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Oxidative Status of DJ-1-dependent Activation of Dopamine Synthesis through Interaction of Tyrosine Hydroxylase and 4-Dihydroxy-L-phenylalanine (L-DOPA) Decarboxylase with DJ-1*[S]

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Shizuma Ishikawa[‡], Takahiro Taira[§], Takeshi Niki[¶], Kazuko Takahashi-Niki[¶], Chinatsu Maita[‡], Hiroshi Maita[¶], Hiroyoshi Ariga[¶1], and Sanae M. M. Iguchi-Ariga[‡]

From the [‡]Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, the [§]Interdisciplinary Graduate School of Medicine and Engineering, Yamanashi University, Chuoh 409-3898, and the [¶]Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo 060-0812, Japan

Parkinson disease (PD) is caused by loss of dopamine, which is synthesized from tyrosine by two enzymes, tyrosine hydroxylase (TH) and 4-dihydroxy-L-phenylalanine decarboxylase (DDC). *DJ-1* is a causative gene for the familial form of PD, but little is known about the roles of DJ-1 in dopamine synthesis. In this study, we found that DJ-1 directly bound to TH and DDC and positively regulated their activities in human dopaminergic cells. Mutants of DJ-1 found in PD patients, including heterozygous mutants, lost their activity and worked as dominant-negative forms toward wild-type DJ-1. When cells were treated with H_2O_2, 6-hydroxydopamine, or 1-methyl-4-phenylpyridinium, changes in activities of TH and DDC accompanied by oxidation of cysteine 106 of DJ-1 occurred. It was found that DJ-1 possessing Cys-106 with SH and SOH forms was active and that DJ-1 possessing Cys-106 with SO_2H and SO_3H forms was inactive in terms of stimulation of TH and DDC activities. These findings indicate an essential role of DJ-1 in dopamine synthesis and contribution of DJ-1 to the sporadic form of PD.

Parkinson disease (PD)[2] is a neurodegenerative disease that occurs by reduction of the dopamine level through dopaminergic cell death in the substantia nigra. Genetic and environmental factors are thought to be triggers for the onset of PD, but the precise molecular mechanisms are still not known. Dopamine is synthesized by the following two steps: tyrosine is converted to L-DOPA by tyrosine hydroxylase (TH) and then L-DOPA is converted to dopamine by L-dopa decarboxylase (DDC). TH is therefore a key enzyme for dopamine synthesis and is used as a marker for dopaminergic neurons.

DJ-1 was first identified by our group as a novel candidate of the oncogene that transformed mouse NIH3T3 cells in cooperation with activated *ras* (1). Deletion and point (L166P) mutations of DJ-1 have been shown to be responsible for onset of familial Parkinson disease, PARK7 (2), and other homozygous and heterozygous mutations of DJ-1 have been identified in patients with familial or sporadic Parkinson disease (3–6). DJ-1 is a multifunctional protein and plays roles in transcriptional regulation and anti-oxidative stress function, and loss of its functions is thought to lead to the onset of Parkinson disease and cancer. DJ-1 has three cysteine residues at positions 46, 53, and 106 (Cys-46, Cys-53, and Cys-106, respectively), and these cysteine residues are oxidized after cells receive oxidative stress, resulting in scavenging of reactive oxidative species (7–12). Although DJ-1 does not directly bind to DNA, DI-1 acts as a co-activator to activate various transcription factors, including the androgen receptor and p53 tumor suppressor, PSF and Nrf2, by sequestering their inhibitory factors (13–18). The anti-oxidative stress function of DJ-1 is therefore thought to be carried out both by self-oxidation of cysteine residues and by activation of redox-related genes.

It has been reported that PSF, a transcription repressor, binds to the promoter region of the *TH* gene to repress its expression and that human DJ-1 binds to PSF to sequester the PSF/co-repressor complex, leading to induction of *TH* gene expression in cultured human cells (16). We have shown that injection of DJ-1 into the substantia nigra of PD model rats, in which dopaminergic neuronal cell death was induced by administration of 6-hydroxydopamine, prevented cell death and locomotion defect, and restored dopamine metabolism of the rats, suggesting that DJ-1 affects dopamine biosynthesis (11). However, it has not been clarified whether DJ-1 regulates the expression of genes or activity of proteins that are related to dopamine biosynthesis.

In this study, we found that DJ-1 directly bonds to TH and DDC to stimulate their activities. Mutants of DJ-1 found in PD patients lost their stimulating activity to TH and DDC, and stimulation occurred in a manner dependent on the oxidative state of Cys-106 of wild-type DJ-1. These findings indicate an

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[1] To whom correspondence should be addressed. Tel.: 81-11-706-3745; Fax: 81-11-706-4988; E-mail: hiro@pharm.hokudai.ac.jp.

[2] The abbreviations used are: PD, Parkinson disease; H_2O_2, hydrogen peroxide; TH, tyrosine hydroxylase; DDC, L-DOPA decarboxylase; RT-PCR, reverse transcription-PCR; GST, glutathione *S*-transferase; 6-OHDA, 6-hydroxydopamine; MPP^+, 1-methyl-4-phenylpyridinium; L-DOPA, 4-dihydroxy-L-phenylalanine; GPDH, glycerol-3-phosphate dehydrogenase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; HPLC, high pressure liquid chromatography.

essential role of DJ-1 in dopamine synthesis and contribution of DJ-1 to the sporadic form of PD.

EXPERIMENTAL PROCEDURES

Cells—Establishment of SH-SY5Y cells with knocking down of the *DJ-1* gene and SH-SY5Y cells harboring a vector was described previously (19). Human SH-SY5Y cells and these cell lines were cultured in Dulbecco's modified Eagle's medium with 10% calf serum.

Establishment of Cell Lines Harboring Wild-type DJ-1 or Mutants of DJ-1—Plasmid pcDNA3 containing FLAG-tagged wild-type or mutant DJ-1s was transfected into SH-SY5Y cells by the calcium phosphate precipitation method, and the cells were cultured in a medium in the presence of 100 μg/ml G418 for 14 days. Cells resistant to the drug were then selected, and expression of FLAG-DJ-1 was examined by Western blotting with an anti-FLAG antibody (M2, Sigma).

RT-PCR and Real Time-PCR—Nucleotide sequences of the oligonucleotide used for RT-PCR and real time primers were as follows: human DJ-1 sense (RT-PCR) 5′-GGTGCAGGCTTG-TAAACATATAAC-3′ and human DJ-1 antisense (RT-PCR) 5′-CTCTAAGTGATCGTCGCAGTTCGC-3′; human TH sense (RT-PCR) 5′-CGGGTCTCTAGATGGTGGATTTT-3′ and human TH antisense (RT-PCR) 5′-GCTGTGGCCTTT-GAGGAGAA-3′; human DDC sense (RT-PCR) 5′-CTG-GAGACTGTGATGATGGA-3 and human DDC antisense (RT-PCR) 5′-GCAAACTCCACTCCATTCA-3; human β-actin sense (RT-PCR) 5′-CCGACAGGATGCAGAAGGAG-3′ and human β-actin antisense (RT-PCR) 5′-GTGGGGTG-GCTTTTAGGATG-3′; human DJ-1 sense (real time-PCR) 5′-TTGTAGGCTGAGAAATCTCTGTG-3′ and human DJ-1 antisense (real time-PCR) 5′-ATCCATTCTCACTGTGT-TCGC-3′; human TH sense (real time-PCR) 5′-GCAGGCA-GAGGCCATCATGT-3′ and human TH antisense (real time-PCR) 5′-GGCGATCTCAGCAATCAGCT-3′; human DDC sense (real time-PCR) 5′-GGGAAGTGCCAGTGAAGCCA-3′ and human DDC antisense (real time-PCR) 5′-GAAGTTGC-CATCTGAGGGG-3′; and human β-actin sense (real time-PCR) 5′-CCCTAAGGCCAACCGRGAAA-3′ and human β-actin antisense (real time-PCR): 5′-ACGACCAGAG-GCATACAGGGA-3′. Total RNAs were prepared from cells and subjected to semi-quantitative RT-PCR and real time-PCR analyses as described previously (20).

Western Blotting—To examine the expression levels of proteins in cells, proteins were extracted from cells with a buffer containing 150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.5% Nonidet P-40. Proteins were then separated on a 12.5% polyacrylamide gel containing SDS and subjected to Western blotting with respective antibodies. Proteins on the membrane were reacted with an IRDye800 (Rockland, Philadelphia, PA) or Alexa Fluor 680-conjugated secondary antibody (Molecular Probes, Eugene, OR) and visualized by using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE). The antibodies used were anti-TH (1:1000, Chemicon, Temecula, CA), anti-DDC (1:500, Sigma), anti-actin (1:4000, Chemicon), anti-phosphorylated TH with a serine residue at amino acid number 19 (1:500, Exalpha Biologicals, Watertown, MA), and anti-DJ-1 (1:4000) antibodies. The anti-DJ-1 antibody was prepared by us as described previously (1).

