ROS, which are harmful to cells. In order to investigate roles of DJ-1 in cells against oxidative stresses, we also established an NIH3T3 cell line, NIH3T3-D2 cells, which are stable DJ-1-knockdown cells expressing siRNA toward DJ-1. The expression level of DJ-1 in D2 cells decreased to about 10% of that in parental NIH3T3 cells (Fig. 3C). Parental NIH3T3 cells, D2 cells, and cell lines expressing various exogenously added mutants of

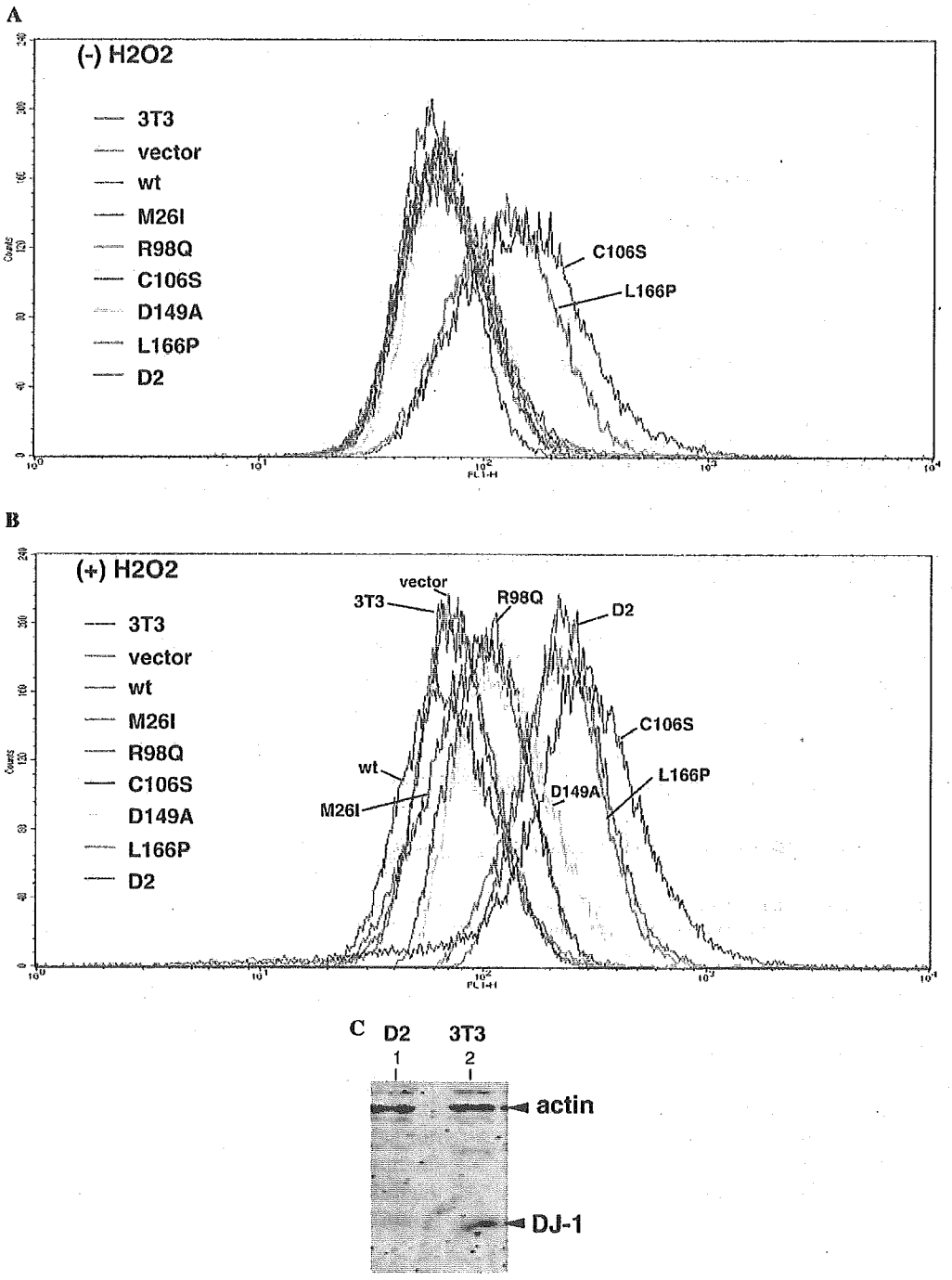

Fig. 3. Elimination of H_2O_2 by DJ-1. (A) Mouse NIH3T3 cell lines harboring FLAG-wild-type DJ-1 and its mutants were treated with 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min and analyzed by flowcytometry. (B) Mouse NIH3T3 cell lines harboring FLAG-wild-type DJ-1 and its mutants were treated with 100 μM H_2O_2 for 60 min and then with DCFH-DA for 30 min and analyzed by flowcytometry. (C) Proteins extracted from NIH3T3 cells (3T3) and DJ-1-knockdown cells (D2) were blotted with anti-mouse DJ-1 and anti-actin antibodies. The proteins that reacted with primary antibodies were visualized with IRDye800-conjugated or Alexa Fluor680-conjugated secondary antibodies using an infrared imaging system (Odyssey, LI-COR).

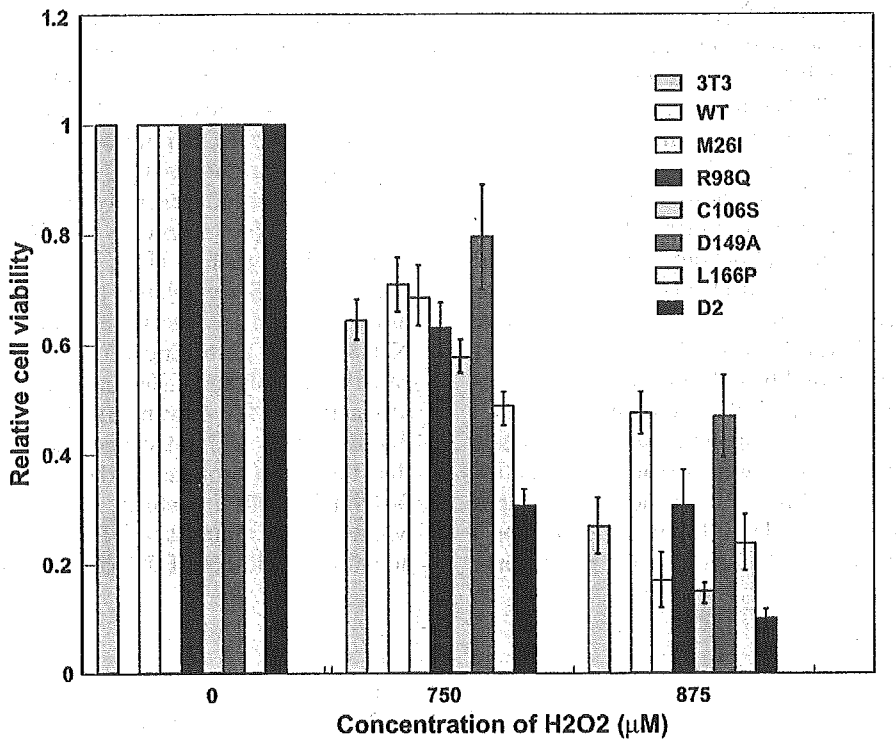

Fig. 4. Acceleration of H_2O_2-induced cell death in cells harboring various mutants of DJ-1. NIH3T3 cell lines were reacted with 750 and 875 μM of H_2O_2 for 3 h and their viabilities were measured by an MTT assay. More than five experiments were carried out.

DJ-1 were cultured without stresses, treated with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min, and analyzed by flowcytometry (Fig. 3A). Cell lines expressing L166P and C106S mutants of DJ-1 were found to have high fluorescence intensity and peaks were shifted to the right (compared to parental 3T3) (Fig. 3A), indicating that L166P and C106S had little activities for scavenging ROS under a condition of no oxidative stress. Of three cysteine residues present in DJ-1, C106 was shown to be the most sensitive cysteine residue to H_2O_2-mediated oxidation [29]. D2 cells, on the other hand, showed curves similar to those of parental NIH3T3 cells (3T3) and cells expressing exogenously added wild-type DJ-1 (wt), suggesting that about 10% of the expression of DJ-1 in parental cells is sufficient for cells to eliminate ROS that are basically produced.

Cell lines were then treated with 100 μM H_2O_2 for 1 h and with DCFH-DA for 30 min, and analyzed by flowcytometry (Fig. 3B). While fluorescence intensities of all of the cells were shifted to the right compared to those in cells without addition of H_2O_2, peaks of D2 cells were dramatically shifted to positions of L166P and C106S cells, indicating that D2 cells have little activity for elimination of exogenously added H_2O_2. Other cell lines expressing DJ-1 mutants, including M26I, R98Q, and D149A, were found to have reduced activities to eliminate exogenously added H_2O_2, suggesting that these mutations of DJ-1 become a risk factor for onset of Parkinson's disease.

Viabilities of these cell lines in the presence of H_2O_2 were then determined by an MTT assay (Fig. 4). Cells harboring exogenously added wild-type DJ-1 were much more resistant to H_2O_2 than were parental cells as described previously [31]. D2 cells, DJ-1-knockdown cells, were found to be most sensitive to H_2O_2. Of the cell lines harboring DJ-1 mutants, on the other hand, L166P and C106S cells were found to be sensitive to H_2O_2, and M26I and R98Q lines followed in terms of sensitivity to H_2O_2. The M26I cell line was much more sensitive to a high dose of H_2O_2 than was the R98Q cell line. In contrast to activity for elimination of H_2O_2, however, the D149A cells were found to be resistant to cell death, and the viability of D149A cells was almost the same as that of the wild-type cells. These results suggest that degrees of resistance of cells to H_2O_2-induced cell death, except for that of D149A cells, are parallel to activities for elimination of H_2O_2.

Discussion

In this study, we examined four DJ-1 mutants found in Parkinson's disease patients in terms of dimer formation, stability, and sensitivities to H_2O_2-induced cell death. The mutants examined are from genes of two

homozygous mutations, L166P [15] and M26I [16], and from genes of two heterozygous mutations, R98Q [16,17] and D149A [16]. R98Q mutation is thought to be gene polymorphism [42]. The results showed that all of the mutants formed heterodimers with wild-type DJ-1 and that all of the mutants except for L166P also formed homodimers. The results also showed that both M26I and L166P, which are gene products of homozygous mutations, were unstable and degraded, in part, by the proteasome system. R98Q and D149A, which are gene products of heterozygous mutations, were much more stable than was wild-type DJ-1 and became much more stable in the presence of MG132, an inhibitor of proteasome. Wild-type DJ-1 and the C106S mutant of DJ-1, a substitution mutant corresponding to the active site of protease activity of DJ-1, were found not to be affected in the presence of MG132. It is therefore thought that all of the mutants of DJ-1 found in Parkinson's disease patients are degraded, in part, by the proteasome system and that heterodimerization of the mutants with wild-type DJ-1 becomes stable in cells.

Reactive oxygen species (ROS) are constantly produced from various organelles in cells, and production of superfluous ROS injures DNA, lipid, and protein, resulting in various diseases, including cancer, infertility, aging, and neurodegenerative diseases such as Parkinson's disease (see recent reviews [43–46] and references therein). ROS such as H_2O_2 induce cell death, and these effects of ROS are related to the onset of Parkinson's disease ([22–24]; see recent review [25]). The results using NIH3T3 cells harboring various mutants of DJ-1 indicate that L166P and C106S mutants of DJ-1 had already lost activities for scavenging ROS that had been produced in cells without exogenously added stresses and that these two proteins worked in a dominant-negative fashion in cells in which wild-type DJ-1 is present, whereas other mutants of DJ-1 did not interfere with the activity of wild-type DJ-1 or still possessed activity to

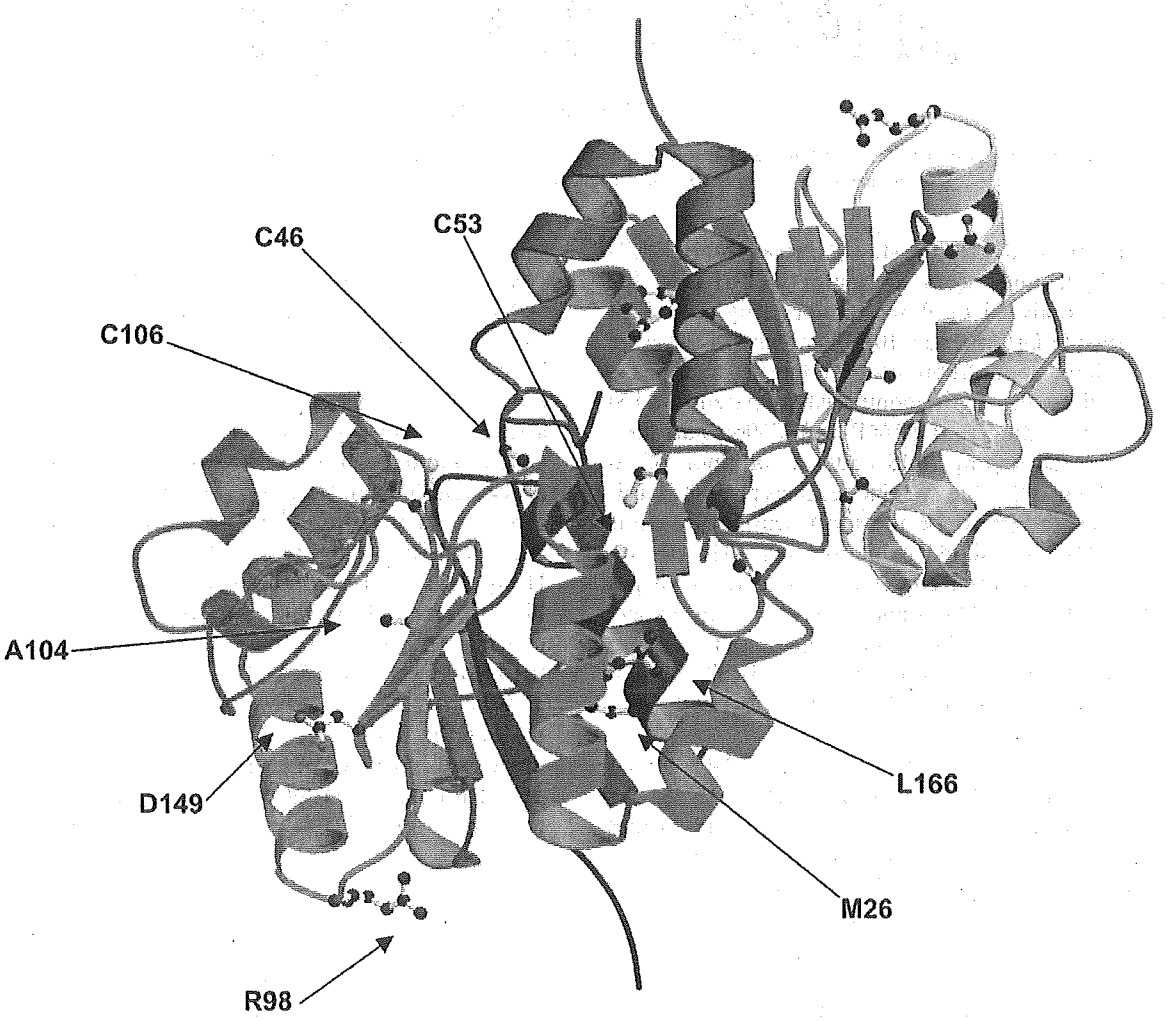

Fig. 5. Ribbon diagrams of dimer forms of DJ-1 and assignment of positions of mutated amino acids. Positions of mutations used in this study were indicated on the ribbon diagrams of dimer forms of DJ-1 using MolScript and Raster3D programs [47,48].

