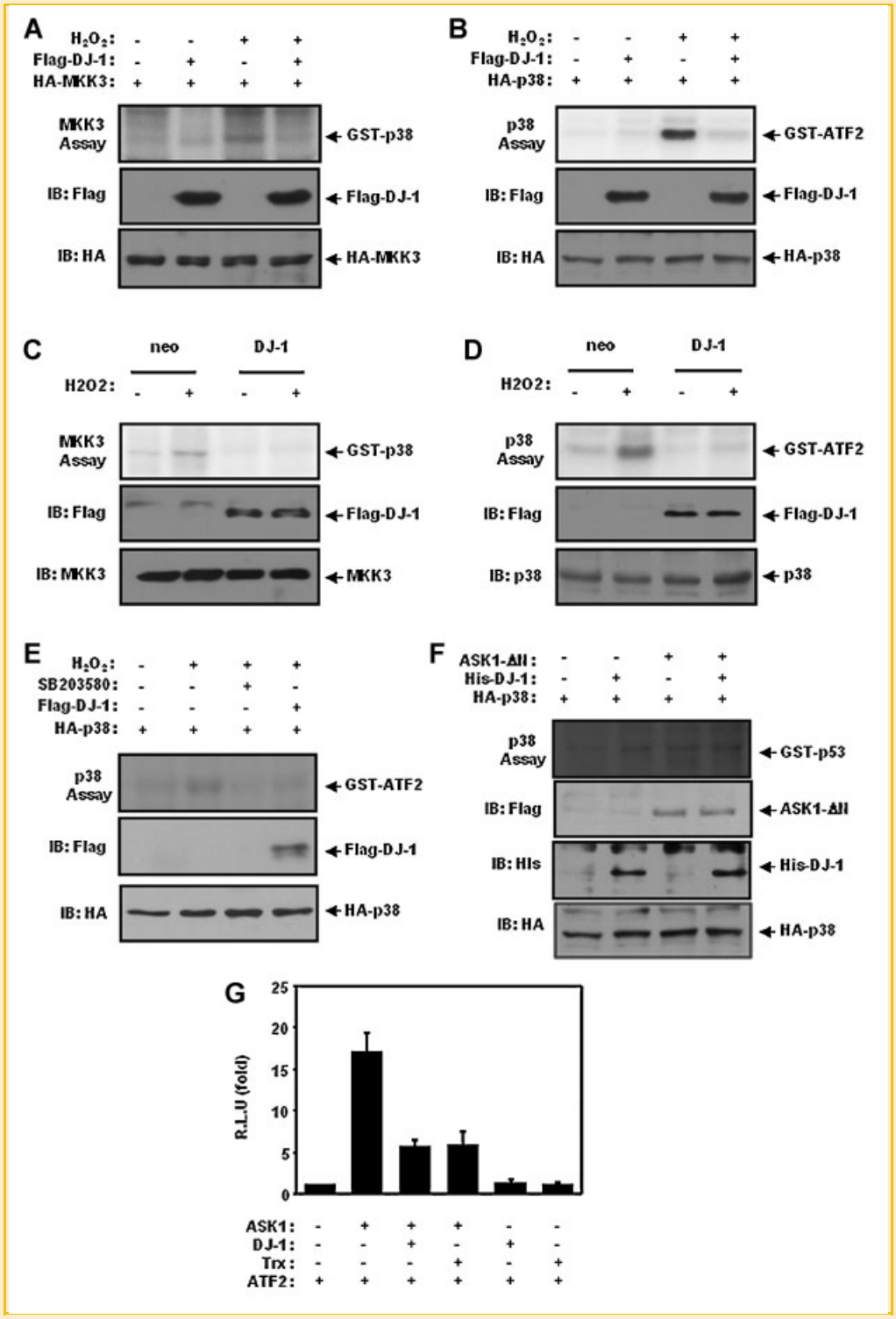

Fig. 2. DJ-1 inhibits MKK3 and p38 activities. A, B: HEK293 cells were transiently transfected with plasmids expressing pCMV-Flag-DJ-1 as indicated. The cells were treated with 2 mM H_2O_2 for 20 min at 37°C, and then assayed for MKK3 or p38 activity via immunocomplex kinase assays. C, D: HEK293-neo or HEK293-DJ-1 cells were treated with 2 mM H_2O_2 for 20 min at 37°C, and then assayed for MKK3 or p38 activity via immunocomplex kinase assays. E: HEK293 cells were transiently transfected with plasmids expressing pCMV-Flag-DJ-1 and pcDNA3-HA-p38 as indicated. The cells were pretreated with 100 mM of SB203580 and then exposed to 2 mM H_2O_2 for 20 min at 37°C. The cells were then assayed for MKK3 or p38 activity via immunocomplex kinase assays. F: HEK293 cells were transiently transfected with plasmids expressing pcDNA3-ASK1ΔN, pCMV-Flag-DJ-1, and pcDNA3-HA-p38 as indicated. The cells were then assayed for MKK3 or p38 activity via immunocomplex kinase assays. The cell lysates were also subjected to immunoblotting analysis with the indicated antibodies. The immunocomplex MKK3 or p38 kinase assays were conducted using GST-p38 or GST-ATF2 as the substrate. G: HEK293 cells were transiently transfected with plasmids expressing pcDNA3-ASK1, pCMV-Flag-DJ-1, pcDNA3-Trx, pFA-ATF2, and pFR-Luc as indicated. After 48 h of transfection, the cells were lysed and the luciferase activity was assayed. Data were normalized with β-galactosidase. These results are expressed as the means ± average deviation of three independent experiments. R.L.U means relative luciferase units. IB, immunoblot.