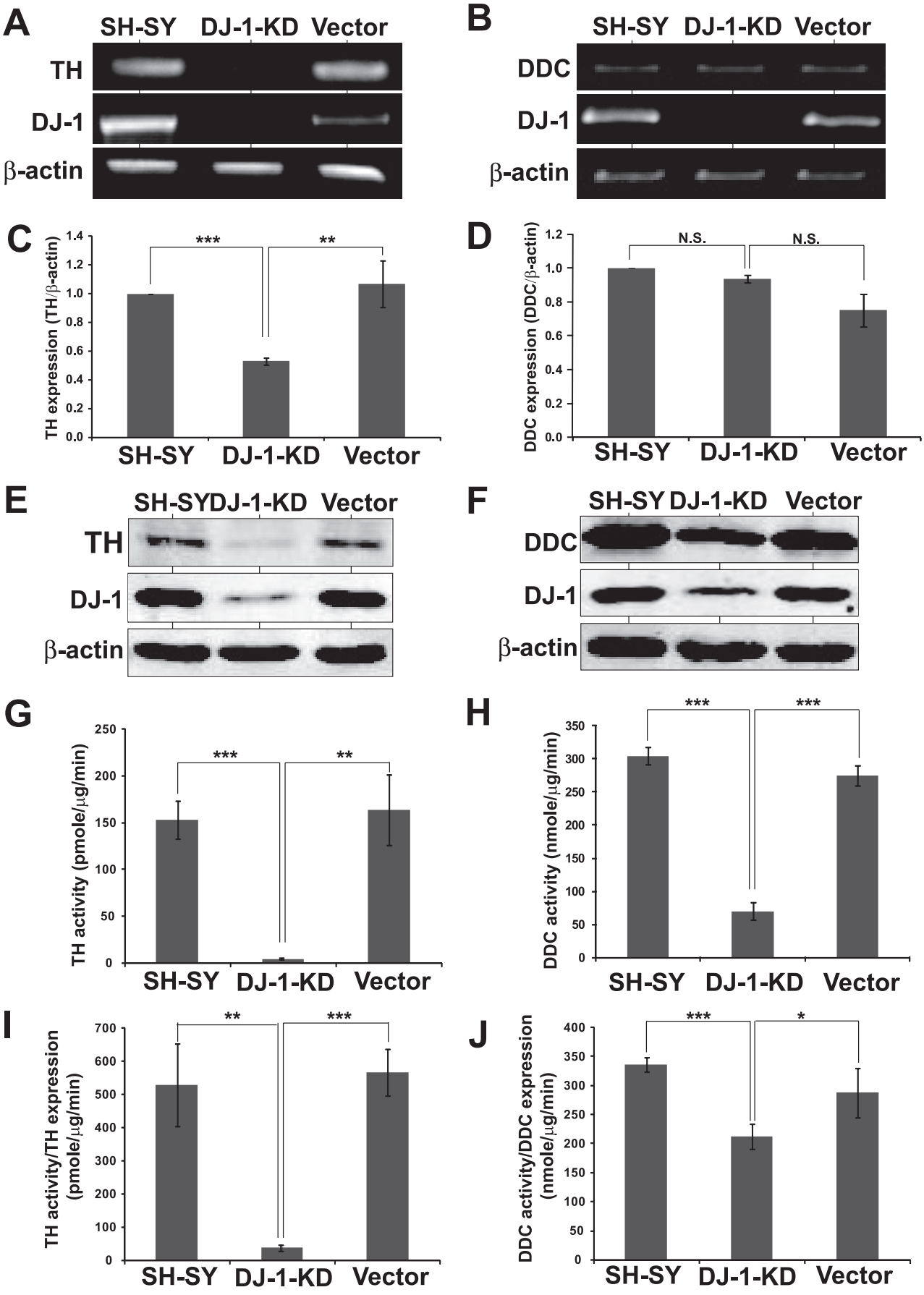
Pulldown Assay—^{35}S-Labeled TH and DDC were synthesized *in vitro* using the reticulocyte lysate of the TnT transcription-translation coupled system (Promega, Madison, WI). Labeled proteins were mixed with GST or GST-DJ-1 expressed in and prepared from *Escherichia coli* at 4 °C for 60 min in a buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5), 0.05% bovine serum albumin, and 0.1% Nonidet P-40. After washing with the same buffer, the bound proteins were separated in a 10% polyacrylamide gel containing SDS and visualized by fluorography.

In Vivo Binding Assay—Proteins were extracted from SH-SY5Y cells by the procedure described previously (13). Proteins were immunoprecipitated with an anti-DJ-1 antibody (1:500) or normal IgG and the precipitates were analyzed by Western blotting with an anti-TH antibody, anti-DDC antibody, or the mouse anti-DJ-1 antibody (1:2000, 3E8, MBL, Nagoya, Japan) as described above.

Luciferase Activity—The promoter region of the human *TH* gene was amplified by RT-PCR using specific primers and total RNA from human SH-SY-5Y cells as templates. Nucleotide sequences of oligonucleotides used for PCR primers are as follows: human TH sense 5′-TCAGAACCTCAGTCCTCG-CATC-3′ and human TH antisense 5′-ggagatctCAACAGG-GACTCAAACACCAGG-3′. Amplified cDNA containing 3416 bp of the human *TH* gene was digested with KpnI and BglII, and fragments obtained were inserted into KpnI and BglII sites of pGL-3 Basic (Promega). This plasmid was named pTH-Luc. SH-SY5Y cells in a 24-well dish were transfected with 0.2 μg of pTH-Luc and various amounts (0–0.2 μg) of pcDNA3-FLAG-DJ-1 together with 25 ng of pCMV-β-gal by the calcium phosphate method (21). Two days after transfection, whole cell extract was prepared by addition of Triton X-100-containing solution from the Pica gene kit (Wako Pure Chemicals, Osaka, Japan) to the cells. About a one-fifth volume of the extract was used for the β-galactosidase assay to normalize the transfection efficiencies as described previously (22), and the luciferase activity due to the reporter plasmid was determined using a luminometer (Luminocounter Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). Proteins in aliquots of the cell extract were analyzed by Western blotting with an anti-FLAG antibody (M2, Sigma) and visualized as described above. The same experiments were repeated three times.

Activities of Tyrosine Hydroxylase and L-Dopa Decarboxylase in Cells—TH and DDC activities in cells were measured according to the published method using HPLC (16, 23). The HPLC system and column used were AKTA explorer 10 S/100 (GE Healthcare), COSMOSIL Cholester Waters (4.6 × 150 mm, Nacalai Tesque, Kyoto, Japan), and the buffer used was a buffer containing 50 mM potassium phosphate (pH 2.6), 0.1 mM EDTA, 0.2 mM heptane sulfonic acid, and 10% methanol.

Assays for TH and DDC Activities in Vitro—Proteins were extracted from SH-SY5Y cells with a buffer containing 150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.5% Nonidet P-40. Twenty μg of proteins was reacted with 100 mM L-tyrosine, 500 μM L-DOPA decarboxylase inhibitor (3-hydroxyben-


A
SH-SY
DJ-1-KD
Vector
TH
DJ-1
β-actin
B
SH-SY
DJ-1-KD
Vector
DDC
DJ-1
β-actin
C
TH expression (TH/β-actin)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
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SH-SY
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Vector
D
DDC expression (DDC/β-actin)
1.2
1.0
0.8
0.6
0.4
0.2
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SH-SY
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TH activity (pmole/μg/min)
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TH activity/TH expression (pmole/μg/min)
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DDC activity/DDC expression (nmole/μg/min)
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SH-SY
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Vector

zylhydrazine dihydrochloride, Acros Organics) for TH, or 250 mM L-DOPA (4-dihydroxy-L-phenylalanine) for DDC, and various amounts of GST-DJ-1 at 37 °C for 15 min, and the reaction was stopped by addition of 0.1 N perchloric acid. After centrifugation of the mixture at 12,000 rpm for 30 min, the supernatant was passed through a 0.45-μm pore size filter (Cosmonice filter W, Nacalai Tesque, Japan) and applied to HPLC as described above.

Identification of the Oxidative States of Cys-106 of DJ-1 by MALDI-TOF/TOF-MS Analysis—Cell extracts from SH-ST5Y cells were prepared as described above and immunoprecipitated with a rabbit anti-DJ-1 polyclonal antibody (final concentration of 4 μg/ml), and immunoprecipitates were separated on a 12.5% polyacrylamide gel. After the gel had been stained with Coomassie Brilliant Blue, a band corresponding to DJ-1 was cut out, reduced, alkylated with a buffer containing iodoacetamide, and digested with trypsin. The peptide solutions were desalted, mixed with α-cyano-4-hydroxycinnamic acid, and applied onto a target plate. MS/MS spectra of the Cys-106-containing peptide spanning 100–122 amino acids were obtained using an Ultraflex (Brucker Daltonics) in reflector mode and analyzed with flex analysis software (Brucker Daltonics). Protein identification was carried out with Mascot software using the National Institute of Biomedical Innovation data base.

Indirect Immunofluorescence—Cells were fixed with 4% paraformaldehyde and reacted with rabbit anti-DJ-1 polyclonal (1:100), mouse anti-TH monoclonal (1:100), and mouse anti-DDC monoclonal antibodies (1:100). The cells were also stained with 4′,6-diamidino-2-phenylindole. The cells were then reacted with a rhodamine-conjugated anti-rabbit IgG or an fluorescein isothiocyanate-conjugated anti-mouse IgG and observed under a Bio-Imaging system (Olympus, FSV100, Tokyo, Japan).