scavenge a low level of ROS. DJ-1-knockdown cells (D2 cells) also possessed activity to scavenge a low level of ROS. Since expressions of other anti-oxidant proteins such as superoxide dismutase, glutathione peroxidase, and catalase were found to increase in NIH3T3 cell line harboring L166P mutant of DJ-1 or D2 cells by the DNA-microarray analysis (data not shown), it is likely that a small amount of DJ-1 in cells is enough to scavenge a low level of ROS but that L166P or C106S mutant of DJ-1 works in a dominant-negative fashion. When cells were exposed to high levels of H_2O_2, however, all of the mutants of DJ-1 were found to be insufficient to eliminate such levels of H_2O_2. High levels of H_2O_2 induce cell death, and DJ-1 has been reported to prevent H_2O_2-induced cell death [31]. The results of this study showed that activities of various mutants of DJ-1 to prevent cell death, except for that of D149A, are parallel to those to eliminate H_2O_2. The reason for the discrepancy in results for activities of D149A is currently not known.

The crystal structure of DJ-1 has been reported by five independent groups, including us [32–36], and positions of mutations used in this study were indicated on ribbon diagrams of dimers formed by DJ-1 using MolScript and Raster3D programs [47,48] (Fig. 5). C46 and C53 are positioned in the dimer interface and C106 is located on the loop as described previously [32]. L166 is located at the middle of α-helix 8 (α8), and mutation from leucine to proline appears to cause a break in the α-helix, resulting in disruption of dimer formation. M26, R98, and D149 are located at the center of α1, at the edge of α4, and at the edge of β sheet 7 (β7), respectively. Both R98 and D149 are located outside DJ-1, and their mutants R98Q and D149A may affect interaction with other DJ-1-binding proteins that affect functions of DJ-1. M26 is located inside DJ-1 and its mutant M26I may change the whole structure of DJ-1, which may result in the relatively high sensitivity to H_2O_2-induced cell death. Since a heterozygous point mutation of A104T located in exon 5 of the DJ-1 gene, homozygous deletion of exons 1–5 [15], and heterozygous deletions of exons 5–7 and exon 5 have been reported in Parkinson's disease patients [49], the position of A104 at the edge of β7 in the DJ-1 structure is also indicated. Although we did not establish NIH3T3 cell line harboring A104T DJ-1 and therefore did not analyze the A104T mutant, A104 and exon 5 might be important for DJ-1 functions.

Recently, DJ-1 has been reported to be colocalized with τ or α-synuclein in fibrillar inclusions in patients with Pick's disease and multiple system atrophy, respectively, suggesting that DJ-1 is related to various neurodegenerative diseases, including Parkinson's disease [20,21]. Although mutations of DJ-1 have not been reported in patients with Pick's disease and multiple system atrophy, a search for mutations of DJ-1 and examination of the level of expression and amounts of oxidized form of DJ-1 in patients with such diseases should help one to elucidate molecular mechanisms of the onset of neurodegenerative diseases, including Parkinson's disease.

Acknowledgments

We thank Yoko Misawa and Kiyomi Takaya for their technical assistance. We also thank Kazuya Honbou for assignment of amino acids in the DJ-1 structure. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Culture, Sports and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan.

References

[1] D. Nagakubo, T. Taira, H. Kitaura, M. Ikeda, K. Tamai, S.M.M. Iguchi-Ariga, H. Ariga, DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras, Biochem. Biophys. Res. Commum. 31 (1997) 509–513.

[2] T. Taira, K. Takahashi, R. Kitagawa, S.M.M. Iguchi-Ariga, H. Ariga, Molecular cloning of human and mouse DJ-1 genes and identification of Sp1-dependent activation of the human DJ-1 promoter, Gene 263 (2001) 285–292.

[3] F. Le Naour, D.E. Misek, M.C. Krause, L. Deneux, T.J. Giordano, S. Scholl, S.M. Hanash, Proteomics-based identification of RS/DJ-1 as a novel circulating tumor antigen in breast cancer, Clin. Cancer Res. 7 (2001) 3328–3335.

[4] K. Miura, E.D. Bowman, R. Simon, A.C. Peng, A.I. Robles, R.T. Jones, T. Katagiri, P. He, H. Mizukami, L. Charboneau, T. Kikuchi, L.A. Liotta, Y. Nakamura, C.C. Harris, Laser capture microdissection and microarray expression analysis of lung adenocarcinoma reveals tobacco smoking- and prognosis-related molecular profiles, Cancer Res. 62 (2002) 3244–3250.

[5] Y. Hod, S.N. Pentyala, T.C. Whyard, M.R. El-Maghrabi, Identification and characterization of a novel protein that regulates RNA–protein interaction, J. Cell. Biochem. 72 (1999) 435–444.

[6] A. Wagenfeld, C.H. Yeung, K. Strupat, T.G. Cooper, Shedding of a rat epididymal sperm protein associated with infertility induced by ornidazole and alpha-chlorohydrin, Biol. Reprod. 8 (1998) 1257–1265.

[7] J.E. Welch, R.R. Barbee, N.L. Roberts, J.D. Suarez, G.R. Klinefelter, SP22: a novel fertility protein from a highly conserved gene family, J. Androl. 19 (1998) 385–393.

[8] A. Wagenfeld, C.H. Yeung, S. Shivaji, V.R. Sundareswaran, H. Ariga, T.G. Cooper, Expression and cellular localization of contraception-associated protein, J. Androl. 21 (2000) 954–963.

[9] G.R. Klinefelter, J.E. Welch, S.D. Perreault, H.D. Moore, R.M. Zucker, J.D. Suarez, N.L. Roberts, K. Bobseine, S.J. Jeffay, Localization of the sperm protein SP22 and inhibition of fertility in vivo and in vitro, Andrology 23 (2002) 48–63.

[10] M. Okada, K. Matsumoto, T. Niki, T. Taira, S.M.M. Iguchi-Ariga, H. Ariga, DJ-1, a target protein for an endocrine disrupter, participates in the fertilization in mice, Biol. Pharm. Bull. 25 (2002) 853–856.

[11] K. Yoshida, Y. Sato, M. Yoshiike, S. Nozawa, H. Ariga, T. Iwamoto, Immunocytochemical localization of DJ-1 in human male reproductive tissue, Mol. Reprod. Dev. 66 (2003) 391–397.

[12] K. Takahashi, T. Taira, T. Niki, C. Seino, S.M.M. Iguchi-Ariga, H. Ariga, DJ-1 positively regulates the androgen receptor by impairing the binding of PIASx alpha to the receptor, J. Biol. Chem. 276 (2001) 37556–37563.

[13] T. Niki, K. Takahashi-Niki, T. Taira, S.M.M. Iguchi-Ariga, H. Ariga, DJBP: a novel DJ-1-binding protein, negatively regulates the androgen receptor by recruiting histone deacetylase complex, and DJ-1 antagonizes this inhibition by abrogation of this complex, Mol. Cancer Res. 1 (2003) 247–261.
[14] T. Taira, S.M.M. Iguchi-Ariga, H. Ariga, Co-localization with DJ-1 is essential for the androgen receptor to exert its transcription activity that has been impaired by androgen-antagonists, Biol. Pharm. Bull. 27 (2004) 574–577.
[15] V. Bonifati, P. Rizzu, M.J. van Baren, O. Schaap, G.J. Breedveld, E. Krieger, M.C. Dekker, F. Squitieri, P. Ibanez, M. Joosse, J.W. van Dongen, N. Vanacore, J.C. van Swieten, A. Brice, G. Meco, C.M. van Duijn, B.A. Oostra, P. Heutink, Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism, Science 299 (2003) 256–259.
[16] P.M. Abou-Sleiman, D.G. Healy, N. Quinn, A.J. Lees, N.W. Wood, The role of pathogenic DJ-1 mutations in Parkinson's disease, Ann. Neurol. 54 (2003) 283–286.
[17] S. Hague, E. Rogaeva, D. Hernandez, C. Gulick, A. Singleton, M. Hanson, J. Johnson, R. Weiser, M. Gallardo, B. Ravina, K. Gwinn-Hardy, A. Crawley, P.H. St. George-Hyslop, A.E. Lang, P. Heutink, V. Bonifati, J. Hardy, A. Singleton, Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation, Ann. Neurol. 54 (2003) 271–274.
[18] K. Hedrich, A. Djarmati, N. Schafer, R. Hering, C. Wellenbrock, P.H. Weiss, R. Hilker, P. Vieregge, L.J. Ozelius, P. Heutink, V. Bonifati, E. Schwinger, A.E. Lang, J. Noth, S.B. Bressman, P.P. Pramstaller, O. Riess, C. Klein, DJ-1 (PARK7) mutations are less frequent than Parkin (PARK2) mutations in early-onset Parkinson disease, Neurology 62 (2004) 389–394.
[19] R. Bandopadhyay, A.E. Kingsbury, M.R. Cookson, A.R. Reid, I.M. Evans, A.D. Hope, A.M. Pittman, T. Lashley, R. Canet-Aviles, D.W. Miller, C. McLendon, C. Strand, A.J. Leonard, P.M. Abou-Sleiman, D.G. Healy, H. Ariga, N.W. Wood, R. de Silva, T. Revesz, J.A. Hardy, A.J. Lees, The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease, Brain 127 (2004) 420–430.
[20] P. Rizzu, D.A. Hinkle, V. Zhukareva, V. Bonifati, L.A. Severijnen, D. Martinez, R. Ravid, W. Kamphorst, J.H. Eberwine, V.M. Lee, J.Q. Trojanowski, P. Heutink, DJ-1 colocalizes with tau inclusions: a link between parkinsonism and dementia, Ann. Neurol. 55 (2004) 113–118.
[21] M. Neumann, V. Muller, K. Gorner, H.A. Kretzschmar, C. Haass, P.J. Kahle, Pathological properties of the Parkinson's disease-associated protein DJ-1 in alpha-synucleinopathies and tauopathies: relevance for multiple system atrophy and Pick's disease, Acta Neuropathol. 107 (2004) 489–496.
[22] R. Heikkila, G. Cohen, Inhibition of biogenic amine uptake by hydrogen peroxide: a mechanism for toxic effects of 6-hydroxydopamine, Science 172 (1971) 1257–1258.
[23] W.J. Nicklas, S.K. Youngster, M.V. Kindt, R.E. Heikkila, MPTP, MPP+ and mitochondrial function, Life Sci. 40 (1987) 721–729.
[24] J. Lotharius, K.L. O'Malley, The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity, J. Biol. Chem. 275 (2000) 38581–38588.
[25] P. Jenner, Oxidative stress in Parkinson's disease, Ann. Neurol. 53 (Suppl. 3) (2003) S26–S36.
[26] A. Mitsumoto, Y. Nakagawa, A. Takeuchi, K. Okawa, A. Iwamatsu, Y. Takanezawa, Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat, Free Radic. Res. 35 (2001) 301–310.
[27] A. Mitsumoto, Y. Nakagawa, DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin, Free Radic. Res. 35 (2001) 885–893.
[28] C. Srisomsap, P. Subhasitanont, A. Otto, E.C. Mueller, P. Punyarit, B. Wittmann-Liebold, J. Svasti, Detection of cathepsin B up-regulation in neoplastic thyroid tissues by proteomic analysis, Proteomics 2 (2002) 706–712.
[29] T. Kinumi, J. Kimata, T. Taira, H. Ariga, E. Niki, Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide mediated oxidation in vivo in human umbilical vein endothelial cells, Biochem. Biophys. Res Commun. 317 (2004) 722–728.
[30] T. Yokota, K. Sugawara, K. Ito, R. Takahashi, H. Ariga, H. Mizusawa, Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition, Biochem. Biophys. Res. Commun. 312 (2003) 1342–1348.
[31] T. Taira, Y. Saito, T. Niki, S.M.M. Iguchi-Ariga, K. Takahashi, H. Ariga, DJ-1 has a role in antioxidative stress to prevent cell death, EMBO Rep. 5 (2004) 213–218.
[32] K. Honbou, N.N. Suzuki, M. Horiuchi, T. Niki, T. Taira, H. Ariga, F. Inagaki, The crystal structure of DJ-1, a protein related to male fertility and Parkinson's disease, J. Biol. Chem. 278 (2003) 31380–31384.
[33] X. Tao, L. Tong, Crystal structure of human DJ-1, a protein associated with early-onset Parkinson's disease, J. Biol. Chem. 278 (2003) 31372–31379.
[34] M.A. Wilson, J.L. Collins, Y. Hod, D. Ringe, G.A. Petsko, The 1.1-Å resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease, Proc. Natl. Acad. Sci. USA 100 (2003) 9256–9261.
[35] Q. Huai, Y. Sun, H. Wang, L.S. Chin, L. Li, H. Robinson, H. Ke, Crystal structure of DJ-1/RS and implication on familial Parkinson's disease, FEBS Lett. 549 (2003) 171–175.
[36] S.J. Lee, S.J. Kim, I.K. Kim, J. Ko, C.S. Jeong, G.H. Kim, C. Park, S.Q. Kang, P.G. Suh, H.S. Lee, S.S. Cha, Crystal structures of human DJ-1 and *Escherichia coli* Hsp31 that share an evolutionarily conserved domain, J. Biol. Chem. 278 (2003) 44552–44559.
[37] M.G. Macedo, B. Anar, I.F. Bronner, M. Cannella, F. Squitieri, V. Bonifati, A. Hoogeveen, P. Heutink, P. Rizzu, The DJ-1L166P mutant protein associated with early onset Parkinson's disease is unstable and forms higher order protein complexes, Hum. Mol. Genet. 12 (2003) 2807–2816.
[38] D.J. Moore, L. Zhang, T.M. Dawson, V.L. Dawson, A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization, J. Neurochem. 87 (2003) 1558–1567.
[39] D.W. Miller, R. Ahmad, S. Hague, M.J. Baptista, R. Canet-Aviles, C. McLendon, D.M. Carter, P.P. Zhu, J. Stadler, J. Chandran, G.R. Klinefelter, C. Blackstone, M.R. Cookson, L166P mutant DJ-1, causative for recessive Parkinson's disease, is degraded through the ubiquitin–proteasome system, J. Biol. Chem. 278 (2003) 36588–36595.
[40] K. Gorner, E. Holtorf, S. Odoy, B. Nuscher, A. Yamamoto, J.T. Regula, K. Beyer, C. Haass, P.J. Kahle, Differential effects of Parkinson's disease-associated mutations on stability and folding of DJ-1, J. Biol. Chem. 279 (2004) 6943–6951.
[41] J.A. Olzmann, K. Brown, K.D. Wilkinson, H.D. Rees, Q. Huai, H. Ke, A.I. Levey, L. Li, L.S. Chin, Familial Parkinson's disease-associated L166P mutation disrupts DJ-1 protein folding and function, J. Biol. Chem. 279 (2004) 8506–8515.
[42] K. Hedrich, N. Schafer, R. Hering, J. Hagenah, A.J. Lanthaler, E. Schwinger, P.L. Kramer, L.J. Ozelius, S.B. Bressman, G. Abbruzzese, P. Martinelli, V. Kostic, P.P. Pramstaller, P. Vieregge, O. Riess, C. Klein, The R98Q variation in DJ-1 represents a rare polymorphism, Ann. Neurol. 55 (2004) 145–146.
[43] A.C. Rego, C.R. Oliveira, Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases, Neurochem. Res. 28 (2003) 1563–7154.
[44] M. Benhar, D. Engelberg, A. Levitzki, ROS, stress-activated kinases and stress signaling in cancer, EMBO Rep. 3 (2002) 420–425.