p38 activity and this effect was inhibited by the overexpression of DJ-1 (Fig. 2B). We then examined H_2O_2-induced endogenous MKK3 and p38 activities in HEK293-DJ-1 and HEK293-neo cells, respectively. The H_2O_2-induced stimulation of endogenous MKK3 (Fig. 2C) and p38 (Fig. 2D) activity was remarkably reduced in the HEK293-DJ-1 cells as compared to the HEK293-neo control cells. These data show that DJ-1 overexpression also blocked H_2O_2-induced MKK3 and p38 activity. To compare the effects of DJ-1 and p38 specific inhibitor on p38 kinase activity, we introduced one specific inhibitor against p38, namely SB203580, to prevent H_2O_2-induced activation of p38. We found that DJ-1 and SB203580 has similar effect on p38 activity (Fig. 2E). Furthermore, co-expression of DJ-1 did not directly influence the activity of p38 on p53 phosphorylation (Fig. 2F). These results suggest that ASK1 signaling pathway was the principal target of DJ-1. The transcription factor ATF2 is a physiological target of ASK1 signaling cascade. Thus, we attempted to determine whether DJ-1 influences the ASK1 signaling-dependent increase in the transcription activity of ATF2, through a luciferase reporter gene assay. ASK1 ectopic expression caused an increase in ATF2-dependent luciferase activity and this effect was inhibited by DJ-1 and thioredoxin (Fig. 2G). This result indicates that DJ-1 inhibits the ASK1-dependent transactivation activity of ATF2.

Furthermore, we attempted to determine the role of DJ-1 in the suppression of ASK1 signaling cascade, using human neuroblastoma SH-SY5Y cells. H_2O_2 was found to markedly stimulate the activities of ectopically expressed ASK1, MKK3, and p38 in the human neuroblastoma SH-SY5Y cells (Fig. 3A–C). As shown in Figure 3, this effect was markedly inhibited in the DJ-1 transfected neuroblastoma cells. These data showed that DJ-1 overexpression also blocked H_2O_2-induced ASK1 signaling activation.