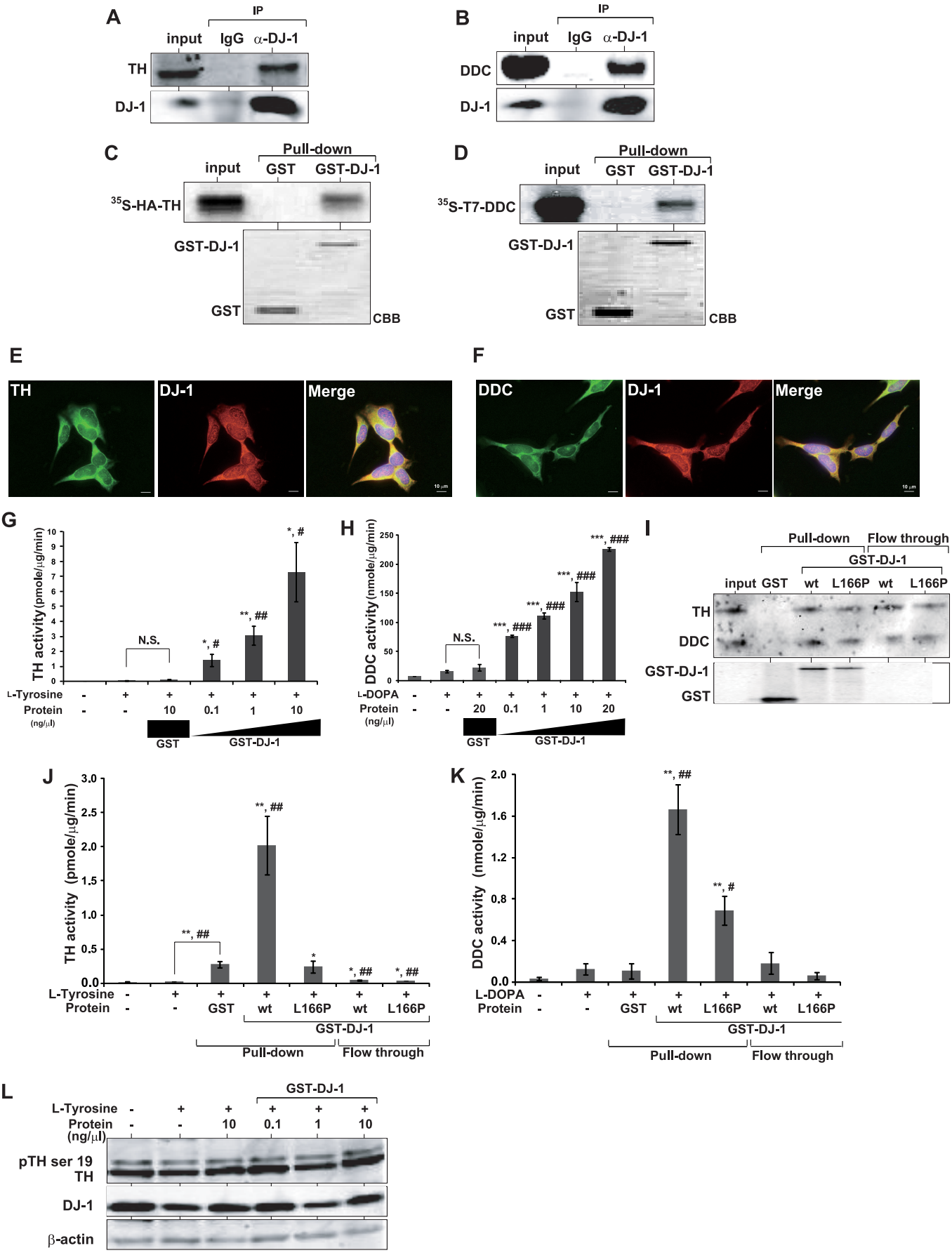
Statistical Analyses—Data are expressed as means ± S.E. Statistical analyses were performed using one-way analysis of variance followed by unpaired Student's *t* test.

RESULTS

Effects of DJ-1 Knockdown on Expression and Activity of TH and DDC—To examine the effects of DJ-1 on expression and activity of TH and DDC, human neuroblastoma SH-SY5Y cells, in which the *DJ-1* gene was knocked down, were established by transfection of a vector containing short hairpin RNA targeting human DJ-1, as described previously (19), and were named "DJ-1 knockdown cells" (19). SH-SY5Y cells harboring the vector without short hairpin RNA were also established and named "Vector cells." Expression levels of DJ-1 and TH mRNAs in parental SH-SY5Y, DJ-1 knockdown and vector cells were examined by semi-quantitative RT-PCR (Fig. 1). β-Actin was used as a loading control. As reported previously (16), the level of TH mRNA in DJ-1 knockdown cells was significantly reduced after DJ-1 expression had been knocked down by short hairpin RNA to ~30% to the level of DJ-1 in parental SH-SY5Y or Vector cells (Fig. 1*A*). To confirm this, quantitative RT-PCR analyses (real time-PCR) were carried out, and results showing 50% reduction of *TH* gene expression in DJ-1 knockdown cells were obtained (Fig. 1*C*). On the other hand, RT-PCR and real time-PCR analyses showed that expression of the *DDC* gene in DJ-1 knockdown cells was not reduced (Fig. 1, *B* and *D*, respectively). Corresponding to the mRNA expression levels of *TH* and *DDC* genes, the protein levels of TH and DDC were found to be reduced and not to be changed, respectively, in DJ-1 knockdown cells by Western blot analyses (Fig. 1, *E* and *F*). TH and DDC activities in DJ-1 knockdown cells were then examined. Although reduced TH activity was observed, DDC activity was also found to be reduced (Fig. 1, *G* and *H*). The same results as those for SH-SY5Y cells were obtained when human SK-N-SH cells were used (data not shown). To clarify whether inhibition of TH and DDC activities in DJ-1 knockdown cells was caused by reduced expression or by modification of enzymes, TH and DDC activities were divided by the expression levels of TH and DDC proteins (Fig. 1, *I* and *J*, respectively). The results showed that TH and DDC activities are reduced in DJ-1 knockdown cells, suggesting that a mechanism other than transcriptional regulation of the *DDC* gene by DJ-1 is responsible for the reduced activity of DDC in DJ-1 knockdown cells.

Positive Regulation of TH and DDC Activities by DJ-1 through Direct Interactions—Proteins extracted from SH-SY5Y cells were immunoprecipitated with an anti-DJ-1 antibody and nonspecific IgG, and precipitates were analyzed by Western blotting with anti-TH and anti-DDC antibodies (Fig. 2). The results showed that TH and DDC were co-immunoprecipitated with DJ-1, indicating complex formation of DJ-1 with TH and with DDC (Fig. 2, *A* and *B*). To determine whether the interaction of DJ-1 with TH and DDC is direct or indirect, pulldown assays were carried out. For these assays, purified GST-DJ-1 or GST alone was reacted with ^{35}S-labeled TH or DDC that had been synthesized *in vitro* using a coupled transcription-translation system. The results clearly showed that GST-DJ-1, but not GST, bound to TH and DDC, indicating direct interaction of DJ-1 with TH and with DDC (Fig. 2, *C* and *D*). SH-SY5Y cells were then immunostained with anti-DJ-1 and anti-TH antibodies or with anti-DJ-1 and anti-DDC antibodies, and DJ-1 and TH or DDC were visualized by fluorescein isothiocyanate- and rhodamine-conjugated secondary antibodies. The results of merged images showed co-localization of DJ-1 with TH and with DDC in cells (Fig. 2, *E* and *F*). It was found that fluores-

FIGURE 1. **Effects of DJ-1 knockdown on expression and activity of TH and DDC.** SH-SY5Y, SH-SY5Y cells with knockdown of DJ-1 expression (DJ-1 knockdown cells), and SH-SH5Y cells harboring pcDNA3 vector (Vector cells) were used. *DJ-1-KD* and *Vector* in the figures indicate DJ-1 knockdown cells and vector cells, respectively. *A* and *B*, expression levels of mRNA of TH (*A*), DDC (*B*), and DJ-1 were analyzed by RT-PCR using specific primers to respective genes and total RNAs as templates as described under "Experimental Procedures." β-Actin was used as a loading control. *C* and *D*, expression levels of mRNA of TH (*C*) and DDC (*D*) were analyzed by real time-PCR as described under "Experimental Procedures." Relative expression of TH and DDC to β-actin is presented. *E* and *F*, expression levels of TH (*E*) and DDC (*F*) and DJ-1 in cells were analyzed by Western blotting with anti-TH, anti-DDC, and anti-DJ-1 antibodies, respectively, as described under "Experimental Procedures." β-Actin was used as a loading control. *G* and *H*, enzyme activity of TH (*G*) and DDC (*H*) in cells was examined as described under "Experimental Procedures." *I* and *J*, enzyme activity of TH (*I*) and DDC (*J*) in *G* and *H* was divided by the expression level of TH and DDC described in *E* and *F*, respectively. Values are mean S.E. ± from three independent experiments. Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *N.S.* means nonspecific.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IP
input
IgG
α-DJ-1
TH
DJ-1
B
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input
IgG
α-DJ-1
DDC
DJ-1
C
Pull-down
input
GST
GST-DJ-1
35S-HA-TH
GST-DJ-1
GST
CBB
D
Pull-down
input
GST
GST-DJ-1
35S-T7-DDC
GST-DJ-1
GST
CBB
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TH
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TH activity (pmole/μg/min)
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L-Tyrosine
Protein (ng/μl)
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DDC activity (nmole/μg/min)
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L-DOPA
Protein (ng/μl)
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Pull-down
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GST-DJ-1
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GST
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L166P
wt
L166P
TH
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TH activity (pmole/μg/min)
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*, ##
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L-Tyrosine
Protein
GST
wt
L166P
wt
L166P
GST-DJ-1
Pull-down
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DDC activity (nmole/μg/min)
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L-DOPA
Protein
GST
wt
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L166P
GST-DJ-1
Pull-down
Flow through
L
GST-DJ-1
L-Tyrosine
Protein (ng/μl)
10
0.1
1
10
pTH ser 19
TH
DJ-1
β-actin