[45] A. Agarwal, R.A. Saleh, M.A. Bedaiwy, Role of reactive oxygen species in the pathophysiology of human reproduction, Fertil. Steril. 79 (2003) 829–843.
[46] T.R. Golden, D.A. Hinerfeld, S. Melov, Oxidative stress and aging: beyond correlation, Aging Cell 1 (2002) 117–123.
[47] P.J. Kraulis, MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures, J. Appl. Crystallogr. 24 (1991) 946–950.
[48] E.A. Merrit, M.E.P. Murphy, Methodological problems in the retrospective computation of responsiveness to change: the lesson of Cronbach, Acta Crystallogr. Sect. D Biol. Crystallogr. 50 (1994) 869–873.
[49] V. Bonifati, B.A. Oostra, P. Heutink, Linking DJ-1 to neurodegeneration offers novel insights for understanding the pathogenesis of Parkinson's disease, J. Mol. Med. 82 (2004) 163–174.

Stimulation of transforming activity of DJ-1 by Abstrakt, a DJ-1-binding protein

AYA SEKITO[1], TAKAHIRO TAIRA[1,3], TAKESHI NIKI[3], SANAE M.M. IGUCHI-ARIGA[2,3] and HIROYOSHI ARIGA[1,3]

[1]Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812; [2]Graduate School of Agriculture, Hokkaido University, Kita 9, Nishi 9, Sapporo 060-8589; [3]CREST, Japan Science and Technology Corporation, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

Received June 21, 2004; Accepted August 25, 2004

Abstract. DJ-1 was identified by us as a novel oncogene in cooperation with activated *ras*. Although over-expression of DJ-1 has been reported in several cancer cells, including cells in breast cancer, lung cancer and prostate cancer, the precise mechanism underlying transformation has not been clarified. In this study, we screened proteins by a yeast two-hybrid method and identified Abstrakt as a DJ-1-binding protein. Abstrakt is an RNA helicase, but it has not yet been characterized. Northern blot analysis showed that human Abstrakt was expressed ubiquitously in all tissues. Abstrakt was then found to bind to and to be colocalized in the nucleus with DJ-1 in human cells. Furthermore, Abstrakt was found to stimulate transforming activity of DJ-1 in rat 3Y1 cells transfected with DJ-1 with activated *ras*. These findings suggest that Abstrakt is a positive regulator for DJ-1.

Introduction

DJ-1 was first identified by our group as a novel oncogene product that transformed mouse NIH3T3 cells in cooperation with activated *ras* (1). The human DJ-1 gene is mapped at chromosome 1p36.2-p36.3, where a hot spot of chromosome abnormalities has been reported in several tumors (2). However, the mechanism underlying cell transformation has not been clarified. Another group has also identified RS, another name for human DJ-1, as a regulatory component of an RNA-binding protein complex (3). Furthermore, CAP-1 or SP22, a rat homologue of human DJ-1, has been identified by other laboratories as a key protein related to infertility of male rats (4-6). DJ-1 was then found to be related to infertility (7,8). It has also been shown that DJ-1 is a circulating tumor antigen in breast cancer, in which DJ-1 is secreted from cells to serum (9), and that DJ-1 is over-expressed in smoker-derived lung adenocarcinoma (10) and prostate cancer (11).

We have shown that DJ-1 acts as a positive regulator for the androgen receptor (AR) by sequestering PIASxα or DJBP (12-14). PIASxα, a member of the PIAS family of proteins, and DJBP, a novel DJ-1-binding protein, function as negative regulators for AR by preventing AR DNA-binding activity and by recruiting histone deacetylase complex, respectively (12,13).

Deletion and point (L166P) mutations of DJ-1 have recently been shown to be responsible for onset of familial Parkinson's disease, PARK7 (15), and other homozygous and heterozygous mutations of DJ-1 have been identified in patients with familial or sporadic Parkinson's disease (16-18). Various lines of evidence also suggest that oxidative stresses contribute to the cascade leading to dopaminergic cell degeneration in PD (19-21; recently reviewed in ref. 22). Expression of DJ-1 was shown to be induced by oxidative stresses (23-25), and cysteine at amino acid number 106 of DJ-1 was found to be oxidized as a form of sulphonic or sulphinic acid (26). A pI shift of DJ-1 towards a more acidic isoform has been observed in PD patients (27). We previously reported that DJ-1 plays a role in an antioxidative stress reaction, in which reactive oxygen species were eliminated *in vitro* and *in vivo* by oxidizing DJ-1 itself, and that mutations of DJ-1, including L166P, lead to cell death (28,29). Other mutants of DJ-1 found in Parkinson's disease patients were also found to possess reduced antioxidative stress activities (30). These findings indicate that DJ-1 is a multi-functional protein in somatic cells and sperm.

In this study, we identified Abstrakt as a DJ-1-binding protein. Abstrakt was found to stimulate transformation activity of DJ-1, suggesting that Abstrakt is one of key players that modulate versatile functions of DJ-1.

Materials and methods

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

Correspondence to: Dr Hiroyoshi Ariga, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812, Japan
E-mail: hiro@pharm.hokudai.ac.jp

Key words: DJ-1, transformation, Abstrakt, oncogene

Construction of plasmids. Nucleotide sequences of the oligonucleotide used for PCR primers were as follows: Abs ATG (EcoRI), 5'-AAAGAATTCATGGAGGAGTCGGAAC CCGA-3'; Abs STOP (SalI), 5'-GGGCTCGAGTCAGAAGT CCATGGAGCTGT-3'. pcDNA3-FLAG-Abs or pcDNA3-HA-Abs: PCR was carried out on an EST clone (accession number: BC015476) obtained from UK HGMP Resource Centra as a template with Abs ATG (EcoRI) and Abs STOP (SalI) as primers. The resultant PCR product was digested with *Eco*RI and *Sal*I and inserted into *Eco*RI-*Xho*I sites of pcDNA3-FLAG or pcDNA3-HA, respectively. Other plasmids used in this study were described previously (1,12,13).

Indirect immunofluorescence. HeLa cells were transfected with 5 μg of pcDNA-Abstrakt-HA by the calcium phosphate precipitation method (31). Forty-eight hours after transfection, cells were fixed with a solution containing 4% paraformaldehyde and reacted with a combination of a mouse anti-HA-monoclonal antibody (12CA5, Roche) and an anti-DJ-1 polyclonal antibody (1). The cells were then reacted with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG and observed under a confocal laser fluorescent microscope.

In vivo binding assay. Two μg of pcDNA3-FLAG-Abstrakt together with 2 μg of pcDNA3-T7-DJ-1 were transfected into human 293T cells 60% confluent in a 10-cm dish by the calcium phosphate precipitation technique (31). Forty-eight hours after transfection, the whole cell extract was prepared by the procedure described previously (12). Approximately 2 mg of the 293T cell proteins was first immunoprecipitated with a mouse anti-FLAG antibody (M2, Sigma) or with a mouse anti-HA antibody (12CA5, Roche) under the same conditions as those described previously (12,13). The precipitates were then separated in a 12% polyacrylamide gel containing SDS, blotted onto a nitrocellulose filter, and reacted with a rabbit anti-T7 antibody (Novagen) and with the mouse anti-FLAG antibody.

Focus forming assay. Rat 3Y1 cells in a 10-cm dish were transfected with 1 μg of pH-ras and/or pcDNA3-F-DJ-1 by the calcium phosphate precipitation method (31). The medium was changed every 3 days, and the cells were stained with Giemsa solution 14 days after transfection.

Results and Discussion

Identification of Abstrakt as a DJ-1-binding protein. We have reported that DJ-1 binds to PIASxα and DJBP and that it regulates AR transcription activity (12,13). In addition to PIASxα and DJBP, cDNA spanning amino acids 419-623 of human Abstrakt was obtained by a yeast two-hybrid screening. After a nucleotide database search, an EST clone of IMAGE no. 3917178 was found to have the entire sequence of human Abstrakt. The *Drosophila Abstrakt* gene was identified genetically by its effect on axon outgrowth and fasciculation of the Bolwig nerve, and it encodes a putative ATP-dependent RNA helicase of the DEAD box protein family (32,33). The human homologue of *Drosophila* Abstrakt was also identified, but its function is unknown.

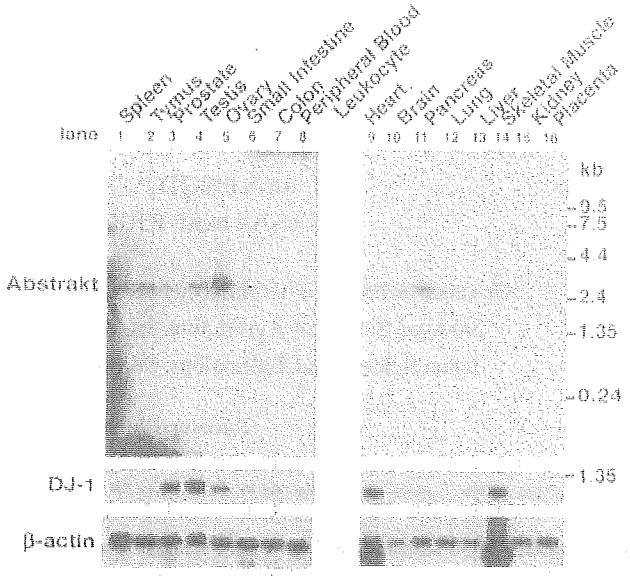

Figure 1. Expressions of Abstrakt mRNA in human tissues. Northern blot analysis was carried out using multiple Northern blot sheets of human tissues (Clontech) with labeled cDNAs of Abstrakt, DJ-1 and ß-actin.