DJ-1 INHIBITS ASK1 ACTIVITY, BUT NOT MKK3 AND p38 ACTIVITIES IN VITRO

The effect of DJ-1 on ASK1 activity was assessed in vitro to determine whether DJ-1 directly targets ASK1. Comparable to endogenously and ectopically expressed DJ-1, a similar level of purified DJ-1 proteins was used in this experiment. Active ASK1 was acquired through the immunoprecipitation of H_2O_2-exposed HEK293 cells using anti-ASK1 antibody. In the in vitro kinase assay, the pretreatment of the active ASK1 with purified DJ-1 protein resulted in the inhibition of ASK1 activity (Fig. 4A). By way of comparison, DJ-1 pretreatment had little effect on the enzyme activity of either MKK3 (Fig. 4B) or p38 (Fig. 4C) in vitro. Furthermore, pretreatment with DJ-1 did not directly influence the activities of any other MAPK3's in vitro, including MLK3 and TAK1 (Fig. 4D). Thus, our data indicate that ASK1 was the principal target protein of DJ-1 in the ASK1-MKK3-p38 signaling cascade. Also, DJ-1 was not phosphorylated by ASK1 (data not shown).

DJ-1 INTERACTS PHYSICALLY WITH ASK1 IN INTACT CELLS AND DISRUPTS ASK1 HOMODIMERIZATION

As our results suggested ASK1 is a target of DJ-1, we next investigated whether these two proteins interact physically in intact