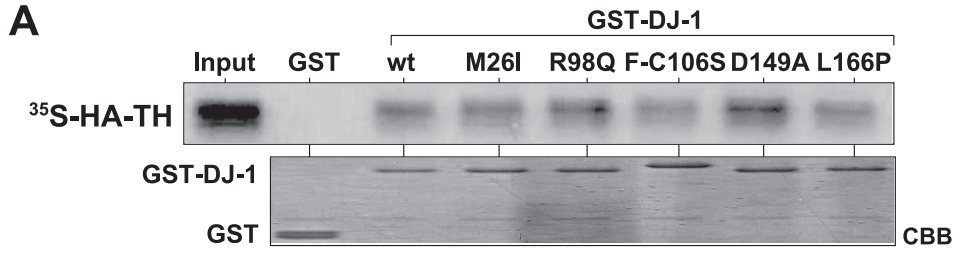
cence patterns were completely or almost completely matched between DJ-1 and TH and between DJ-1 and DDC, respectively, indicating co-localization of DJ-1 with TH and with DDC. To examine a ternary complex among DJ-1, TH, and DDC, FLAG-tagged TH was transfected into SH-SY5Y cells. Forty eight h after transfection, proteins extracted from cells were immunoprecipitated with an agarose-conjugated anti-FLAG antibody or with IgG, and half of the precipitates were analyzed by Western blotting with anti-FLAG, anti-DJ-1, and anti-DDC antibodies. The other half of the precipitates was eluted with a FLAG peptide, and proteins eluted were further immunoprecipitated with an anti-DJ-1 antibody followed by Western blotting (supplemental Fig. 1*A*). Both DDC and DJ-1 were immunoprecipitated with the anti-FLAG antibody, indicating that FLAG-TH was associated either with DDC or with DJ-1. Double immunoprecipitation of proteins with anti-FLAG and anti-DJ-1 antibodies also precipitated DDC and DJ-1, indicating a ternary complex among DJ-1, TH, and DDC in SH-SY5Y cells. Pulldown assays in which purified GST-TH or GST alone was reacted with ^{35}S-labeled DDC that had been synthesized *in vitro* were carried out to examine the direct interaction of TH with DDC. The results clearly showed that GST-TH, but not GST, bound to DDC, indicating direct interaction of TH with DDC (supplemental Fig. 1*B*).

We then examined the effect of DJ-1 on TH and DDC activities. To do that, purified GST-DJ-1 and GST were reacted with proteins extracted from SH-SY5Y cells, which are enzyme sources for TH and DDC, in the presence of tyrosine or L-DOPA, substrates for TH and DDC, respectively, and the quantity of L-DOPA or dopamine was measured (Fig. 2, *G* and *H*). The results clearly showed that GST-DJ-1, but not GST, stimulated TH and DDC activities in a dose-dependent manner. To determine the specificity of this reaction, protein extracts were first reacted with an anti-TH antibody, anti-DDC antibody, or IgG, and TH and DDC activities were then measured in the presence of purified GST-DJ-1. The results clearly showed that the anti-TH or anti-DDC antibody, but not IgG, almost completely inhibited the enzyme activities (supplemental Fig. 2, *A* and *B*). When proteins extracted from DJ-1 knockdown SH-SY5Y cells were used as enzyme sources, TH and DDC activities lower than those using proteins from parental SH-SY5Y cells were observed in the presence of various concentrations of GST-DJ-1, suggesting that, in addition to exogenously added GST-DJ-1, DJ-1 present in protein extracts from cells also affected enzyme activities of TH and DDC (supplemental Fig. 2, *C* and *D*). To distinguish the effect of DJ-1 on activities of DJ-1-bound TH/DDC or DJ-1-unbound TH/DDC, proteins extracted from SH-SY5Y cells were mixed with GST, GST-DJ-1, or GST-L166P-DJ-1 and applied to glutathione-Sepharose columns. DJ-1-bound (pull-down) and DJ-1-unbound proteins (flow-through) were then separated (Fig. 2*I*). Almost all of the GST-DJ-1 and GST-L166P-DJ-1 were found to bind to glutathione-Sepharose columns under this condition, and almost equal amounts of TH or DDC were obtained as DJ-1-bound and DJ-1-unbound forms. When activities of DJ-1-bound and DJ-1-unbound forms of TH and DDC were measured, it was found that wild-type DJ-1-bound TH and DDC gave high activities but that L166P DJ-1-bound TH and DDC or DJ-1-unbound TH and DDC gave low or no activities, respectively (Fig. 2, *J* and *K*). These results clearly show that direct binding of DJ-1 to TH and DDC stimulates their activities.

As it is known that the activity of TH is affected by its phosphorylation, the phosphorylation level of TH at serine 19 in the reaction mixture used in the experiment for which results are shown in Fig. 2*G* was examined. No change in the phosphorylation level of TH was found, suggesting that DJ-1 stimulates TH activity without modulation of TH protein (Fig. 2*L*).

Effect of Mutations of DJ-1 on TH and DDC Activities—Because several mutations in the *DJ-1* gene have been found in PD patients whose TH or DDC activity is supposed to be reduced, we examined the effects of mutations of DJ-1 on TH and DDC activities. M26I, E64D, and L166P DJ-1 are derived from homozygous mutations, and R98Q and D149A DJ-1 are from heterozygous mutations of the *DJ-1* gene. Because oxidation of DJ-1 at Cys-106 is important for DJ-1 to exert its functions (24), C106S DJ-1 was also used. We first examined binding activities of these DJ-1 mutants to TH or DDC by *in vitro* pulldown assays. All of the mutants and wild-type DJ-1 were found to bind to TH and DDC, although there were some variations in binding activity of DJ-1 mutants to TH and DDC (Fig. 3, *A* and *B*). Effects of DJ-1 mutants on TH and DDC activities were then examined using the *in vitro* system. Although wild-type DJ-1 stimulated TH activity, activity to TH was not stimulated by any of the mutants (Fig. 3*C*, see lanes in which 20 ng of GST-mutant DJ-1 was added to mixtures). Furthermore, all of the DJ-1 mutants inhibited activity that had been obtained by wild-type DJ-1 in a dose-dependent manner. It is notable that when the same amount of mutant DJ-1 as that of wild-type DJ-1 was