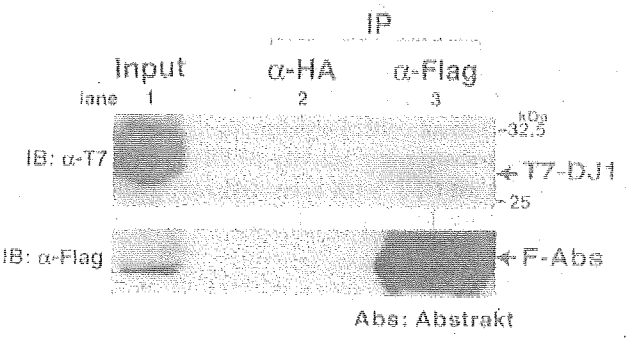

Figure 2. Association of DJ-1 with Abstrakt. DJ-1 and Abstrakt were tagged with either T7 or FLAG, and their expression vectors were transfected into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG antibody or anti-HA antibody. The proteins in the precipitates were separated in a 12% polyacrylamide gel and analyzed by Western blotting with anti-FLAG and anti-T7 antibodies.

Since there is no report on human Abstrakt, the expression of Abstrakt mRNA was first examined by Northern blot analysis in various human tissues using a probe of Abstrakt cDNA. In human tissues, mRNA of 2.6 kb corresponding to that of human Abstrakt was expressed ubiquitously in all of the tissues with strong expression in the ovary, and ubiquitous expression of DJ-1 in tissues was also shown (Fig. 1).

Associations of DJ-1 with Abstrakt in cells were then examined. To do this, FLAG-tagged Abstrakt was co-transfected with T7-tagged DJ-1 into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared from transfected cells, proteins in the extracts were immunoprecipitated with an anti-FLAG antibody or an anti-HA antibody, and the precipitates were analyzed by Western blotting with an anti-T7 or anti-Flag antibody (Fig. 2). The

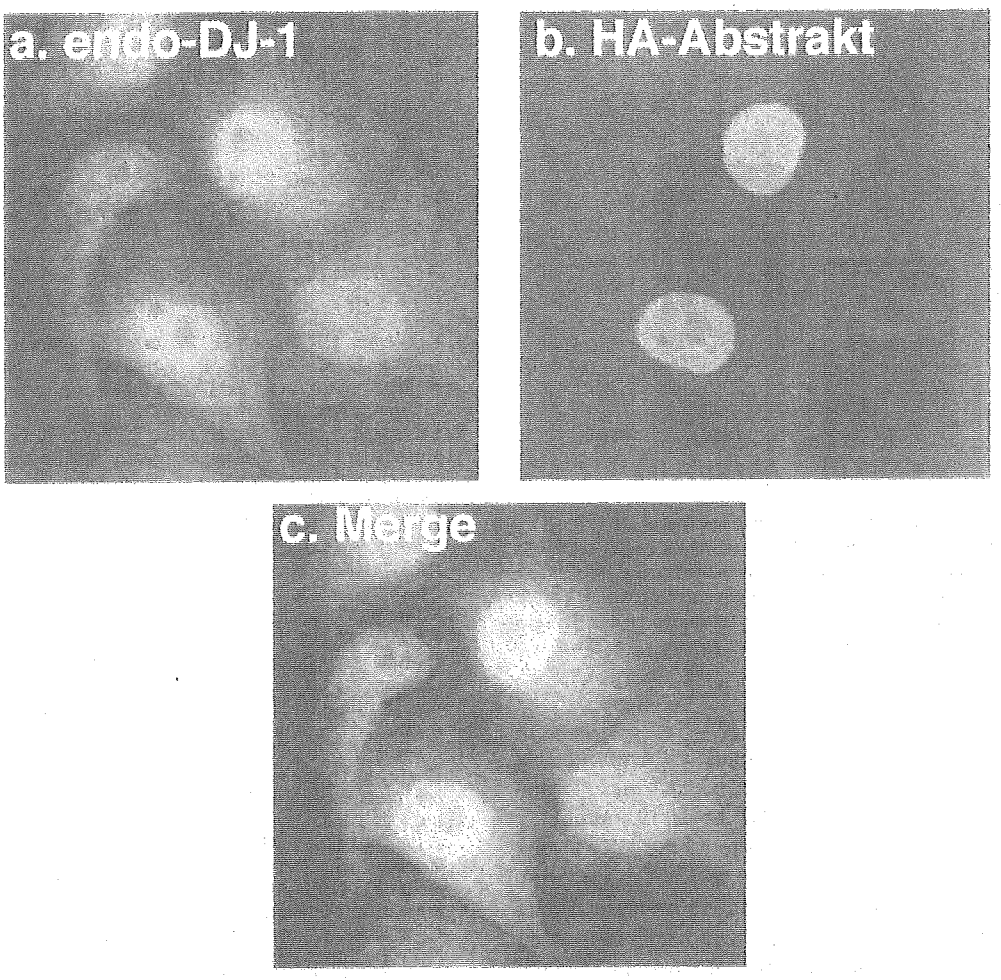

Figure 3. Colocalization of DJ-1 and Abstrakt. HeLa cells were transfected with HA-Abstrakt by the calcium phosphate precipitation technique (31). Forty-eight hours after transfection, cells were fixed, reacted with an anti-DJ-1 polyclonal antibody and an anti-HA monoclonal antibody, and visualized with a FITC-conjugated anti-rabbit antibody and rhodamine-conjugated anti-mouse antibody. The two figures were merged (Merge).

anti-FLAG antibody, but not the anti-HA antibody, was first confirmed to precipitate FLAG-tagged Abstrakt (Fig. 2, lower part). The results also showed that T7-DJ-1 was co-immunoprecipitated with FLAG-Abstrakt (Fig. 2, upper part), indicating that DJ-1 is associated with Abstrakt in 293T cells.

Previous studies have shown that DJ-1 is localized both in the cytoplasm and nucleus in human HeLa cells and is translocated from the cytoplasm to nuclei during the S-phase of the cell cycle upon induction by mitogen (1). Localization of Abstrakt in cells, however, has not been reported. To determine the cellular localization of Abstrakt and DJ-1, an expression vector for HA-tagged Abstrakt was transfected into human HeLa cells. Two days after transfection, the cells were stained with anti-DJ-1 and anti-FLAG antibodies to stain endogenously expressed DJ-1 and ectopically expressed Abstrakt, respectively, and the proteins were detected by FITC- and rhodamine-conjugated second antibodies, respectively, and then visualized under a confocal laser microscope (Fig. 3). Endogenously expressed DJ-1 (green) and Abstrakt (red) were co-localized in the nucleus as shown by the yellow color (Fig. 3C, Merge).

Stimulation of transforming activity of DJ-1 by Abstrakt. Since DJ-1 possesses transforming activity in collaboration of activated *ras*, the effect of Abstrakt on cell transforming activity of DJ-1 was also examined. Rat 3Y1 cells were transfected with expression vectors for H-*ras* and DJ-1 with or without the expression vector for Abstrakt and cultured for 14 days. The cells were then stained with the Giemsa solution, and foci due to transformed cells were counted (Fig. 4). As reported previously (1), DJ-1 or ras alone yielded small numbers of foci (data not shown) and DJ-1 in combination with ras yielded large numbers of foci (Fig. 4). No Abstrakt yielded foci of transformed cells by itself (data not shown). Co-introduction of Abstrakt in addition to DJ-1 and ras, however, reproducibly increased the number of transformed cell foci by 1.5-fold (Fig. 4). The results suggest that Abstrakt is a positive regulator for DJ-1.

In this study, we identified human Abstrakt as a DJ-1-binding protein and found that Abstrakt stimulated transforming activity of DJ-1. The *Abstrakt* gene in *Drosophila* has been identified by a genetic screening for mutations affecting the projection of the larval optic nerve and was found by genetic methods to regulate aspects of cell polarity

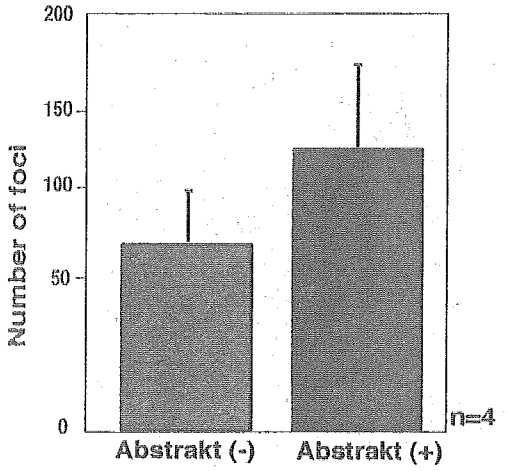

Figure 4. Effect of Abstrakt on the focus forming activity of DJ-1. Rat 3Y1 cells in a 10-cm dish were transfected with 1 μg of pH-ras and/or pcDNA3-F-DJ-1 by the calcium phosphate precipitation method (31), and the medium was changed every 3 days. Fourteen days after transfection, the cells were stained with Giemsa solution and the foci due to transformed cells were counted. The average numbers of 4 independent experiments are shown.

in oocytes and embryos and also to regulate the inscuteable levels and asymmetric division of neural and mesodermal progenitors in *Drosophila* (32-34). Abstrakt encodes a putative ATP-dependent RNA helicase of the DEAD box protein family and was found to bind to RNA, suggesting that Abstrakt functions in mRNA splicing or translational control (34). Although these findings have been observed in *Drosophila*, biochemical characteristics of Abstrakt have not been clarified and there has been no report on a human homolog of *Drosophila* Abstrakt. Since DJ-1/RS has been found to be a component of the RNA polymerase complex (3), it is likely that DJ-1 is associated with Abstrakt. Multiple steps, including DNA replication and activation or inactivation of genes that are related to cell cycle progression, are required for cells to be transformed. It is therefore thought that RNA helicase activity contributes to reactions in these steps. Although functions of Abstrakt in human cells are not known, the findings in this study suggest that Abstract is a positive regulator for some functions of DJ-1.

Acknowledgments

We thank Yoko Misawa and Kiyomi Takaya for their technical assistance. This study was supported by grants-in-aid from the Ministry of Education, Science, Culture, Sports and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan.

References

1. Nagakubo D, Taira T, Kitaura H, Ikeda M, Tamai K, Iguchi-Ariga SMM and Ariga H: DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem Biophys Res Commun 31: 509-513, 1997.
2. Taira T, Takahashi K, Kitagawa R, Iguchi-Ariga SMM and Ariga H: Molecular cloning of human and mouse DJ-1 genes and identification of Sp1-dependent activation of the human DJ-1 promoter. Gene 263: 285-292, 2001.
3. Hod Y, Pentyala SN, Whyard TC and El-Maghrabi MR: Identification and characterization of a novel protein that regulates RNA-protein interaction. J Cell Biochem 72: 435-444, 1999.
4. Wagenfeld A, Yeung CH, Strupat K and Cooper TG: Shedding of a rat epididymal sperm protein associated with infertility induced by ornidazole and alpha-chlorohydrin. Biol Reprod 8: 1257-1265, 1998.
5. Welch JE, Barbee RR, Roberts NL, Suarez JD and Klinefelter GR: SP22: a novel fertility protein from a highly conserved gene family. J Androl 19: 385-393, 1998.
6. Wagenfeld A, Yeung CH, Shivaji S, Sundareswaran VR, Ariga H and Cooper TG: Expression and cellular localization of contraception-associated protein. J Androl 21: 954-963, 2000.
7. Klinefelter GR, Welch JE, Perreault SD, Moore HD, Zucker RM, Suarez JD, Roberts NL, Bobseine K and Jeffay S: Localization of the sperm protein SP22 and inhibition of fertility *in vivo* and *in vitro*. J Androl 23: 48-63, 2002.
8. Okada M, Matsumoto K, Niki T, Taira T, Iguchi-Ariga SMM and Ariga H: DJ-1, a target protein for an endocrine disrupter, participates in the fertilization in mice. Biol Pharm Bull 25: 853-856, 2002.
9. Le Naour F, Misek DE, Krause MC, Deneux L, Giordano TJ, Scholl S, Hanash SM: Proteomics-based identification of RS/DJ-1 as a novel circulating tumor antigen in breast cancer. Clin Cancer Res 7: 3328-3335, 2001.
10. Miura K, Bowman ED, Simon R, Peng AC, Robles AI, Jones RT, Katagiri T, He P, Mizukami H, Charboneau L, Kikuchi T, Liottac LA, Nakamura Y and Harris CC: Laser capture microdissection and microarray expression analysis of lung adenocarcinoma reveals tobacco smoking- and prognosis-related molecular profiles. Cancer Res 62: 3244-3250, 2002.
11. Grzmil M, Voigt S, Thelen P, Hemmerlein B, Helmke K and Burfeind P: Up-regulated expression of the MAT-8 gene in prostate cancer and its siRNA-mediated inhibition of expression induces a decrease in proliferation of human prostate carcinoma cells. Int J Oncol 24: 97-105, 2004.
12. Takahashi K, Taira T, Niki T, Seino C, Iguchi-Ariga SMM and Ariga H: DJ-1 positively regulates the androgen receptor by impairing the binding of PIASx alpha to the receptor. J Biol Chem 276: 37556-37563, 2001.
13. Niki T, Takahashi-Niki K, Taira T, Iguchi-Ariga SMM and Ariga H: DJBP: a novel DJ-1-binding protein, negatively regulates the androgen receptor by recruiting histone deacetylase complex, and DJ-1 antagonizes this inhibition by abrogation of this complex. Mol Cancer Res 1: 247-261, 2003.
14. Taira T, Iguchi-Ariga SMM and Ariga H: Co-localization with DJ-1 is essential for the androgen receptor to exert its transcription activity that has been impaired by androgen-antagonists. Biol Pharm Bull 27: 574-577, 2004.
15. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA and Heutink P: Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 299: 256-259, 2003.
16. Abou-Sleiman PM, Healy DG, Quinn N, Lees AJ and Wood NW: The role of pathogenic DJ-1 mutations in Parkinson's disease. Ann Neurol 54: 283-286, 2003.
17. Hague S, Rogaeva E, Hernandez D, Gulick C, Singleton A, Hanson M, Johnson J, Weiser R, Gallardo M, Ravina B, Gwinn-Hardy K, Crawley A, St George-Hyslop PH, Lang AE, Heutink P, Bonifati V, Hardy J and Singleton A: Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. Ann Neurol 54: 271-274, 2003.
18. Hedrich K, Djarmati A, Schafer N, Hering R, Wellenbrock C, Weiss PH, Hilker R, Vieregge P, Ozelius LJ, Heutink P, Bonifati V, Schwinger E, Lang AE, Noth J, Bressman SB, Pramstaller PP, Riess O and Klein C: DJ-1 (PARK7) mutations are less frequent than Parkin (PARK2) mutations in early-onset Parkinson disease. Neurology 62: 389-394, 2004.
19. Heikkila R and Cohen G: Inhibition of biogenic amine uptake by hydrogen peroxide: a mechanism for toxic effects of 6-hydroxydopamine. Science 172: 1257-1258, 1971.
20. Nicklas WJ, Youngster SK, Kindt MV and Heikkila RE: MPTP, MPP+ and mitochondrial function. Life Sci 40: 721-729, 1987.
21. Lotharius J and O'Malley KL: The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity. J Biol Chem 275: 38581-38588, 2000.