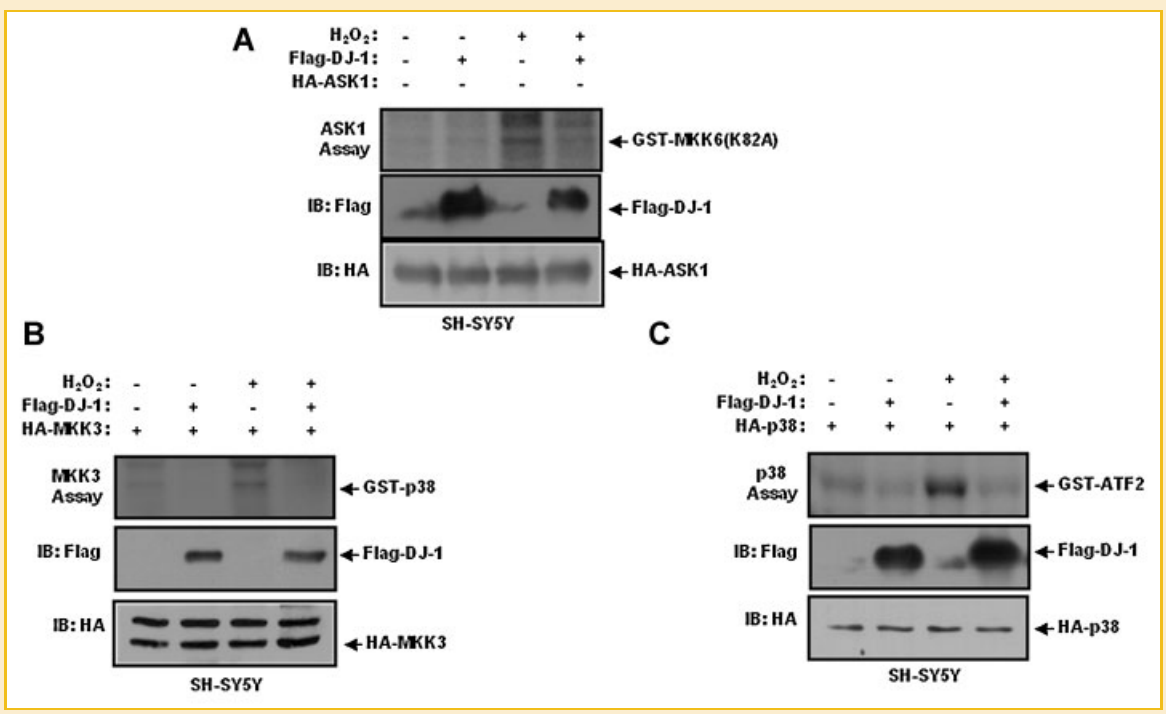

Fig. 3. DJ-1 inhibits downstream components of the ASK1 signaling pathway in human neuroblastoma SH-SY5Y cells. A–C: SH-SY5Y cells were transiently transfected with plasmids expressing pCMV-Flag-DJ-1, pcDNA3-HA-ASK1, pcDNA3-HA-MKK3, and pcDNA3-HA-p38 as indicated. The cells were treated with 2 mM H_2O_2 for 20 min at 37°C, and then assayed for ASK1, MKK3, or p38 activity via immunocomplex kinase assays. The cell lysates were also subjected to immunoblotting analysis with the indicated antibodies. The immunocomplex ASK1, MKK3, or p38 kinase assays were conducted using GST-MKK6(K82A), GST-p38, or GST-ATF2 as the substrate. IB, immunoblot.

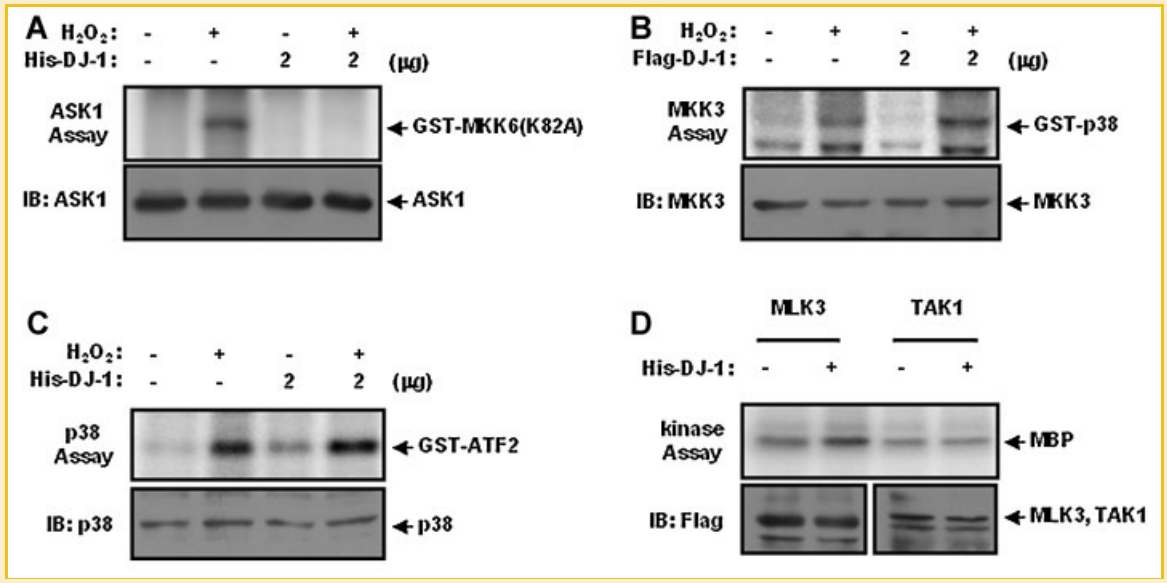

Fig. 4. DJ-1 inhibits ASK1 activity, but not MKK3 and p38 activities in vitro. A–C: HEK293 cells were treated with 2 mM H_2O_2 for 20 min at 37°C, and then subjected to immunoprecipitation using anti-ASK1, anti-MKK3, or anti-p38 antibodies as indicated. D: DJ-1 had no effect on MLK3 and TAK1. The HEK293 cells were transiently transfected with pcDNA3-HA-MLK3 or pcDNA3-HA-TAK1, and then subjected to immunoprecipitation using anti-Flag antibody as indicated. A–D: The immunopellets were incubated with purified DJ-1 protein in 50 μl of HEPES buffer at a pH of 7.4 for 1 h at room temperature, washed twice with HEPES buffer, and then assayed for ASK1, MKK3, or p38 activity using GST-MKK6 (K82A), GST-p38, or GST-ATF2 as the substrates, respectively. The immunocomplex kinase assays for MLK3 and TAK1 were conducted using myelin basic protein (MBP) as a universal substrate. The cell lysates were also subjected to immunoblotting analysis with the indicated antibodies. IB, immunoblot.