FIGURE 2. **Stimulation of TH and DDC activities by DJ-1.** *A* and *B*, proteins extracted from SH-SY5Y cells were immunoprecipitated (*IP*) with a rabbit anti-DJ-1 polyclonal antibody or IgG, and precipitates were analyzed by Western blotting with an anti-TH (*A*) or anti-DDC (*B*) antibody and a mouse anti-DJ-1 monoclonal antibody (3E8, MBL, Nagoya, Japan). *C* and *D*, GST or GST-DJ-1 was expressed in and prepared from *E. coli* and reacted with ^{35}S-labeled HA-TH (*C*) or T7-DDC (*D*) that had been synthesized *in vitro* using a coupled transcription-translation system (Promega). *CBB*, Coomassie Brilliant Blue. After the reaction, the mixture was subjected to pulldown assays as described under "Experimental Procedures." *E* and *F*, SH-SY5Y cells were fixed with 4% paraformaldehyde and reacted with anti-TH and anti-DJ-1 antibodies (*E*) and with anti-DJ-1 and anti-DDC antibodies (*F*). After cells had been reacted with fluorescein isothiocyanate- or rhodamine-conjugated secondary antibody, proteins were visualized using a Bio-imaging system (Olympus, FSV100). *G*, cell extracts were prepared from SH-SY5Y cells, and 100 mM L-tyrosine, 500 μM L-DOPA decarboxylase inhibitor (3-hydroxybenzylhydrazine dihydrochloride), and various amounts of GST-DJ-1 were added. After reaction at 37 °C for 10 min, TH activity was measured as described under "Experimental Procedures." *N.S.* means nonspecific. *H*, cell extracts were prepared from SH-SY5Y cells and 250 mM L-DOPA, and various amounts of GST-DJ-1 were added. After reaction at 37 °C for 10 min, DDC activity was measured as described under "Experimental Procedures." *I–K*, 500 μg of proteins extracted from SH-SY5Y was applied to 200 ng of GST-DJ-1. *wt*, wild type. After reaction at 4 °C for 120 min, the mixture was applied to glutathione-Sepharose 4B, and proteins that bound to GST-DJ-1 and proteins that did not bind to GST-DJ-1 were obtained. An aliquot of these proteins was analyzed by Western blotting with anti-TH and anti-DDC antibodies (*I*), and the other aliquots were used to measure TH and DDC activities as described under "Experimental Procedures" (*J* and *K*, respectively). *L*, proteins in reaction mixtures for TH activity as shown in *G* were analyzed by Western blotting with anti-phosphorylated TH with a serine residue at amino acid number 19 (*pTH ser 19*), anti-TH, anti-DJ-1, and anti-β-actin antibodies. Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with an amount of "lysate + substrate," and #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with the amount of "lysate + substrate + GST."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Input
GST
wt
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F-C106S
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L166P
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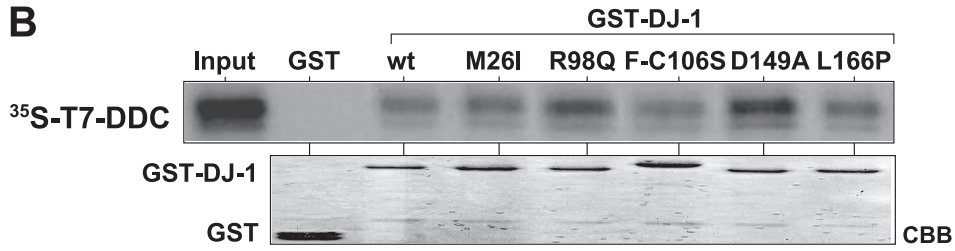
GST
CBB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Input
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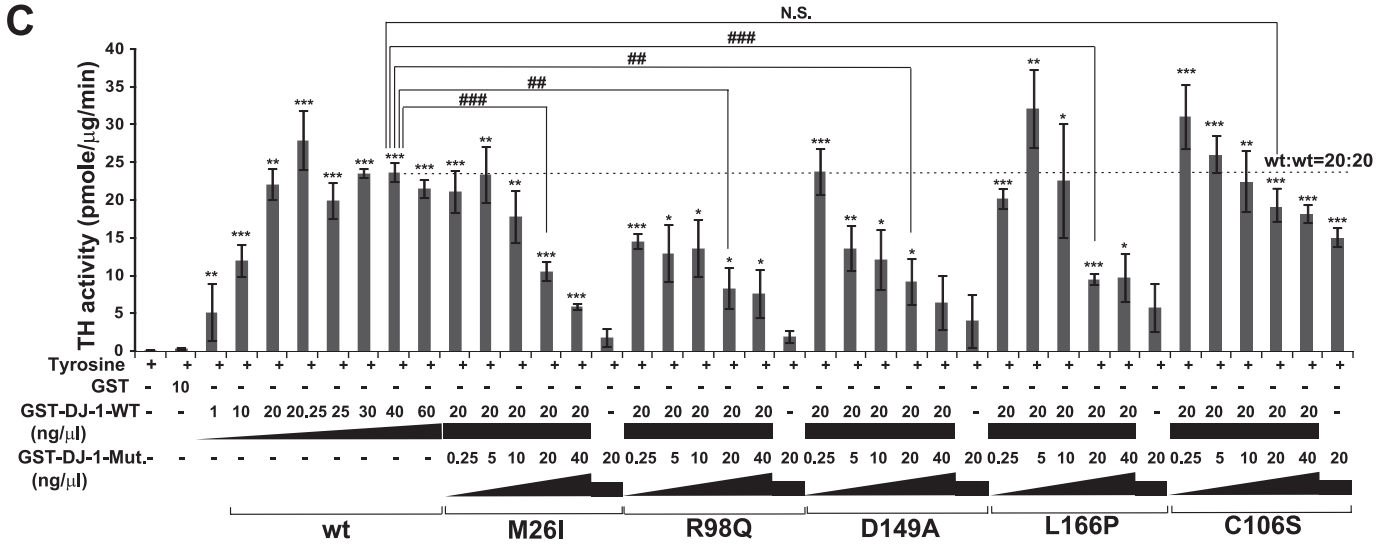
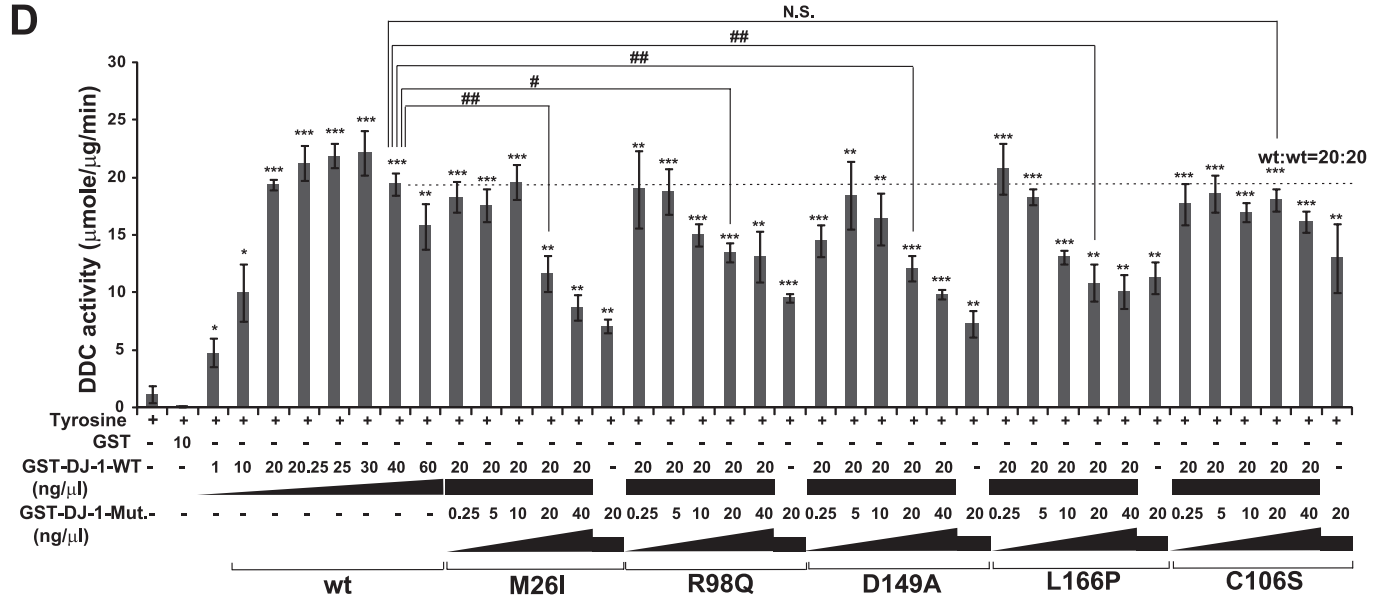

C
N.S.
###
##
##
###
wt:wt=20:20
TH activity (pmole/μg/min)
Tyrosine
GST
GST-DJ-1-WT (ng/μl)
GST-DJ-1-Mut. (ng/μl)
wt
M26I
R98Q
D149A
L166P
C106S

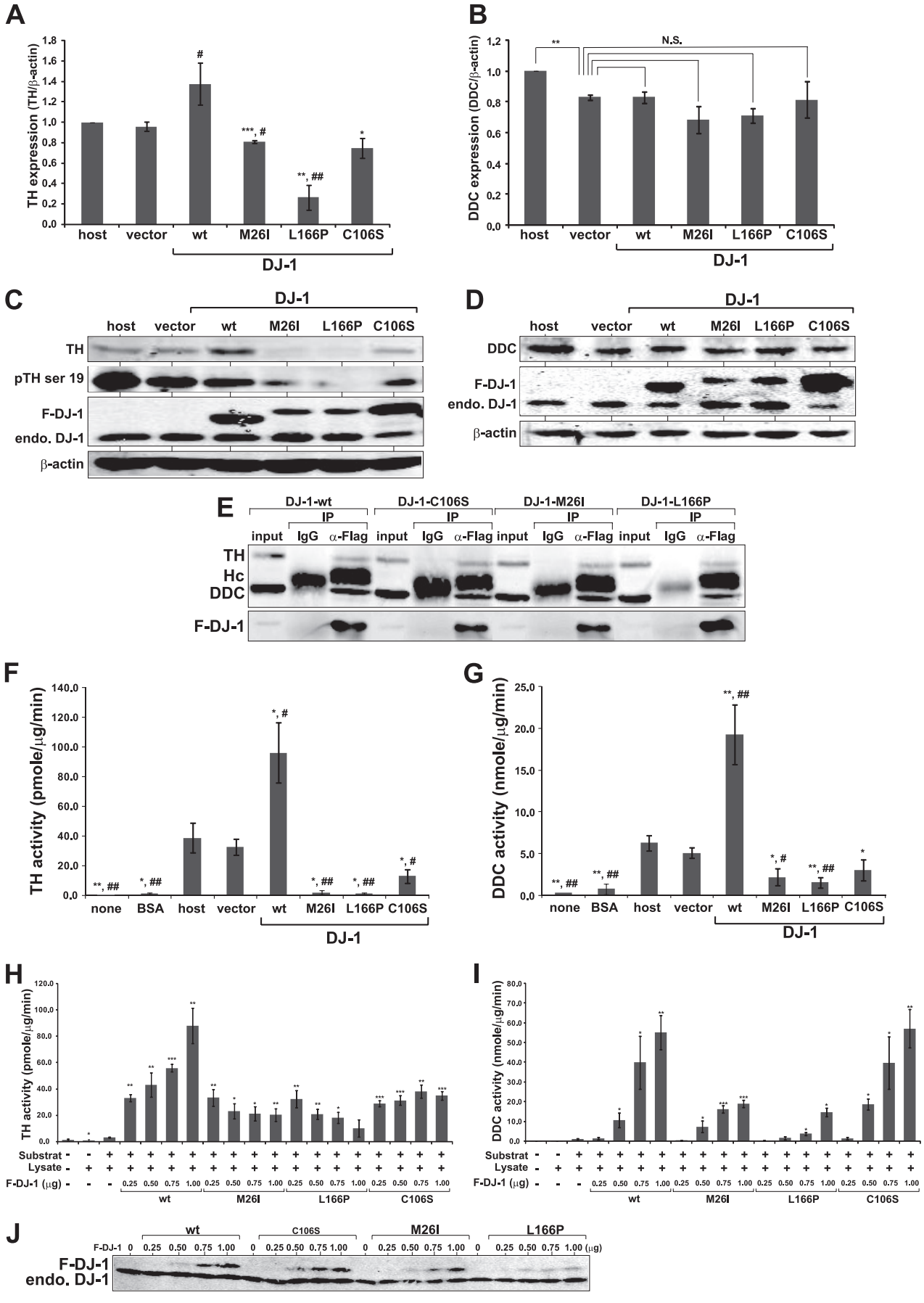

D
N.S.
##
##
#
##
wt:wt=20:20
DDC activity (μmole/μg/min)
Tyrosine
GST
GST-DJ-1-WT (ng/μl)
GST-DJ-1-Mut. (ng/μl)
wt
M26I
R98Q
D149A
L166P
C106S

present in the mixture, activity of less than half of that obtained by wild-type DJ-1 alone was observed (Fig. 3*C*, see lanes in which 20 ng each of wild-type and mutant of DJ-1 was added), indicating that all of the mutants, even mutants from heterozygous mutations of the *DJ-1* gene, acted as a dominant-negative form against wild-type DJ-1. In the case of DDC, on the other hand, all of the mutants of DJ-1 were found to have weak stimulating activity toward DDC compared with the activity of wild-type DJ-1 and to inhibit the activity of wild-type DJ-1 without a dominant-negative effect (Fig. 3*D*).