22. Jenner P: Oxidative stress in Parkinson's disease. Ann Neurol 53 (Suppl. 3): S26-S36, 2003.
23. Mitsumoto A, Nakagawa Y, Takeuchi A, Okawa K, Iwamatsu A and Takanezawa Y: Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic Res 35: 301-310, 2001.
24. Mitsumoto A and Nakagawa Y: DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 35: 885-893, 2001.
25. Srisomsap C, Subhasitanont P, Otto A, Mueller EC, Punyarit P, Wittmann-Liebold B and Svasti J: Detection of cathepsin B up-regulation in neoplastic thyroid tissues by proteomic analysis. Proteomics 2: 706-712, 2002.
26. Kinumi T, Kimata J, Taira T, Ariga H and Niki E: Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide mediated oxidation *in vivo* in human umbilical vein endothelial cells. Biochem Biophys Res Commun 317: 722-728, 2004.
27. Bandopadhyay R, Kingsbury AE, Cookson MR, Reid AR, Evans IM, Hope AD, Pittman AM, Lashley T, Canet-Aviles R, Miller DW, McLendon C, Strand C, Leonard AJ, Abou-Sleiman PM, Healy DG, Ariga H, Wood NW, De Silva R, Revesz T, Hardy JA and Lees AJ: The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. Brain 127: 420-430, 2004.
28. Yokota T, Sugawara K, Ito, K, Takahashi R, Ariga H and Mizusawa H: Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. Biochem Biophys Res Commun 312: 1342-1348, 2003.
29. Taira T, Saito Y, Niki T, Iguchi-Ariga SMM, Takahashi K and Ariga H: DJ-1 has a role in antioxidative stress to prevent cell death. EMBO Rep 5: 213-218, 2004.
30. Takahashi-Niki K, Niki T, Taira T, Iguchi-Ariga SMM and Ariga H: Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients. Biochem Biophys Res Commun 320: 389-397, 2004.
31. Graham FL and van der Eb AJ: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456-467, 1973.
32. Irion U and Leptin M: Developmental and cell biological functions of the *Drosophila* DEAD-box protein abstrakt. Curr Biol 9: 1373-1381, 1999.
33. Schmucker D, Vorbruggen G, Yeghiayan P, Fan HQ, Jackle H and Gaul U: The *Drosophila* gene abstrakt, required for visual system development, encodes a putative RNA helicase of the DEAD box protein family. Mech Dev 91: 189-196, 2000.
34. Irion U, Leptin M, Siller K, Fuerstenberg S, Cai Y, Doe CQ, Chia W and Yang X: Abstrakt, a DEAD box protein, regulates Insc levels and asymmetric division of neural and mesodermal progenitors. Curr Biol 14: 138-144, 2004.

DJ-1 restores p53 transcription activity inhibited by Topors/p53BP3

YUMI SHINBO[1], TAKAHIRO TAIRA[1,3], TAKESHI NIKI[3], SANAE M.M. IGUCHI-ARIGA[2,3] and HIROYOSHI ARIGA[1,3]

[1]Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812; [2]Graduate School of Agriculture, Hokkaido University, Kita 9, Nishi 9, Sapporo 060-8589; [3]CREST, Japan Science and Technology Corporation, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

Received July 20, 2004; Accepted September 16, 2004

Abstract. DJ-1 is a multi-functional protein that plays roles in transcriptional regulation and anti-oxidative stress, and loss of its function is thought to result in onset of Parkinson's disease. Here, we report that DJ-1 bound to Topors/p53BP3, a ring finger protein binding to both topoisomerase I and p53, *in vitro* and *in vivo* and that both proteins were colocalized in cells. DJ-1 and p53 were then found to be sumoylated by Topors in cells. It was also found that DJ-1 bound to p53 *in vitro* and *in vivo* and that colocalization with and its binding to p53 were stimulated by UV irradiation of cells. Transcription activity of p53 was found to be abrogated by Topors concomitant with sumoylation of p53 in a dose-dependent manner, and DJ-1 restored its repressed activity by releasing the sumoylated form of p53. These findings suggest that DJ-1 positively regulates p53 through Topors-mediated sumoylation.

Introduction

DJ-1 was first identified by our group as a novel candidate of the oncogene product that transformed mouse NIH3T3 cells in cooperation with activated *ras* (1). The human DJ-1 gene is mapped at chromosome 1p36.2-p36.3, where a hot spot of chromosome abnormalities has been reported in several tumors (2). DJ-1 has been reported to be overexpressed in breast cancer (3) and in smoker-derived lung adenocarcinoma (4). DJ-1 has also been reported to be a regulatory component of an RNA-binding protein complex (5) and to be related to infertility (6-11). We have shown that DJ-1 acts as a positive regulator for the androgen receptor (AR) by sequestering PIASxα or DJBP (12-14). PIASxα, a member of the PIAS family of proteins, and DJBP, a novel DJ-1-binding protein, function as negative regulators for AR by preventing AR DNA-binding activity and by recruiting histone-deacetylase complex, respectively (12,13). PIAS family proteins, which include PIAS1, PIAS3, PIASxα, PIASxß and PIASy, were found to function as transcriptional coregulators in various other cellular pathways, including the p53 pathway. PIAS proteins were then found to act as E3 SUMO-1 ligases that stimulate SUMO-1 conjugation to proteins, including p53 (15-17). We found that DJ-1 was also sumoylated with PIASxα and PIASy to exert its full activities (Shinbo *et al*, unpublished data).

Deletion and point (L166P) mutations of DJ-1 have recently been shown to be responsible for onset of familial Parkinson's disease (PD), PARK7 (18), and other homozygous and heterozygous mutations of DJ-1 have been identified in patients with familial or sporadic PD (19-21). Various lines of evidence also suggest that oxidative stresses contribute to the cascade leading to dopaminergic cell degeneration in PD (22-24; for review see ref. 25), and expression of DJ-1 was shown to be induced by oxidative stresses (26-30). A pI shift of DJ-1 towards a more acidic isoform has been observed in PD patients (31). We previously reported that DJ-1 plays a role in an anti-oxidative stress reaction, in which reactive oxygen species were eliminated *in vitro* and *in vivo* by oxidizing DJ-1 itself, and that mutations of DJ-1, including L166P, lead to cell death (29,32).

In this study, we found that Topors/p53BP3 is a DJ-1-binding protein and that Topors sumoylated both DJ-1 and p53. DJ-1 restored p53 transcription activity that had been abrogated by Topors-induced sumoylation.

Correspondence to: Dr Hiroyoshi Ariga, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Sapporo 060-0812, Japan
E-mail: hiro@pharm.hokudai.ac.jp

Key words: DJ-1, p53, Topors, SUMO-1, transcription

Materials and methods

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

Construction of plasmids. Nucleotide sequences of the oligonucleotide used for PCR primers were as follows: Topors ATG (MfeI), 5'-GGGGAATTCGACCCTGTCACCGAGACC-3';

Topors STOP (XhoI), 5'-CCTGGTAGAGACTGTGATGTG-3'; p53 ATG (EcoRI), 5'-GGGGAATTCATGACTGCCAT GGGAGGAG-3'; p53 STOP (XhoI), 5'-GGGCTCGAGT CAGTCTGAGTCAGGCCC-3'; pcDNA3-FLAG-Topors or pcDNA3-FLAG-p53: PCR was carried out on an EST clone (accession no.: AW106470) obtained from UK HGMP Resource Centra and pEF-p53 (33) as templates either with Topors ATG (MfeI) and Topors STOP (XhoI) or with p53 ATG (EcoRI) and p53 STOP (XhoI) as primers. The resultant PCR product was digested with *Mfe*I and *Xho*I or with *Eco*RI and *Xho*I and inserted into *Eco*RI-*Xho*I sites of pcDNA3-FLAG or pcDNA3-T7, respectively. Other plasmids used in this study were described previously (1,12,13,29).

SUMO-1 conjugation to DJ-1 in vivo. H1299 cells were transfected with 5 μg of pcDNA-FLAG-DJ-1, 2 μg of pcDNA3-T7-SUMO-1 and 5 μg of pcDNA-F-Topors by the calcium phosphate precipitation method (34). Forty-eight hours after transfection, proteins were extracted from human H1299 cells with a buffer containing 0.1% NP-40, 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl and by sonication. Proteins were then immunoprecipitated with an anti-FLAG monoclonal antibody-conjugated agarose (M2, Sigma), and the precipitates were dissolved with a sample buffer containing 30 mM Tris-HCl (pH 6.8), 6% SDS, 0.006% bromophenol blue, 12% 2-mercaptoethanol and 50% glycerol, boiled for 15 min, and subjected to Western blotting using the anti-FLAG monoclonal antibody or an anti-SUMO-1 polyclonal antibody (FL-101, Santa Cruz). Proteins were then visualized by using ECL system (Amersham BioScience).

Indirect immunofluorescence. HeLa cells were transfected with 5 μg of pcDNA-FLAG-Topors by the calcium phosphate precipitation method (34). Forty-eight hours after transfection, cells were fixed with a solution containing 4% paraformaldehyde and reacted with a combination of a mouse anti-FLAG monoclonal antibody (M2, Sigma) and an anti-DJ-1 polyclonal antibody (1). The cells were then reacted with an fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG and observed under a confocal laser fluorescent microscope. ME180 cells were irradiated with 20 J/m^2 of ultraviolet (UV) light. At 30 min after UV irradiation, cells were treated as described above using an anti-p53 monoclonal antibody (Pab240, Santa Cruz) and the anti-DJ-1 polyclonal antibody.

In vitro binding assay. ^{35}S-labeled Topors or p53 was synthesized *in vitro* using the reticulocyte lysate of the TnT-transcription-translation coupled system (Promega) and pcDNA3-FLAG-Topors or pcDNA3-FLAG-p53 as a template. Labeled proteins were mixed with GST or GST-DJ-1 expressed in and prepared from *E. coli* at 4°C for 60 min in a buffer containing 150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.5% NP-40. After washing with the same buffer, the bound proteins were separated in a 12% polyacrylamide gel containing SDS and visualized by fluorography.

In vivo binding assay. Two μg of pcDNA3-FLAG-Topors or pcDNA3-FLAG-p53 together with 2 μg of pcDNA3-DJ-1-HA were transfected into human 293T cells 60% confluent in a 10-cm dish by the calcium phosphate precipitation technique (34). Forty-eight hours after transfection, the whole cell extract was prepared by the procedure as described previously (12). Approximately 2 mg of the 293T cell proteins was first immunoprecipitated with a mouse anti-FLAG antibody (M2, Sigma) or with non-specific mouse IgG under the same conditions as those used in the *in vitro* binding assay as described above. After washing with the same buffer, the precipitates were separated in a 12% polyacrylamide gel containing SDS, blotted onto a nitrocellulose filter, and reacted with a rabbit anti-HA antibody (MBL) and with the mouse anti-FLAG antibody.

Luciferase assay. One hundred ng of PG-13-Luc, a reporter plasmid, 50 ng each of pcDNA3-FLAG-p53 and pcDNA3-T7-SUMO-1, various amounts of pcDNA3-FLAG-Topors, and various amounts of pcDNA3-FLAG-DJ-1 together with 50 μg of pCMV-ß-gal, a ß-galactosidase expression vector, were transfected into H1299 cells ~60% confluent in a 6-cm dish by the calcium phosphate method (34). Forty-eight hours after transfection, whole cell extracts were prepared by the addition of Triton X-100-containing solution from a Pica gene kit (Wako Pure Chemicals Co. Ltd., Kyoto, Japan) to the cells. About a one-fifth volume of the extract was used for the ß-galactosidase assay to normalize the transfection efficiency as described previously (12), and the luciferase activity due to the reporter plasmid was determined using a Pica gene kit and a luminometer, Lumat LB 9507 (EG & G Berthold). The same experiments were repeated three to five times.

Results

Identification of Topors as a DJ-1-binding protein. We have reported that DJ-1 binds to PIASxα and DJBP and that it regulates the AR transcription activity (12,13). In addition to PIASxα and DJBP, cDNA spanning amino acids 439-1045 of human Topors (Fig. 1) was obtained by a yeast two-hybrid screening. After a nucleotide database search, an EST clone of IMAGE no. 2225874 was found to be a mouse homolog of human Topors. Since the entire sequence of this clone had not been determined, we determined its sequence and deposited it to the DDBJ databank (accession no. AB072395). Topors, a protein also named p53BP3 or Lun, was comprised of four PEST domains, a leucine zipper-like domain and a ring finger motif, and it is identified as topoisomerase I- or p53-binding protein (35-37) (Fig. 1A). The cDNA initially obtained in a two-hybrid screening covers both p53 and topoisomerase I-binding regions. Association of DJ-1 with full-sized Topors was first confirmed by using a two-hybrid method (data not shown).