cells. Here, HEK293 cells were co-transfected with vectors encoding HA epitope-tagged ASK1 (HA-ASK1) and Flag epitope-tagged wild-type DJ-1, and were then subjected to co-immunoprecipitation analysis (Fig. 5A). Immunoblot analysis using anti-Flag antibody of HA immunoprecipitates from the transfected cells revealed that HA-ASK1 physically associated with Flag-DJ-1 in the cells. Conversely, under conditions identical to those described above, immunoblot analysis of the Flag immunoprecipitates with an anti-HA antibody also showed the interaction between the two proteins (Fig. 5B). We also attempted to characterize the interaction occurring between ASK1 and the pathogenic L166P mutant form of DJ-1. We transfected the cells with a Flag epitope-tagged DJ-1 L166P and HA epitope-tagged ASK1, the interaction between ASK1 and the mutant was much weaker than its interaction with the wild-type DJ-1 (Fig. 5C). The homo-oligomerization of ASK1 is one mechanism for ASK1 activation. Therefore, we examined whether DJ-1 would interfere with ASK1 oligomerization. Here, the HEK293 cells were transfected with Flag-epitope-tagged ASK1 (Flag-ASK1) and HA epitope-tagged ASK1 (HA-ASK1) in the presence or absence of His epitope-tagged DJ-1 (His-DJ-1) and thioredoxin. Co-immunoprecipitation analysis indicated that HA-ASK1 was associated with Flag-ASK1 in the transfected cells. This ASK1 homo-oligomerization was inhibited by co-expression of DJ-1 and thioredoxin (Fig. 5D). These results suggest that DJ-1 may inhibit ASK1 activation, at least in part, by the suppression of ASK1 homo-oligomerization.

Furthermore, we also attempted to confirm the effects of H_2O_2 on the physical association occurring between ASK1 and DJ-1. We found that H_2O_2 stimulates the association between ASK1 and DJ-1 (Fig. 5E). Next, we conducted confocal analysis with immune-fluorescence staining whether ASK1 was co-localized with DJ-1 in the presence and absence of H_2O_2. ASK1 and DJ-1 is located predominantly within the cytoplasm (Fig. 5F). The image overlay revealed the cytoplasm co-localization of ASK1 and DJ-1 in the presence and absence of H_2O_2 (Fig. 5F). These results suggest that H_2O_2 does not significantly regulate the distribution of ASK1 and DJ-1.

DISCUSSION

DJ-1 expression was previously determined to be induced via oxidative stress [Mitsumoto and Nakagawa, 2001; Mitsumoto et al., 2001; Srisomsap et al., 2002]. Thus far, DJ-1 has been implicated in the modulation of several signaling processes associated with the regulation of cell death. A recent report demonstrated that the H_2O_2-quenching ability of DJ-1 may not be sufficient to account for its overall cytoprotective functions [Junn et al., 2005]. Collectively, DJ-1 prevents cell death triggered by a variety of apoptotic stimuli, including H_2O_2, dopamine, UV irradiation, and MPP^+ [Yokota et al., 2003; Taira et al., 2004; Shinbo et al., 2006] by three different mechanisms. Firstly, the loss of DJ-1 function leads to impairments of PI3k/Akt signaling. Secondly, DJ-1 sequesters Daxx within the nucleus and prevents it from gaining access to the cytoplasm, which keeps Daxx from binding to and activating its effector kinase, ASK1. Thirdly, DJ-1 modulates the JNK signaling pathway by negative regulation of MEKK1 via physical interaction. In the modulation of ASK1 signaling by DJ-1, one should consider a Daxx-independent signaling pathway to reveal the role of DJ-1 in the suppression of ASK1 signaling in PD.