To examine the effect of mutation of DJ-1 on TH and DDC activities in cells, SH-SY5Y cells expressing FLAG-tagged wild-type and mutants of DJ-1, M26I, L166P, and C106S were established, and expression levels of introduced DJ-1 mutants and endogenously expressing wild-type DJ-1 were analyzed by Western blotting with anti-FLAG and anti-DJ-1 antibodies, respectively (Fig. 4, *C* and *D*). M26I DJ-1 and L166P DJ-1 were found to be expressed lower than that of endogenous mouse DJ-1, and wild-type DJ-1 and C106S DJ-1 were expressed at higher levels than that of endogenous mouse DJ-1. First, the expression levels of TH and DDC mRNA in these cell lines were examined by semi-quantitative PCR (data not shown) and quantitative PCR (real time-PCR) (Fig. 4, *A* and *B*). Intensity of bands in RT-PCR was quantified (supplemental Fig. 3). Although the expression levels of DDC mRNAs in cells expressing wild-type and mutants of DJ-1 were not significantly changed, the levels of TH mRNA in cells expressing wild-type DJ-1 and mutants of DJ-1 were increased and decreased, respectively, compared with the levels in cells harboring the vector alone or parental SH-SY5Y cells (host). Expression levels of TH and DDC proteins were parallel to those of their mRNA; reduced levels of TH but not DDC were observed in cells expressing M26I, L166P, and C106S DJ-1 (Fig. 4, *C* and *D*). To examine association of mutant DJ-1s with TH or DDC, cell extracts were immunoprecipitated with an anti-FLAG antibody, and the precipitates were analyzed by Western blotting with an anti-TH or DDC antibody (Fig. 4*E*). The results showed that all of the mutants of DJ-1 bound to TH and DDC. TH and DDC activities in these cells were examined, and reduced levels of their activities to less than 50% of the levels in parental (host) or vector-containing cells were found in cells expressing mutants of DJ-1 (Fig. 4, *F* and *G*). To examined the effect of mutant DJ-1s on wild-type DJ-1 toward TH and DDC activities, SH-SY5Y cells were transfected with various amounts of expression vectors for FLAG-tagged C106S, M26I, and L166P DJ-1. Forty two h after transfection, cells were cultured in the presence of L-tyrosine and DDC inhibitor or in the presence of L-DOPA for 6 h. Expression levels of endogenous DJ-1 and exogenously added FLAG-tagged mutants of DJ-1 were then examined by Western blotting with anti-DJ-1 and anti-FLAG antibodies, respectively (Fig. 4*J*). One μg of expression vectors for mutants of DJ-1 gave levels similar to or less than that of endogenous DJ-1. Although wild-type DJ-1 stimulated TH and DDC activities in a dose-dependent manner, M26I and L166P DJ-1, but not C106S DJ-1, were found to inhibit TH and DDC activities to less than half of the activities in wild-type DJ-1-transfected cells (Fig. 4, *H* and *I*), suggesting a negative role of pathogenic mutants of DJ-1 toward TH and DDC.

Oxidative Status of DJ-1-dependent Regulation of TH and DDC—Because oxidation of Cys-106 is important for DJ-1 to exert its function, the oxidative status of Cys-106 in cells exposed to H_2O_2, 6-hydroxydopamine (6-OHDA), and 1-methyl-4-phenylpyridinium (MPP^+) was examined. 6-OHDA and MPP^+ are neurotoxins and are known to induce dopaminergic cell death in cultured cells and animals. SH-SY5Y cells were exposed to various amounts of H_2O_2, 6-OHDA, or MPP^+ for 1 h and then L-tyrosine or L-DOPA was added for TH or DDC assays, respectively. One h after addition of L-tyrosine or L-DOPA, the cells were divided into 4 parts and used for analyses of the oxidative status of Cys-106, expression levels of mRNA and protein, and activities of TH and DDC. To examine the oxidative status of Cys-106, proteins extracted from cells were immunoprecipitated with an anti-DJ-1 antibody, and precipitates were separated on a polyacrylamide gel. A protein band corresponding to DJ-1 was cut out, digested with trypsin, and subjected to MALDI-TOF/TOF-MS analysis (Fig. 5*A*). Without oxidative stress, ~80% of Cys-106 of DJ-1 was a reduced form, and this form was decreased in a dose-dependent manner accompanied by an increase in SO_2H and SO_3H forms after SH-SY5Y cells were exposed to H_2O_2, 6-OHDA, and MPP^+. Under these conditions, expression levels of mRNA of TH and DJ-1 were examined by RT-PCR (data not shown) and real time-PCR (Fig. 5, *B* and *C*). Both assays showed that although expression of DJ-1 mRNA was increased with increased doses of H_2O_2, 6-OHDA, and MPP^+ and gradually decreased at doses over 50 μM, the expression of TH mRNA showed a peak at 10 μM and was then decreased. Promoter activity of the *TH* gene was further examined. For this reaction, SH-SY5Y cells were transfected with a plasmid containing the luciferase gene-linked promoter region up to −3000 bp from the transcriptional start site of the human *TH* gene. At 46 h after transfection, cells were exposed to various concentrations of H_2O_2, 6-OHDA, and MPP^+ for 2 h, and their luciferase activity was measured (Fig. 5*D*). The results showed that promoter activity gave a curve similar to that of mRNA expression of the *TH* gene. Expression levels of proteins of TH, DDC, and DJ-1 were also examined by Western blotting (Fig. 5*E*). The results showed that although the level of DDC was not changed during treatment of cells with the three drugs, the TH level first increased up to drug concentration of 10 μM and then decreased as in the case of TH mRNA level (Fig. 5*E*). When TH activities in SH-SY5Y cells exposed to H_2O_2, 6-OHDA, and MPP^+ were examined, patterns similar to those of mRNA and protein were observed; TH activity was maximal at drug concentrations of 10 μM and then decreased to a level

FIGURE 3. **Effects of mutations of DJ-1 on TH and DDC activities *in vitro*.** *A* and *B*, GST, GST-wild-type DJ-1, or GST mutants of DJ-1 were expressed in and prepared from *E. coli* and reacted with ^{35}S-labeled HA-TH (*A*) or T7-DDC (*B*) that had been synthesized *in vitro* using a coupled transcription-translation system (Promega). After the reaction, the mixture was subjected to pulldown assays as described under "Experimental Procedures." *CBB*, Coomassie Brilliant Blue. *C* and *D*, effects of wild-type (*wt*) DJ-1 and various mutants of DJ-1 on TH (*C*) and DDC (*D*) activities were examined *in vitro* as described in the legends of Fig. 1, *G* and *H*. Values are mean ± S.E. from three independent experiments. Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with an amount of "no protein," and #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with the amount of "*wt:wt = 20:20*." *N.S.* means nonspecific.


A
TH expression (TH/β-actin)
host
vector
wt
M26I
L166P
C106S
DJ-1
B
DDC expression (DDC/β-actin)
N.S.
C
TH
pTH ser 19
F-DJ-1
endo. DJ-1
β-actin
D
DDC
E
DJ-1-wt
DJ-1-C106S
DJ-1-M26I
DJ-1-L166P
IP
input
IgG
α-Flag
Hc
F
TH activity (pmole/μg/min)
none
BSA
G
DDC activity (nmole/μg/min)
H
Substrat
Lysate
F-DJ-1 (μg)
I
J

lower than that without drugs. Although expression levels of DDC mRNA and protein were not changed during the exposure of cells to drugs, activity of DDC like TH showed a biphasic pattern during the course of drug treatment (Fig. 5*F*). To examine the specificity of oxidative state-dependent activity of DJ-1 to TH and DDC, SH-SY5Y cells were treated with various concentrations of H_2O_2, 6-OHDA, and MPP^+ for 2 h, and the activity of glycerol-3-phosphate dehydrogenase (GPDH), which does not bind to DJ-1, was examined (Fig. 5*G*). Also, SH-SY5Y cells that had been transfected with an empty vector and expression vectors for FLAG-tagged wild-type DJ-1 or FLAG-tagged mutants of DJ-1 were treated with 200 μM H_2O_2 for 2 h at 46 h after transfection, and then GPDH activity was examined (Fig. 5*H*). Both experiments showed no change of GPDH activity. These results suggest that oxidation-dependent activation and repression of TH and DDC activities may correlate with the oxidative status of Cys-106 of DJ-1.