Associations of DJ-1 with Topors in cells were then examined. To do this, FLAG-Topors was cotransfected with DJ-1-HA into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared from transfected cells, proteins in the extracts were immunoprecipitated with an anti-FLAG antibody or non-specific IgG, and the precipitates were blotted with an anti-DJ-1 or anti-Flag antibody (Fig. 1B). The FLAG antibody but not IgG was first confirmed to precipitate FLAG-tagged Topors (data not shown). The results also showed that DJ-1-HA and endogenously expressed DJ-1,

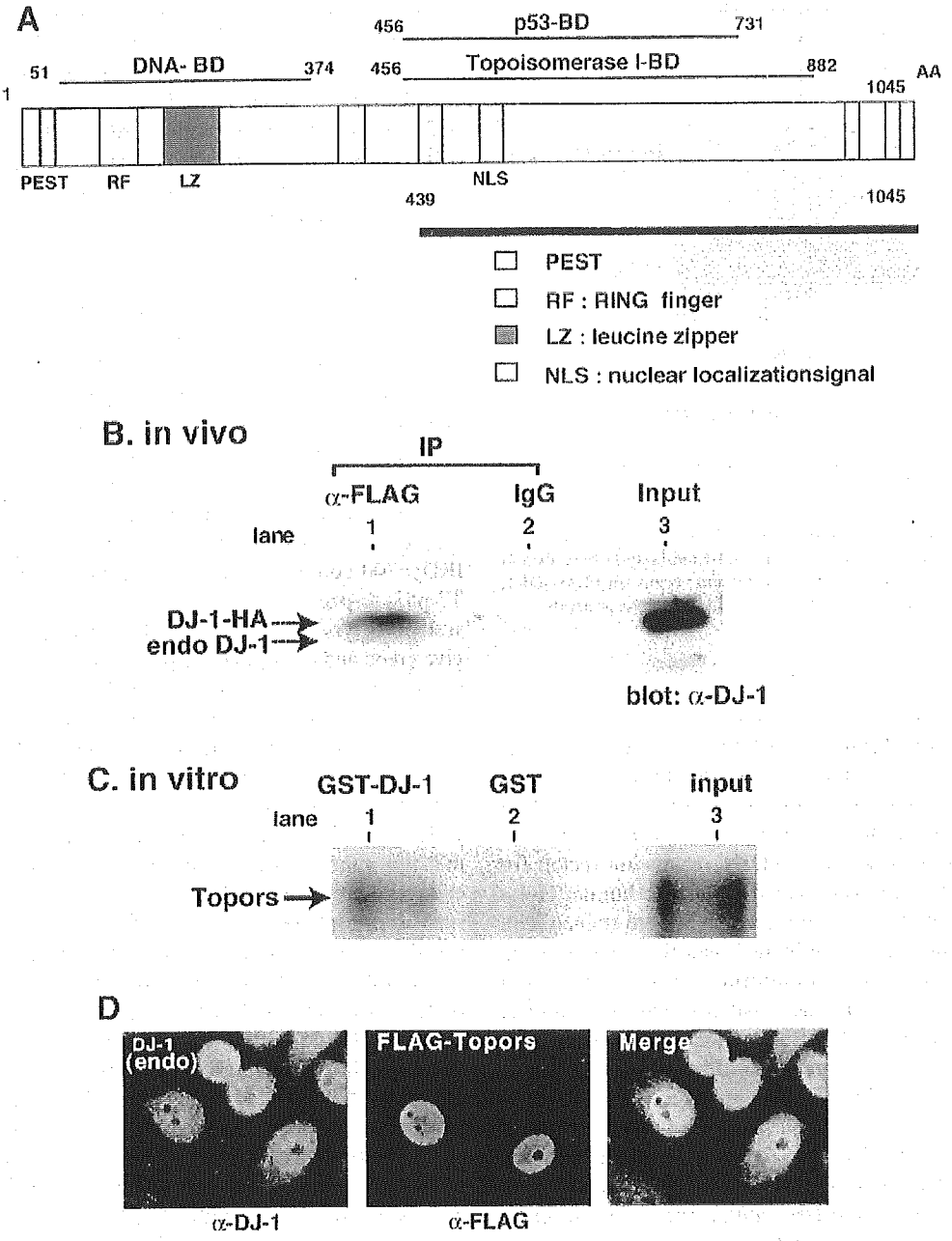

Figure 1. Association of DJ-1 with Topors. (A), Schematic drawings of Topors. RE, NLS and LZ indicate regions of the ring finger domain, nuclear localization signal and leucine zipper region, respectively. (B), DJ-1 and Topors were tagged with either HA or FLAG, and their expression vectors were transfected into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG antibody or non-specific IgG. The proteins in the precipitates were separated in a 12% polyacrylamide gel and blotted with an anti-DJ-1 antibody. (C), GST or GST-DJ-1 was expressed in *E. coli* BL21(DE3) and applied to glutathione-Sepharose 4B. ^{35}S-labeled Topors synthesized *in vitro* in a coupled transcription/translation system was then applied to the column. The labeled proteins that had bound to the column were separated in a gel and visualized by fluorography. (D), HeLa cells were transfected with FLAG-Topors by the calcium phosphate precipitation technique. Forty-eight hours after transfection, cells were fixed, reacted with an anti-DJ-1 polyclonal antibody and an anti-FLAG monoclonal antibody, and visualized with a FITC-conjugated anti-rabbit antibody and a rhodamine-conjugated anti-mouse antibody. Two figures were merged (Merge).

which migrated below DJ-1-HA, were co-immunoprecipitated with FLAG-Topors (Fig. 1B, lane 1), indicating that DJ-1 is associated with Topors in 293T cells. To examine direct bindings of DJ-1 with Topors , *in vitro* pull-down assays were then carried out. GST or GST-DJ-1 expressed in and prepared from *E. coli* was mixed with ^{35}S-labeled Topors synthesized *in vitro* in a reticulocyte lysate, and bound proteins were separated in a gel and visualized by fluorography (Fig. 1C). The results showed that GST-DJ-1, but not GST, directly bound to Topors (Fig. 1C, lanes 1 and 2, respectively). These results from *in vivo* and *in vitro* binding assays suggest that DJ-1 forms complexes with Topors.

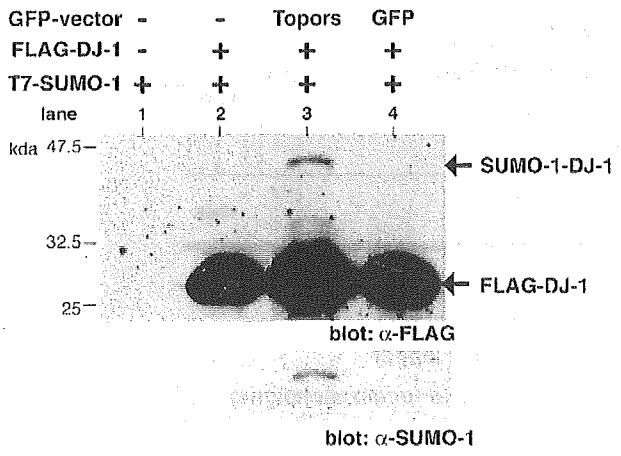

Figure 2. SUMO-1 conjugation to DJ-1 by Topors. H1299 cells were co-transfected with FLAG-DJ-1, T7-SUMO-1, and GFP-Topors or GFP alone. Forty-eight hours after transfection, proteins extracted from transfected cells were immunoprecipitated with an anti-FLAG antibody and blotted with the anti-FLAG antibody or an anti-SUMO-1 antibody as described in Materials and methods. The amounts of expression vectors for FLAG-DJ-1, T7-SUMO-1, and GFP-Topors or GFP were 5, 2 and 5 μg, respectively.

Previous studies have shown that DJ-1 is localized both in the cytoplasm and nucleus in human HeLa cells and is translocated from the cytoplasm to nuclei during the S-phase of the cell cycle upon induction by mitogen (1) and that Topors is located in the nucleus (35-37). To determine the cellular localization of Topors and DJ-1, expression vectors for FLAG-tagged Topors were transfected into human HeLa cells. Two days after transfection, the cells were stained with anti-DJ-1 and anti-FLAG antibodies, and the proteins were detected by FITC- and rhodamine-conjugated second antibodies, respectively, and then visualized under a confocal laser micro-scope (Fig. 1D). Endogenously expressed DJ-1 (green) and Topors (red) were co-localized in the nucleus as shown by the yellow color (Fig. 1D, Merge).

SUMO-1 conjugation to DJ-1 by Topors. Topors contains a ring finger domain that has been observed in E3-ubiquitin ligase or E3-SUMO-1 ligase, and *Drosophila* Topors has recently been shown to possess E3-ubiquitin ligase activity (38). Since DJ-1 is known not to be degraded by the ubiquitin-proteasome system, we investigated whether DJ-1 is sumoylated by Topors. To do this, human H1299 cells were cotransfected with FLAG-DJ-1 and GFP-SUMO-1 or GFP vector alone. Forty-eight hours after transfection, cells were lysed in SDS-containing buffer, boiled, and immunoprecipitated with an anti-FLAG antibody. Precipitates were blotted with the anti-FLAG antibody or an anti-SUMO-1 antibody (Fig. 2). In addition to a 26-kDa band corresponding to FLAG-DJ-1, a 44-kDa band appeared in the immunoprecipitate with the anti-FLAG antibody in the case of cotransfection with Topors but not GFP (Fig. 2, upper panel, lanes 3 and 4, respectively). Since SUMO-1 conjugation to proteins is known to give an addition of approximately 20 kDa to proteins in SDS-polyacrylamide gel and since the 44-kDa band reacted with the anti-SUMO-1 antibody, the results clearly showed that DJ-1 was sumoylated *in vivo* in H1299 cells (Fig. 2, lower panel, lane 3).

Binding of DJ-1 to p53. Since Topors is also a p53-binding protein (36) and expression of both DJ-1 and p53 was induced by several stresses (26-30, for review see ref. 39, refs. therein), it is possible that DJ-1 also binds to p53. T7-p53 was co-transfected with FLAG-DJ-1 into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared from transfected cells, proteins in the extracts were immunoprecipitated with an anti-FLAG antibody or non-specific IgG, and the precipitates were blotted with an anti-p53 polyclonal or anti-Flag monoclonal antibody (Fig. 3A-a). The FLAG antibody, but not IgG, was first confirmed to precipitate FLAG-DJ-1 (data not shown). Two bands with strong and weak intensities were also observed in the lane indicating proteins coimmunoprecipitated with FLAG-DJ-1, and four bands were observed in the input lane (Fig. 3A-a). To characterize these bands, 293T cells were transfected with T7-p53 and proteins in cells were subjected to Western blotting with the anti-p53 antibody. Proteins on the filter were then reacted with IRDye800-conjugated anti-rabbit IgG and IRDye700-conjugated anti-mouse IgG to detect all p53 and T7-p53, respectively, and visualized using an infrared imaging system (Odyssey, LI-COR), in which IRDye800 and IRDye700 give green and red signals, respectively (Fig. 3A-b). The results showed that while an anti-T7 antibody detected two bands only in T7-p53 transfected cells, the anti-p53 antibody detected four bands and two bands in transfected and non-transfected cells, respectively (Fig. 3A-b, lanes 3, 4, 1 and 2, respectively). When these two figures were merged, the upper two bans turned yellow and the lower two bands remained green, indicating that the upper and lower bands correspond to T7-p53 and endogenously expressed p53. It is known that modifications such as phosphorylation and acetylation of 53 occur in cells and these appear as bands with different mobilities on poly-acrylamide gel. Since phosphorylated p53 runs slowly compared to non-modified p53, it is thought that the upper and lower bands in this figure correspond to phosphorylated and non-phosphorylated p53, respectively. Although the precise modification of p53 was not clear in this experiment, the results suggest that DJ-1 was preferentially associated with modified forms of T7-p53 and endogenous p53 in cells. Association of Topors with p53 was also examined. 293T cells were transfected with FLAG-Topors and T7-p53, and proteins were analyzed as described above (Fig. 3A-c). As described previously (36,40), Topors were found to bind to both T7-p53 and endogenous p53, and modified and non-modified forms of p53 were found to bind to Topors.

To examine direct binding of DJ-1 with p53, *in vitro* pull-down assays were then carried out. GST or GST-DJ-1 expressed in and prepared from *E. coli* was mixed with ^{35}S-labeled p53 synthesized *in vitro* in a reticulocyte lysate, and bound proteins were separated in a gel and visualized by fluorography (Fig. 3B). The results showed that GST-DJ-1 but not GST directly bound to p53 (Fig. 3B, lanes 2 and 3, respectively) but that two bands that may correspond to modified and non-modified forms of p53 bound to GST-DJ-1. Although the reason for this discrepancy in the between *in vivo* and *in vitro* results is not known at present, the results suggest that DJ-1 forms complexes with p53.