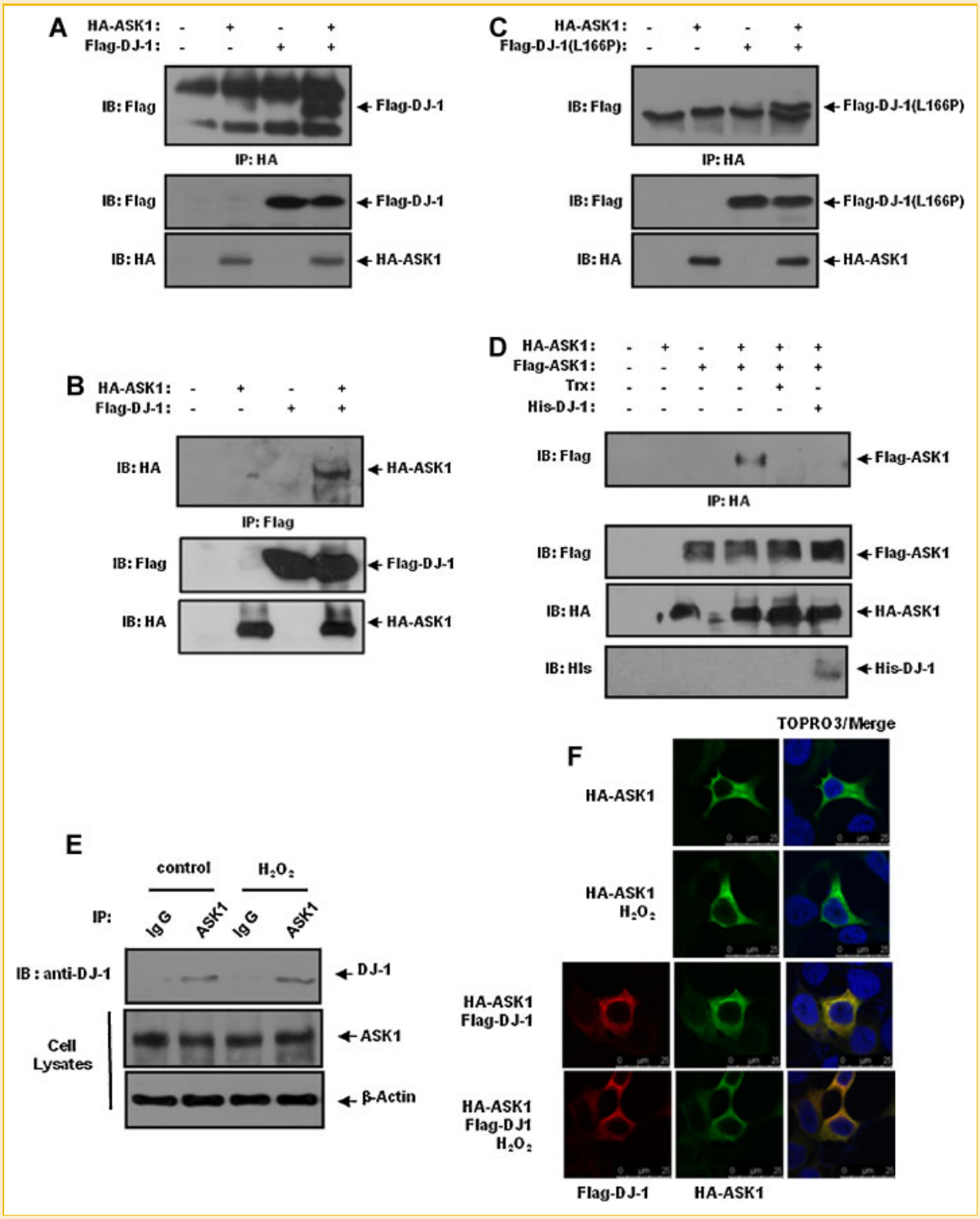

Fig. 5. DJ-1 interacts physically with ASK1 in intact cells and disrupts ASK1 homodimerization. A, B: HEK293 cells were transiently transfected with pcDNA3-HA-ASK1 or pcDNA-Flag-DJ-1. After 48 h of transfection, the cells were lysed and subjected to immunoprecipitation with anti-HA or anti-Flag antibody as indicated. The immunoprecipitates were immunoblotted with anti-Flag or anti-HA antibody. C: HEK293 cells were transiently transfected with pcDNA3-HA-ASK1 or pcDNA-Flag-DJ-1 (L166P). After 48 h of transfection, the cells were lysed and subjected to immunoprecipitation with anti-HA antibody as indicated. The immunoprecipitates were immunoblotted with anti-Flag antibody. A–C: The expressions of ASK1, wild-type DJ-1, or the L166P mutant DJ-1 were analyzed via immunoblotting using anti-HA or anti-Flag monoclonal antibody, respectively. D: HEK293 cells were transiently transfected with expression vectors encoding empty vector, pcDNA3-HA-ASK1, pcDNA3-Flag-ASK1, pcDNA3-thioredoxin, and pcDNA3-His-DJ-1. After 48 h of transfection, the cell lysates were subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitates were then immunoblotted with anti-Flag antibody. The cell lysates were also immunoblotted with anti-Flag, anti-HA, and anti-His antibodies, and subjected to immunoblotting analysis. E: HEK293 cells were treated with 2 mM H_2O_2 for 20 min at 37°C, and then subjected to immunoprecipitation using IgG or anti-ASK1 antibodies as indicated. The immunoprecipitates were immunoblotted with anti-DJ-1 antibody. The expressions of ASK1 or DJ-1were analyzed via immunoblotting using anti-ASK1 or anti-DJ-1 antibody, respectively. F: HEK293 cells were transiently transfected with expression vectors encoding empty vector, pcDNA3-HA-ASK1 and pcDNA3-Flag-DJ-1 and then cells were treated with 2 mM H_2O_2 for 20 min at 37°C. The cells were then fixed and DJ-1 and ASK1 were stained with Alexa 546 (red) and Alexa 488 (green). The overlays are shown in the right panels, and the color yellow denotes co-localization. IB, immunoblot. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