To directly assess this possibility, purified recombinant DJ-1 was exposed to H_2O_2 and then dialyzed to remove H_2O_2. Aliquots of dialyzed DJ-1 were used for analysis of the oxidative status of Cys-106 and also for testing its activity toward TH and DDC activities *in vitro* (Fig. 6). Without H_2O_2 treatment, almost all of the DJ-1 possessed a reduced form (SH form) of Cys-106, and the sum of SH and SOH forms was 97.9% of total forms. After treatment of DJ-1 with 1 mM H_2O_2 for 10 min, the amount of the SOH form increased, but the sum of the SH and SOH forms of Cys-106 was still more than 80% of total forms. After treatment of DJ-1 with 5 and 10 mM H_2O_2 for 30 min, the sum of the SH and SOH forms of Cys-106 decreased to 56.1 and 28.9%, respectively, and forms with SO_2H and SO_3H increased to more than 50% of total forms (Fig. 6*A*). Both TH and DDC activity levels, on the other hand, were high when DJ-1 was treated with 0 and 1 mM H_2O_2 for 10 min and were lost with 5 mM H_2O_2 (Fig. 6*B*), suggesting that DJ-1 possessing Cys-106 with SH and SOH forms is active and that DJ-1 possessing Cys-106 with SO_2H and SO_3H forms is inactive in terms of stimulation of TH and DDC activities, and that DJ-1 possessing Cys-106 with SO_2H and SO_3H forms acts as a dominant-negative form against active DJ-1.

Complex formation of TH with DJ-1 or with DDC in cells that had been treated with H_2O_2 was then examined. SH-SY5Y cells expressing FLAG-tagged TH were treated with 100 and 200 μM H_2O_2 for 2 h, and proteins in cell extracts were immunoprecipitated with an anti-FLAG antibody followed by Western blotting. Intensities of precipitated protein bands were quantified, and relative binding activity of DJ-1 or DDC to TH is shown (supplemental Fig. 4). The results showed that the amount of complex of DJ-1 with TH or that of DDC with TH was increased in a dose-dependent manner. These results suggest that although a protein complex between DJ-1, TH, and DDC was formed under oxidative stress conditions, excessive oxidation of Cys-106 of DJ-1 abrogates the stimulating activity of DJ-1 toward TH and DDC.

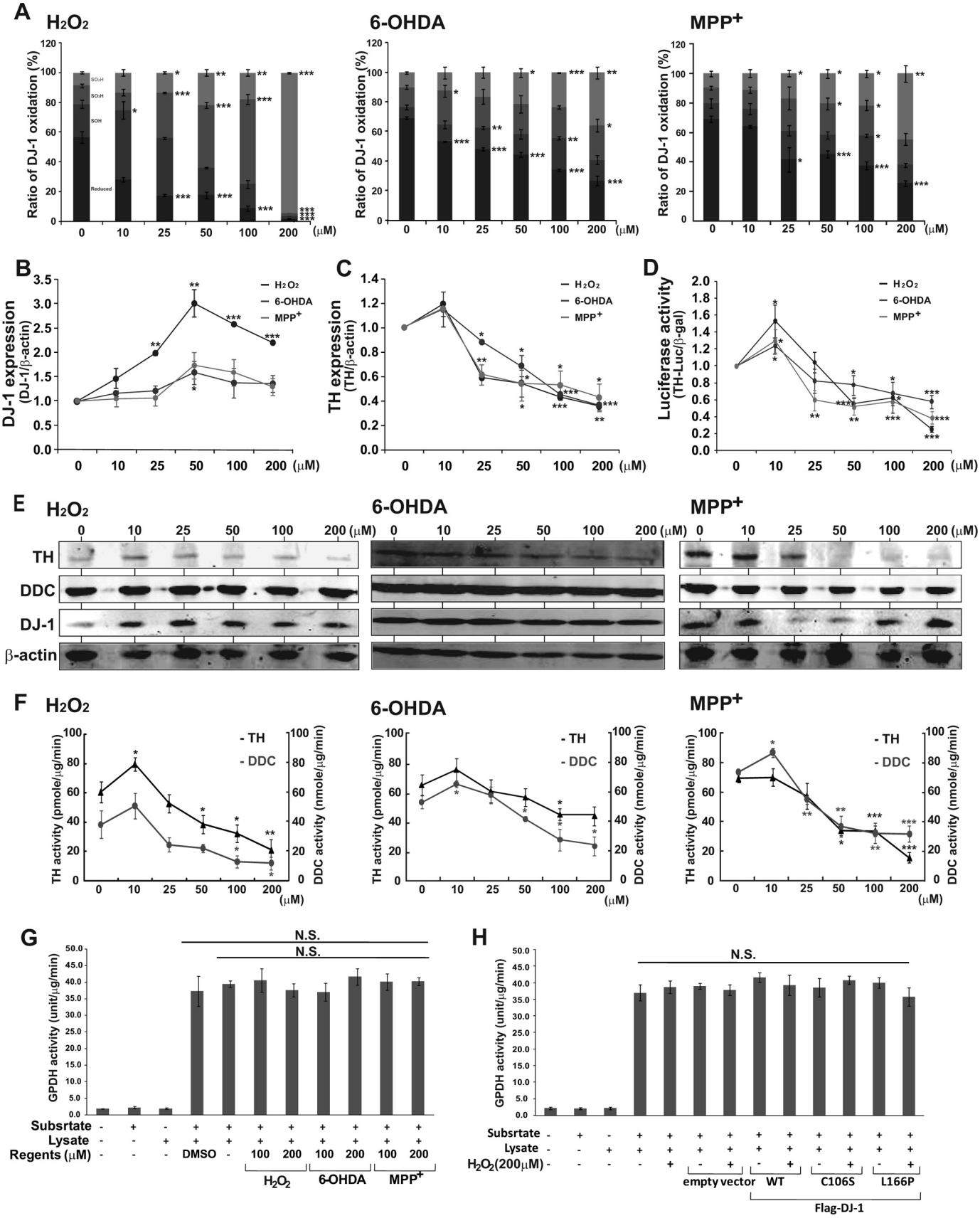
DISCUSSION

In this study, we showed that DJ-1 activates enzymes that are involved in dopamine synthesis in human cells through direct interaction of DJ-1 with TH and DDC. Although mutants of DJ-1 found in patients with familial and sporadic forms of PD, like wild-type DJ-1, bound to TH and DDC, the stimulating activities of these mutants were greatly diminished and worked as a dominant-negative form against wild-type DJ-1. Furthermore, we found that the stimulating activity of DJ-1 requires the presence of the reduced and SOH forms of Cys-106, which account for more than 50% of total forms. Although mechanisms how mutants of DJ-1 and highly oxidized DJ-1 inhibit TH and DDC are currently not clear, it is possible that the conformation of wild-type DJ-1, which is needed for DJ-1 to stimulate TH and DDC activities, is changed by mutation or by excess oxidation, resulting in loss of its stimulating activity.

It has been reported that DJ-1 stimulates transcription of the *TH* gene by sequestering PSF, a negative factor for *TH* gene expression, through inhibiting its sumoylation (16). We have confirmed this, but transcription of the *DDC* gene was not affected by DJ-1 knockdown (Fig. 1, *A–D*). DJ-1 therefore stimulates TH activity both by up-regulation of *TH* gene expression and by protein-protein interaction, and DJ-1 stimulates DDC activity only by protein-protein interaction. Phosphorylation of Ser-19 of TH by various kinases stimulates TH activity (25–28). Introduction of DJ-1 *in vitro* and *in vivo*, however, did not stimulate phosphorylation of TH, although its activity was increased (Fig. 2*L* and Fig. 4*C*), suggesting that DJ-1 stimulates TH activity without causing modification of TH. It has been reported that α-synuclein was associated with DDC and inhibited DDC activity in dopaminergic cells (23). Because DJ-1 inhibits aggregation of α-synuclein (16), it is interesting that DJ-1 and α-synuclein mutually antagonize their functions in dopamine synthesis.

Although expression levels of TH and DDC and their activities in PD patients harboring DJ-1 mutations have not been reported, the level of dopamine and dopaminergic cell death in these patients must be decreased and increased, respectively. In

FIGURE 4. **Effects of mutations of DJ-1 on TH and DDC activities *in vivo*.** SH-SY5Y cells (*host*), SH-SY5Y cells harboring pcDNA3 vector (*vector*), and SH-SY5Y cells expressing wild-type (*wt*) or various mutants of DJ-1 were used. *A* and *B*, expression levels of mRNA of TH (*A*) and DDC (*B*) genes were analyzed by real time-PCR as described in the legends of Fig. 1, *C* and *D*. Relative expression of TH and DDC to β-actin is shown. Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with an amount of host, and #, $p < 0.05$; ##, $p < 0.01$; and ###, $p < 0.001$ compared with the amount of vector. *C* and *D*, expression levels of TH, phosphorylated TH with a serine residue at amino acid number 19 (*pTH ser 19*), FLAG-DJ-1, endogenous DJ-1, and β-actin were analyzed as described in the legends of Fig. 1, *E* and *F*, and Fig. 2*L*. *E*, proteins extracted from SH-SY5Y cells expressing wild-type (*wt*) or various mutants of DJ-1 were immunoprecipitated (*IP*) with an anti-FLAG antibody or with nonspecific (*N.S.*) IgG, and precipitates were analyzed by Western blotting with anti-FLAG, anti-TH, and anti-DDC antibodies. *F* and *G*, TH and DDC activities in cells were measured as described in the legends of Fig. 1, *G* and *H*. Significance: *, $p < 0.05$; **, $p < 0.01$ compared with an amount of host, and #, $p < 0.05$; ##, $p < 0.01$ compared with the amount of vector. *H* and *I*, SH-SY5Y cells were transfected with various amounts of expression vectors for FLAG-tagged wild-type DJ-1 and mutants of DJ-1. Forty two h after transfection, cells were cultures in the presence of L-tyrosine and DDC inhibitor or in the presence of L-DOPA for 6 h, and then their TH and DDC activities were measured as described under "Experimental Procedures." Values are mean ± S.E. from three independent experiments. Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with an amount of *substrate* + *lysate*. *J*, proteins were extracted from SH-SY5Y cells described in *H* and *J* and analyzed with Western blotting with anti-FLAG and anti-DJ-1 antibodies.