Expression of p53 and DJ-1 has been shown to be induced by UV irradiation (Shinbo *et al*, unpublished data). We there-

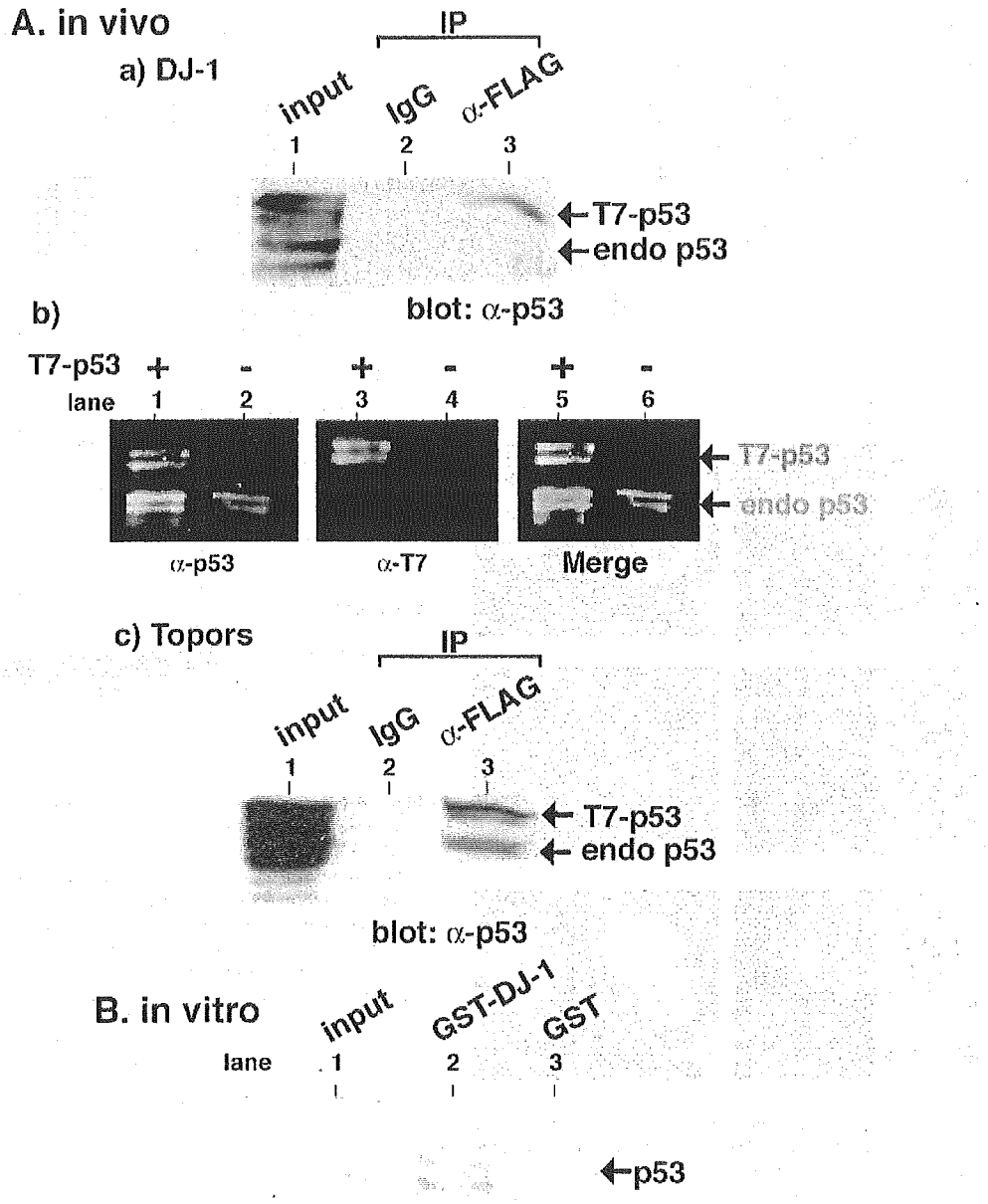

Figure 3. Association of DJ-1 with p53. (A-a), DJ-1 and p53 were tagged with either FLAG or T7, and their expression vectors were transfected into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG antibody or non-specific IgG. The proteins in the precipitates were separated in a 10% polyacrylamide gel and blotted with an anti-p53 antibody. (A-b), 293T cells were transfected with T7-p53. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were blotted with both a rabbit anti-p53 antibody and a mouse T7-antibody. Filters were then reacted with an IRDye800-conjugated anti-rabbit IgG and an IRDye700-conjugated anti-mouse IgG and visualized using an infrared imaging system (Odyssey, LI-COR). (A-c), Expression vectors for FLAG-Topors and T7-p53 were transfected into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were subjected to immunoprecipitation (IP) with an anti-FLAG antibody or non-specific IgG, followed by Western blotting with an anti-p53 antibody as described in (A). (B), GST or GST-DJ-1 was expressed in *E. coli* BL21(DE3) and applied to glutathione-Sepharose 4B. ^{35}S-labeled p53 synthesized *in vitro* in a coupled transcription/translation system was then applied to the column. The labeled proteins that had bound to the column were separated in a gel and visualized by fluorography.

fore investigated the effect of UV irradiation on complex formation and cellular localization of DJ-1 and p53. H1299 cells, which are p53-negative cells, were transfected with FLAG-p53. Forty-eight hours after transfection, cells were irradiated with 20 J/m^2 of UV and cultured for another 30 min. Protein extracts from cells were then immunoprecipitated with an anti-FLAG antibody or non-specific IgG and the immunoprecipitates were blotted with an anti-DJ-1 antibody (Fig. 4A). DJ-1-p53 complex was precipitated with the anti-FLAG antibody but not IgG, and the amount of the complex was found to be increased by UV irradiation (Fig. 4A, lanes 2 and 4). Mouse ME180 cells, which are p53-positive cells, were irradiated with 20 J/m^2 of UV. Thirty minutes after UV irradiation, cells were fixed with anti-DJ-1 and anti-p53

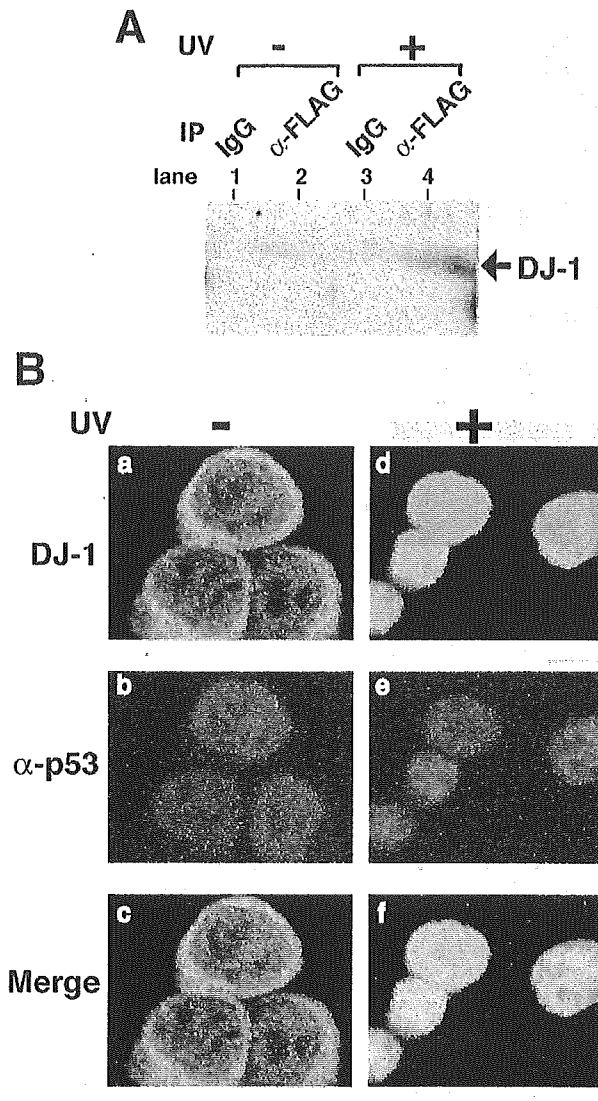

Figure 4. Stimulation of DJ-1-p53 complex formation by UV irradiation. (A), H1299 cells were transfected with FLAG-p53 by the calcium phosphate precipitation technique. Forty-eight hours after transfection, cells were irradiated with 20 J/m^2 of UV. Cell extracts were prepared at 30 min after UV irradiation, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG antibody or non-specific IgG. The proteins in the precipitates were separated in a 12% polyacrylamide gel and blotted with an anti-DJ-1 antibody. (B), ME180 cells were irradiated with 20 J/m^2 of UV. Thirty minutes after UV irradiation, cells were fixed, reacted with an anti-DJ-1 polyclonal antibody and an anti-p53 monoclonal antibody, and visualized with a FITC-conjugated anti-rabbit antibody and an rhodamine-conjugated anti-mouse antibody. ME180 cells that had not been subjected to UV irradiation were also treated in the same manner as UV-irradiated cells. Two figures were merged (c and f).

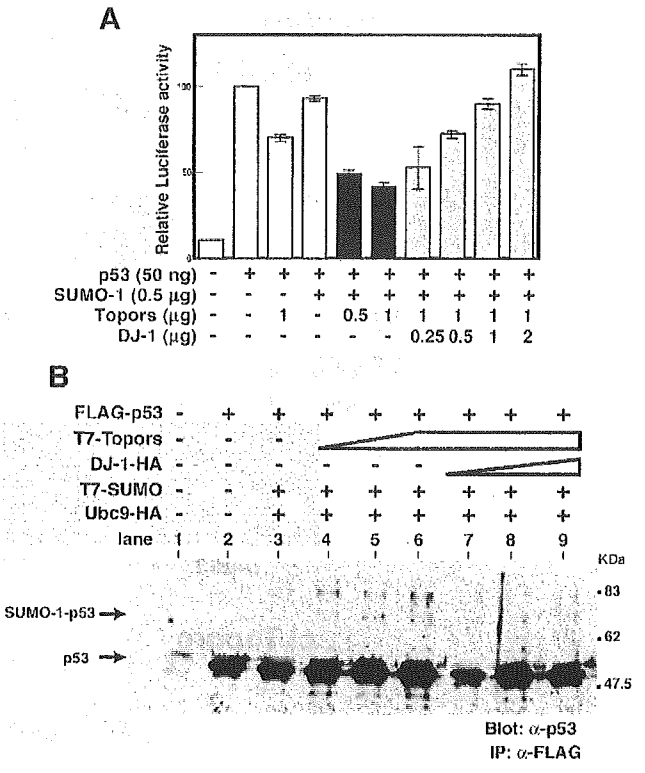

Figure 5. Effect of transcription activity of p53 by Topors and DJ-1. (A), 293T cells were transfected with various combinations of pcDNA3-FLAG-p53, pcDNA3-T7-Topors, pcDNA3-DJ-1-HA and pcDNA3-T7-SUMO-1 together with 100 ng of PG13-Luc. Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured. (B), H1299 cells were transfected with various combinations of FLAG-p53, T7-Topors, DJ-1-HA, T7-SUMO-1 and UBC9-HA. Forty-eight hours after transfection, proteins extracted from transfected cells were immunoprecipitated with an anti-FLAG antibody and blotted with an anti-p53 antibody or an anti-SUMO-1 antibody as described in Materials and methods. The amounts of expression vectors for FLAG-p53, T7-SUMO-1 and UBC9-HA were 5, 2 and 2 μg, respectively, those for T7-Topors and DJ-1-HA were 2.5, 5.0 and 7.5 μg in lanes 4, 5 and 6-9, and those for DJ-1-HA were 2.5, 5.0 and 7.5 μg, respectively.

antibodies, and the proteins were detected by fluorescein isothiocyanate- and rhodamine-conjugated second antibodies, respectively, and then visualized under a confocal laser microscope (Fig. 4B). While p53 was located in the nucleus before and after UV irradiation, DJ-1 was found to be localized strongly and weakly in the cytoplasm and nucleus, respectively, and to be partially colocalized with p53 in the nucleus before UV irradiation (Fig. 4B, a-c). After UV irradiation of cells, DJ-1 was strongly expressed and translocated to the nucleus, in which DJ-1 (green) and p53 (red) were co-localized as shown by the yellow color (Fig. 4B, d-f). Under this condition, expressions of DJ-1 and p53 began to be induced at 30 min and 60 min after UV irradiation, respectively (Shinbo *et al*, unpublished data). These results indicate that DJ-1 was associated with p53 in the nucleus.

Restoration of p53 transcription activity repressed by Topors. We then examined the effects of Topors and DJ-1 on transcription activity of p53. Since DJ-1 was sumoylated by Topors, the effect of SUMO-1 was also examined. H1299 cells, which are p53-negative cells, were transfected with various combinations of FLAG-p53, T7-SUMO-1 and DJ-1-HA together with PG13-Luc, which is a luciferase reporter construct containing a 13 x p53-responsive element (41). Forty-eight hours after transfection, cells were lysed and their luciferase activities were measured (Fig. 5A). Topors was found to repress the p53 transcription activity to 75% of that without transfection of Topors and was found to marginally repress p53 activity. When both Topors and SUMO-1 were

cotransfected, both proteins repressed the transcription activity of p53 in a dose-dependent manner, and about 60% of the p53 activity was repressed after 1 μg of pcDNA3-FLAG-Topors was transfected into cells (Fig. 5A), suggesting that Topors acts as a negative regulator of p53 and that its activity was stimulated by SUMO-1. DJ-1-HA was then cotransfected into H1299 cells together with 1 μg of pcDNA3-FLAG-Topors and 0.5 μg of pcDNA3-T7-SUMO-1, and the luciferase activities were determined (Fig. 5A). The results showed that DJ-1 restored p53 transcription activity repressed by Topors in a dose-dependent manner. Transfection of pEGFP-N1 or pcDNA3 vector into cells did not restore the p53 activity repressed by Topors and SUMO-1 (data not shown).

Since Topors sumoylated DJ-1 and p53 was reported to be sumoylated by PIAS family proteins, the effect of Topors on SUMO-1 conjugation to p53 was then examined. H1299 cells were cotransfected with various combinations of FLAG-p53, T7-SUMO-1, UBC9-HA and DJ-1-HA together with PG13-Luc. Forty-eight hours after transfection, the cell extracts were immunoprecipitated with an anti-FLAG antibody and the precipitates were subjected to Western blotting using an anti-p53 antibody or an anti-SUMO-1 antibody (Fig. 5B). The results showed that in addition to a 53-kDa band corresponding to FLAG-p53, a 73-kDa band appeared in the immunoprecipitate with the anti-FLAG antibody in the case of cotransfection with Topors and that intensity of this band increased in a dose-dependent manner (Fig. 5B, lanes 4-6). The 73-kDa band reacted with the anti-SUMO-1 antibody (data not shown), suggesting that p53 was sumoylated *in vivo* in H1299 cells. In the case of cotransfection of DJ-1-HA with T7-Topors into cells, however, the 73-kDa band disappeared (Fig. 5B, lanes 7-9). These results suggest that sumoylation of p53 by Topors represses the p53 transcription activity and that DJ-1 restored its repressed activity through SUMO-1 conjugation.