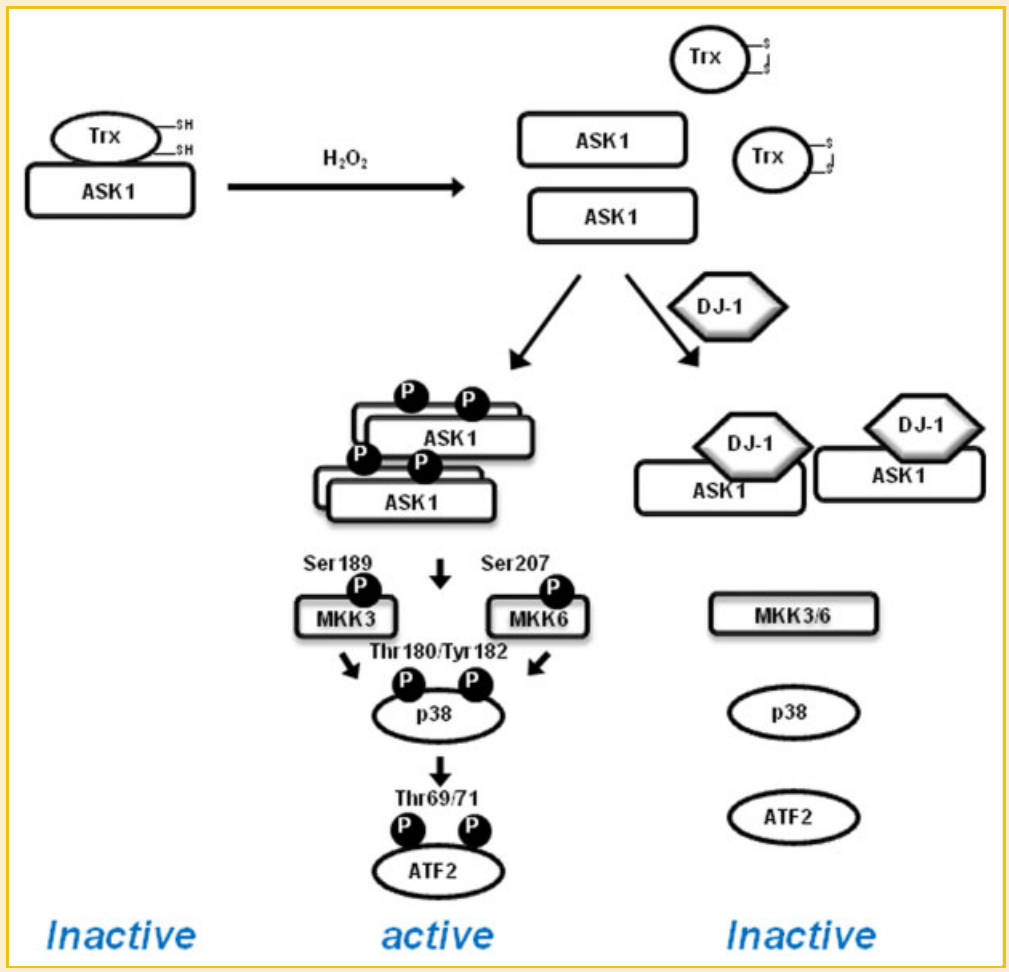

Fig. 6. Model for the role of DJ-1 in the regulation of ASK1 signaling cascade. Oxidative stress affects activation of ASK1 and association with thioredoxin. In the presence of oxidative stress, thioredoxin is dislodged from ASK1–Trx complex and maintained in an active state. Activated ASK1 activates downstream MKK3, MKK6, and ultimately p38 MAPK. This leads to increased activation of the ATF2 transcription factor. In the presence of DJ-1, ASK1 activity was downregulated through physical binding of DJ-1 to ASK1.

In recent work, we determined that DJ-1 physically interacts with MEKK1 and inhibits MEKK1 activation, resulting in the suppression of MEKK1-mediated cell death. Furthermore, here we determined that DJ-1 prevents ASK1 signaling through physical interaction. ASK1 is closely linked to doparminergic neuronal cell death, and DJ-1 is a potent inhibitor of the oxidative stress-induced cell death signaling pathway. These observations indicate that the direct inhibition of ASK1 is an important mechanism by which DJ-1 operates as a negative regulator of the ASK1 signaling pathway (Fig. 6).

As indicated above, the Mouradian group has suggested that DJ-1 may interact with Daxx, sequestering it within the nucleus and preventing it from gaining access to the cytoplasm, thereby blocking Daxx-mediated ASK1 activation [Junn et al., 2005]. Several studies have proposed that dopaminergic neuronal death is increased via the activation of the ASK1 signaling pathway [Cassarino et al., 2000; Saporito et al., 2000; Chun et al., 2001; Xia et al., 2001; Ouyang and Shen, 2006; Pan et al., 2007]. These observations indicate that ASK1 may exert an important effect in the mediation of dopaminergic neuronal death, and that the blockage of ASK1 signaling via specific inhibitors may prevent or effectively retard the progression of PD and other neurodegenerative diseases.

Our data now reveal a new mechanism: wild-type DJ-1 directly interacts with and thereby inhibits ASK1 activation, thus resulting in suppression of the ASK1 signaling pathway. Considering that ASK1 is a MAP3K that participates in the ASK1 signaling cascade, DJ-1's inhibition of ASK1 activation may be an important mechanism by which it inhibits the ASK1 signaling pathway in the progression of PD.

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