A
H2O2
6-OHDA
MPP+
Ratio of DJ-1 oxidation (%)
SO2H
SO3H
SOH
Reduced
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B
DJ-1 expression (DJ-1/β-actin)
C
TH expression (TH/β-actin)
D
Luciferase activity (TH-Luc/β-gal)
H2O2
6-OHDA
MPP+
E
TH
DDC
DJ-1
β-actin
F
TH activity (pmole/μg/min)
DDC activity (nmole/μg/min)
- TH
- DDC
G
GPDH activity (unit/μg/min)
N.S.
Subsrtate
Lysate
Regents (μM)
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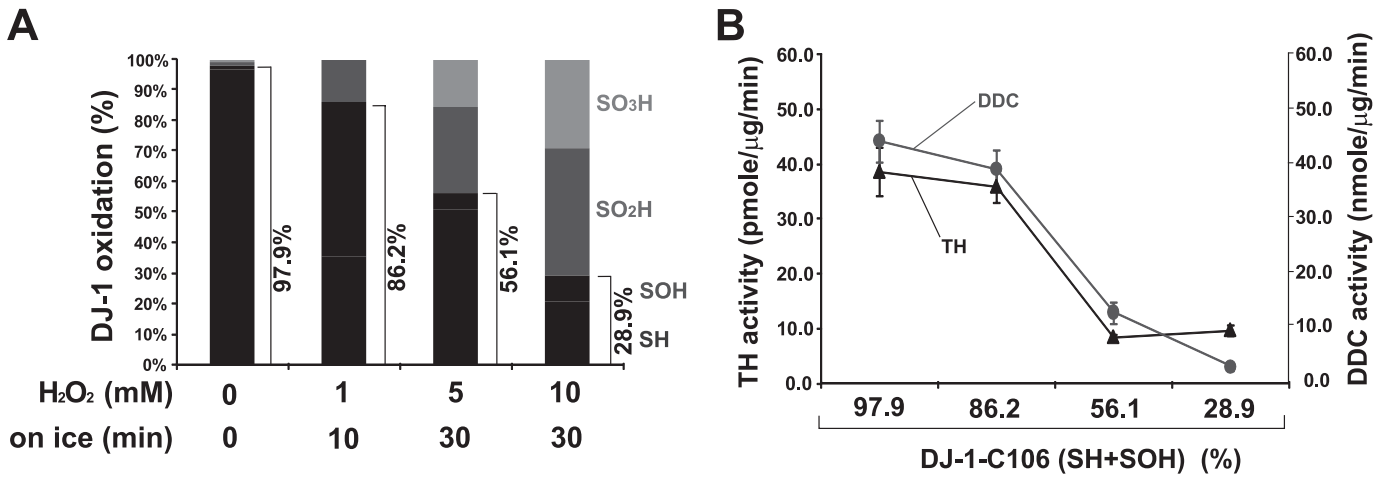

FIGURE 6. **Effect of oxidation of DJ-1 on TH and DDC activities *in vitro*.** *A,* 1 μg of recombinant DJ-1 was reacted with various concentrations of H_2O_2 for 10 or 30 min, dialyzed against 1× PBS (−) containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, digested with trypsin, and subjected to TOF-MS analysis as described under "Experimental Procedures." *B,* half of DJ-1 as described in *A* was mixed with extracts from SH-SY5Y cells, L-tyrosine, and a DDC inhibitor for TH assays or with extracts from SH-SY5Y cells and L-DOPA for DDC assays. Fifteen min after the reaction, the amount of L-DOPA or dopamine was measured using HPLC as described under "Experimental Procedures."

DJ-1 knock-out mice, on the other hand, dopaminergic cell death did not occur, resulting in no reduction of dopamine content (29–31). Age-dependent motor deficits and dopaminergic dysfunction in DJ-1 knock-out mice have however been reported (31). We also confirmed no reduction of expression levels and activities of TH and DDC in DJ-1 knock-out mice (data not shown). Why do these differences occur in humans and mice? We found that DJ-1 stimulated transcription of the human *TH* gene but not the mouse *TH* gene (data not shown). This finding may be one of the reasons for these differences.

In cells expressing pathogenic mutants of DJ-1 found in PD patients, the expression level of the *TH* gene, but not that of the *DDC* gene, was decreased (Fig. 4), indicating loss of transcriptional activity of mutants of DJ-1. Although these mutants bound to TH and DDC, stimulating activities of TH and DDC were severely impeded. Furthermore, the dominant-negative effect of heterozygous mutants, R98Q and D149A, against wild-type DJ-1 on TH and DDC activities suggests that heterozygous mutation of the *DJ-1* gene affects onset of PD, although PARK7/DJ-1 is a recessive mutation in familial PD.

Oxidative stress is thought to be a cause of PD. Absence of the reduced form of DJ-1 and the presence of abnormally oxidized DJ-1 have been found in brains of patients with PD (32), and accumulation of highly oxidized DJ-1 has been found in patients with Parkinson disease and in patients with Alzheimer disease (33). Of the three cysteine residues of DJ-1, Cys-106 is the most sensitive to oxidative stress and is oxidized as SOH, SO_2H, and SO_3H (8). Zhou *et al.* (34) has reported that DJ-1 at Cys-106 with SO_2H is an active form in terms of chaperone activity toward α-synuclein. Which type of oxidized form of Cys-106 determines inactivation of DJ-1 is, however, not clear. We found that the expression level of the *TH* gene was first increased and then decreased in a dose-dependent manner in cells that had been treated with H_2O_2, 6-OHDA, and MPP^+ (Fig. 5), indicating that transcriptional activity of DJ-1 also depends on its oxidative status. Parallel to transcriptional activity of DJ-1, the stimulating activity of DJ-1 toward TH and DDC also showed a bi-phasic pattern, and TOF-MS analyses showed that the stimulating activity of DJ-1 requires the presence of the reduced and SOH forms of Cys-106, which account for more than 50% of total forms. A ternary complex among DJ-1, TH, and DDC was found, and binding of TH with DJ-1 or with DDC was increased with increase in the doses of H_2O_2 to which SH-ST5Y cells were exposed (supplemental Figs. 1 and 4), suggesting accumulation of an inactive complex in cells under an oxidative stress condition. The sporadic form of PD is an age-dependent disease, and age-dependent oxidation of DJ-1 in flies, mice, and humans has been reported (35). It is therefore thought that inactivation of DJ-1 with aging abrogates the dopamine synthesis system, leading to the onset of PD.

FIGURE 5. **Effect of oxidation of cells on TH and DDC activities *in vivo*.** *A,* SH-SY5Y cells were exposed to various concentrations of H_2O_2, 6-OHDA, and MPP^+ for 1 h, and proteins were extracted from the cells. Proteins in cell extracts were then immunoprecipitated with an anti-DJ-1 antibody and separated on an SDS-containing polyacrylamide gel. After staining proteins in gels, a protein band corresponding to DJ-1 was subjected to TOF-MS analysis as described under "Experimental Procedures." Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with an amount of "0 μM". *B* and *C,* expression levels of mRNA of DJ-1 and TH in SH-SY5Y cells that had been exposed to various concentrations of H_2O_2, 6-OHDA, and MPP^+ for 1 h were by real time-PCR (*B* and *C*) as described in the legends of Fig. 1, *A–D*. Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with an amount of 0 μM. *D,* SH-SY5Y cells were transfected with a TH gene promoter-luciferase construct. At 46 h after transfection, cells were exposed to various concentrations of H_2O_2, 6-OHDA, and MPP^+ for 1 h, and their luciferase activity was measured. Significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with an amount of 0 μM. *E,* expression levels of TH, DDC, DJ-1, and β-actin in cells exposed to H_2O_2, 6-OHDA, and MPP^+ were analyzed as described in the legends of Fig. 1, *E* and *F*. *F,* TH and DDC activities in cells exposed to H_2O_2, 6-OHDA, and MPP^+ were measured as described in the legends of Fig. 1, *G* and *H*. Significance: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with an amount of "0 μM". *G,* GPDH activity in SH-SY5Y cells exposed to H_2O_2, 6-OHDA, and MPP^+ for 2 h was measured according to the manufacturer's protocol (MK426, Takara Bio, Kyoto, Japan). Significance: *N.S.* (nonspecific) compared with an amount of *DMSO or substrate + lysate*. Values are mean S.E. ± from three independent experiments. *H,* SH-SY5Y cells were transfected with expression vectors for FLAG-tagged wild-type DJ-1, C106S DJ-1, and L166P DJ-1. Forty six h after transfection, cells were exposed to 200 μM H_2O_2 for 2 h, and their GPDH activity was measured as described in *G*. Significance: *N.S.* (nonspecific) compared with an amount of *substrate + lysate*. Values are mean S.E. ± from three independent experiments.

The oxidative stress function of DJ-1 has been reported by us and other groups (7, 9–11), and DJ-1 knock-out mice have been shown to be highly sensitive to oxidative stresses (29–31, 36). Taken together, the results suggest that functions of DJ-1 in terms of oxidative stress reaction and dopamine synthesis greatly contribute to the pathogenesis of Parkinson disease.

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