Discussion

In this study, we found that DJ-1 bound to both Topors and p53 and that SUMO-1 conjugation of DJ-1 and p53 were stimulated by Topors. We then found that the transcription activity of p53 was inhibited by Topors and SUMO-1 concomitant with an increase in the sumoylated form of p53 and that DJ-1 restored the repressed activity of p53. These findings, together with the fact that Topors was sumoylated (42), suggest that there is a SUMO-1 conjugation network between p53 and DJ-1. p53 has been shown to be sumoylated by PIAS1 or PIASxα as an E3 ligase (16,17), and controversial results regarding the effects of sumoylation on p53 transcription activity have been reported (15). We found that DJ-1 bound to PIASxα and was sumoylated by PIASxα and PIASy as E3 ligases to exert its full activities (12; Shinbo *et al*, unpublished data). Expression of DJ-1, like that of p53, is stimulated by several stresses, and DJ-1 plays a role in anti-oxidative stress (29). DJ-1 and p53 therefore seem to have similar characters, suggesting that DJ-1 and p53 use the same machinery through the SUMO-1 conjugation system and that both proteins function mutually against stresses. We are now examining whether Topors works as a bona fide SUMO-1 E3 ligase both for DJ-1 and p53.

The mechanisms to restore repressed transcription activity of p53 by DJ-1 are not known at present. One possibility is that DJ-1 disrupts p53-Topors interaction by absorbing Topors. In this case, DJ-1 may use Topors as an E3 SUMO-1 ligase for DJ-1 instead of p53 or DJ-1 may degrade Topors due to DJ-1 protease activity (43; Niki *et al*, unpublished data). In either case, we do not have experimental data yet.

Several kinds of modification, including phosphorylation, acetylation, sumoylation and methylation, occur on p53 and these modifications affect p53 activity positively or negatively. DJ-1 was found to preferentially bind to a modified form of p53 in cells but to have potential activity to bind to modified and non-modified forms of p53 *in vitro*. These results suggest that a specific signal such as oxidative stress triggers modification of p53 and/or translocation of DJ-1 from the cytoplasm to nucleus to prevent cells from undergoing apoptosis or senescence. DJ-1 is a multi-functional protein and loss of its function may trigger cell death. Studies on the functional interaction between DJ-1 and p53 are therefore needed.

Acknowledgements

We are grateful to B. Vogelstein for providing PG13-Luc. We also thank Yoko Misawa and Kiyomi Takaya for their technical assistance. This study was supported by Grants-in-Aid from the Ministry of Education, Science, Culture, Sports and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan.

References

1. Nagakubo D, Taira T, Kitaura H, Ikeda M, Tamai K, Iguchi-Ariga SMM and Ariga H: DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem Biophys Res Commun 31: 509-513, 1997.
2. Taira T, Takahashi K, Kitagawa R, Iguchi-Ariga SMM and Ariga H: Molecular cloning of human and mouse DJ-1 genes and identification of Sp1-dependent activation of the human DJ-1 promoter. Gene 263: 285-292, 2001.
3. Le Naour F, Misek DE, Krause MC, Deneux L, Giordano TJ, Scholl S and Hanash SM: Proteomics-based identification of RS/DJ-1 as a novel circulating tumor antigen in breast cancer. Clin Cancer Res 7: 3328-3335, 2001.
4. Miura K, Bowman ED, Simon R, Peng AC, Robles AI, Jones RT, Katagiri T, He P, Mizukami H, Charboneau L, Kikuchi T, Liotta LA, Nakamura Y and Harris CC: Laser capture microdissection and microarray expression analysis of lung adenocarcinoma reveals tobacco smoking- and prognosis-related molecular profiles. Cancer Res 62: 3244-3250, 2002.
5. Hod Y, Pentyala SN, Whyard TC and El-Maghrabi MR: Identification and characterization of a novel protein that regulates RNA-protein interaction. J Cell Biochem 72: 435-444, 1999.
6. Wagenfeld A, Yeung CH, Strupat K and Cooper TG: Shedding of a rat epididymal sperm protein associated with infertility induced by ornidazole and alpha-chlorohydrin. Biol Reprod 8: 1257-1265, 1998.
7. Welch JE, Barbee RR, Roberts NL, Suarez JD and Klinefelter GR: SP22: a novel fertility protein from a highly conserved gene family. J Androl 19: 385-393, 1998.
8. Wagenfeld A, Yeung CH, Shivaji S, Sundareswaran VR, Ariga H and Cooper TG: Expression and cellular localization of contraception-associated protein. J Androl 21: 954-963, 2000.
9. Klinefelter GR, Welch JE, Perreault SD, Moore HD, Zucker RM, Suarez JD, Roberts NL, Bobseine K and Jeffay S: Localization of the sperm protein SP22 and inhibition of fertility *in vivo* and *in vitro*. J Androl 23: 48-63, 2002.
10. Okada M, Matsumoto K, Niki T, Taira T, Iguchi-Ariga SMM and Ariga H: DJ-1, a target protein for an endocrine disrupter, participates in the fertilization in mice. Biol Pharm Bull 25: 853-856, 2002.

11. Yoshida K, Sato Y, Yoshiike M, Nozawa S, Ariga H and Iwamoto T: Immunocytochemical localization of DJ-1 in human male reproductive tissue. Mol Reprod Dev 66: 391-397, 2003.
12. Takahashi K, Taira T, Niki T, Seino C, Iguchi-Ariga SMM and Ariga H: DJ-1 positively regulates the androgen receptor by impairing the binding of PIASx alpha to the receptor. J Biol Chem 276: 37556-37563, 2001.
13. Niki T, Takahashi-Niki K, Taira T, Iguchi-Ariga SMM and Ariga H: DJBP: a novel DJ-1-binding protein, negatively regulates the androgen receptor by recruiting histone deacetylase complex, and DJ-1 antagonizes this inhibition by abrogation of this complex. Mol Cancer Res 1: 247-261, 2003.
14. Taira T, Iguchi-Ariga SMM and Ariga H: Co-localization with DJ-1 is essential for the androgen receptor to exert its transcription activity that has been impaired by androgen-antagonists. Biol Pharm Bull 27: 574-577, 2004.
15. Seeler JS and Dejean A: Nuclear and unclear functions of SUMO. Nat Rev Mol Cell Biol 4: 690-699, 2003.
16. Kahyo T, Nishida T and Yasuda Y: Involvement of PIAS1 in the sumoylation of tumor suppressor p53. Mol Cell 8: 713-718, 2001.
17. Schmidt D and Muller S: Members of the PIAS family act as SUMO ligases for c-Jun and p53 and repress p53 activity. Proc Natl Acad Sci USA 99: 2872-7287, 2002.
18. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA and Heutink P: Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 299: 256-259, 2003.
19. Abou-Sleiman PM, Healy DG, Quinn N, Lees AJ and Wood NW: The role of pathogenic DJ-1 mutations in Parkinson's disease. Ann Neurol 54: 283-286, 2003.
20. Hague S, Rogaeva E, Hernandez D, Gulick C, Singleton A, Hanson M, Johnson J, Weiser R, Gallardo M, Ravina B, Gwinn-Hardy K, Crawley A, St George-Hyslop PH, Lang AE, Heutink P, Bonifati V, Hardy J and Singleton A: Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. Ann Neurol 54: 271-274, 2003.
21. Hedrich K, Djarmati A, Schafer N, Hering R, Wellenbrock C, Weiss PH, Hilker R, Vieregge P, Ozelius LJ, Heutink P, Bonifati V, Schwinger E, Lang AE, Noth J, Bressman SB, Pramstaller PP, Riess O and Klein C: DJ-1 (PARK7) mutations are less frequent than Parkin (PARK2) mutations in early-onset Parkinson disease. Neurology 62: 389-394, 2004.
22. Heikkila R and Cohen G: Inhibition of biogenic amine uptake by hydrogen peroxide: a mechanism for toxic effects of 6-hydroxydopamine. Science 172: 1257-1258, 1971.
23. Nicklas WJ, Youngster SK, Kindt MV and Heikkila RE: MPTP, MPP^+ and mitochondrial function. Life Sci 40: 721-729, 1987.
24. Lotharius J and O'Malley KL: The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity. J Biol Chem 275: 38581-38588, 2000.
25. Jenner P: Oxidative stress in Parkinson's disease. Ann Neurol 53 (Suppl 3): S26-S36, 2003.
26. Mitsumoto A, Nakagawa Y, Takeuchi A, Okawa K, Iwamatsu A and Takanezawa Y: Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic Res 35: 301-310, 2001.
27. Mitsumoto A and Nakagawa Y: DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 35: 885-893, 2001.
28. Srisomsap C, Subhasitanont P, Otto A, Mueller EC, Punyarit P, Wittmann-Liebold B and Svasti J: Detection of cathepsin B up-regulation in neoplastic thyroid tissues by proteomic analysis Proteomics 2: 706-712, 2002.
29. Taira T, Saito Y, Niki T, Iguchi-Ariga SMM, Takahashi K and Ariga H: DJ-1 has a role in antioxidative stress to prevent cell death. EMBO Rep 5: 213-218, 2004.
30. Kinumi T, Kimata J, Taira T, Ariga H and Niki E: Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide mediated oxidation *in vivo* in human umbilical vein endothelial cells. Biochem Biophys Res Commun 317: 722-728, 2004.
31. Bandopadhyay R, Kingsbury AE, Cookson MR, Reid AR, Evans IM, Hope AD, Pittman AM, Lashley T, Canet-Aviles R, Miller DW, McLendon C, Strand C, Leonard AJ, Abou-Sleiman PM, Healy DG, Ariga H, Wood NW, De Silva R, Revesz T, Hardy JA and Lees AJ: The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. Brain 127: 420-430, 2004.
32. Yokota T, Sugawara K, Ito K, Takahashi R, Ariga H and Mizusawa H: Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. Biochem Biophys Res Commun 312: 1342-1348, 2003.
33. Takahashi C, Harada Y, Ariga H and Iguchi-Ariga SMM: Involvement of *Pim*-1 in DNA fragmentation in mouse NS-1 derived cells. Biochem Biophys Res Comm 215: 538-546, 1995.
34. Graham FL and van der Eb AJ: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456-467, 1973.
35. Haluska P Jr, Saleem A, Rasheed Z, Ahmed F, Su EW, Liu RF and Rubin EH: Interaction between human topoisomerase I and a novel RING finger/arginine-serine protein. Nucleic Acids Res 27: 2538-2544, 1999.
36. Zhou R, Wen H and Ao SZ: Identification of a novel gene encoding a p53-associated protein. Gene 235: 93-101, 1999.
37. Chu D, Kakazu N, Gorrin-Rivas MJ, Lu HP, Kawata M, Abe T, Ueda K and Adachi Y: Cloning and characterization of LUN, a novel ring finger protein that is highly expressed in lung and specifically binds to a palindromic sequence. J Biol Chem 276: 14004-14013, 2001.
38. Secombe J and Parkhurst SM: *Drosophila* Topors is a RING finger-containing protein that functions as a ubiquitin-protein isopeptide ligase for the hairy basic helix-loop-helix repressor protein. J Biol Chem 279: 17126-17133, 2004.
39. Hofseth LJ, Hussain SP and Harris CC: p53: 25 years after its discovery. Trends Pharmacol Sci 25: 177-181, 2004.
40. Saleem A, Dutta J, Malegaonkar D, Rasheed F, Rasheed Z, Rajendra R, Marshall H, Luo M, Li H and Rubin EH: The topoisomerase I- and p53-binding protein topors is differentially expressed in normal and malignant human tissues and may function as a tumor suppressor. Oncogene 23: 5293-300, 2004.
41. El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW and Vogelstein B: Definition of a consensus binding site for p53. Nat Genet 1: 45-49, 1992.
42. Weger S, Hammer E and Engstler M: The DNA topoisomerase I binding protein topors as a novel cellular target for SUMO-1 modification: characterization of domains necessary for subcellular localization and sumolation. Exp Cell Res 290: 13-27, 2003.
43. Olzmann JA, Brown K, Wilkinson KD, Rees HD, Huai Q, Ke H, Levey AI, Li L and Chin LS: Familial Parkinson's disease-associated L166P mutation disrupts DJ-1 protein folding and function J Biol Chem 279: 8506-8515, 2004.

Free Radical Research, October 2005; 39(10): 1091–1099

Taylor & Francis
Taylor & Francis Group

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

HONG MEI LI[1,2], TAKESHI NIKI[1], TAKAHIRO TAIRA[1,3], SANAE M. M. IGUCHI-ARIGA[1,2], & HIROYOSHI ARIGA[1,3]

[1]*CREST, Japan Science and Technology Corporation, Saitama 332-0012, Japan,* [2]*Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan, and* [3]*Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*

Accepted by Professor E. Niki

(Received 31 May 2005; in revised form 7 July 2005)

Abstract

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

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

Introduction

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

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

Correspondence: H. Ariga, Graduate School of Pharmaceutical Sciences, Hokkaido University, kita 12, Nishi 6, Kita-ku, Sapporo 060-0812, Japan. Tel: 81 11 707 3745. Fax: 81 11 706 4988. E-mail: hiro@pharm.hokudai.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis
DOI: 10.1080/10715760500260348