厚生労働科学研究費補助金
こころの健康科学研究事業

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パーキンソン病 PARK7 の原因遺伝子 DJ-1 の機能と創薬応用

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研究要旨

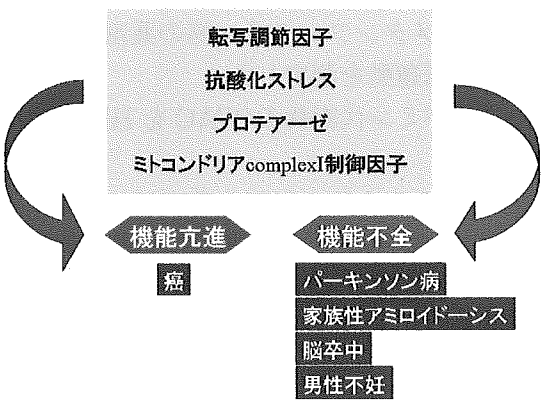
本研究室で新規癌遺伝子として単離同定した DJ-1 は一昨年度家族性パーキンソン病 PARK7 の原因遺伝子として同定され、現在まで１３箇所の DJ-1 遺伝子変異がパーキンソン病患者で報告されている。DJ-1 の機能解析と DJ-1 が関与するパーキンソン病発症機構の解明を目的として本研究がスタートした。DJ-1 は細胞増殖、受精、癌、不妊、パーキンソン病と多機能を有するが、本来の機能は転写調節、抗酸化ストレス、プロテアーゼ、更にミトコンドリア complex 1 の制御因子であることが明らかとし、これらのいずれかの欠損もパーキンソン病発症原因となる。また、弧発性パーキンソン病患者脳では不活性型と考えられる異常酸化型の DJ-1 が観察された。更に、パーキンソン病モデルラットの DJ-1 タンパク質を注射すると劇的にドーパミン神経細胞死の阻止とそれに伴う行動異常が改善された。更に、虚血性脳卒中ラットも同様に DJ-1 は抑制した。DJ-1 活性は 106 番目のシステイン(C106)の酸化状態で制御されるが、この C106 領域に結合する低分子化合物を in silico で同定した。これらの DJ-1 結合化合物は DJ-1 C106 の酸化を押さえることにより、活性型 DJ-1 を維持することで、酸化ストレス誘導神経細胞死を抑制した。ことから、DJ-1 と DJ-1 結合化合物はパーキンソン病および他の神経変性疾患の創薬ターゲットおよび治療薬になることが示された。

A. 研究目的

申請者が新規癌遺伝子として1997年に単離し研究をしてきたDJ-1は家族性パーキンソン病(PARK7)の原因遺伝子であることが報告され本研究プログラムが始まった。現在までにパーキンソン病患者で１３箇所のDJ-1遺伝子変異（欠損、点突然変異）が報告されている。パーキンソン病は酸化ストレス、それに伴う異常タンパク質の凝集、ミトコンドリアのcomplex 1の機能阻害が原因と考えられているが詳細な分子機構は明らかでなかった。我々は以前及び本プログラムによって、DJ-1は転写調節、抗酸化ストレス、プロテアーゼの３つの機能を有し、その機能破綻はパーキンソン病などの脳神経変性疾患、男性不妊、細胞癌化の原因となることを明らかとした。

そこで、DJ-1の機能解明を行うことでパーキンソン病の発症機構を解明し、同時に、パーキンソン病治療薬への展開を志向した。

DJ-1の機能と疾患



B. 研究方法

１．DJ-1の抗酸化ストレス機能

マウスNIH3T3細胞に野生型及びパーキンソン病に見られる各種変異体DJ-1を定常的に発現している細胞株、ドパミン性神経細胞株SH-SY5Y細胞に過酸化水素、6-ヒドロキシドパミンなどを細胞に加えた場合の細胞内活性酸素量をDCFH添加後、フローサイトメーターで定量した。

上術した各種細胞株、及びDJ-1遺伝子をターゲットとしたsiRNAを導入DJ-1ノックダウン細胞D2に過酸化水素を1-2 時間作用させ、生細胞数をMTT法で定量した。

2．DJ-1結合タンパク質とDJ-1との相互作用

既に同定しているHIPK1, Hsp70などのシャペロンとの相互作用を、免疫沈降→沈降物をWestern blotting解析で同定した。また、細胞内の共局在を免疫染色法で解析した。HIPK1に対する機能解析のために、H1299細胞にHIPK1に対するsiRNAを作用後、過酸化水素処理を行い、DJ-1発現と細胞死をMTTアッセイで測定した。

3．DJ-1のプロテアーゼ活性

精製DJ-1と各種タンパク質を反応させ、反応物をSDS-電気泳動で展開させた後、Western blottingでそれらのタンパク質の分解を検討した。また、パエル受容体、トランスサイレチン(TTR), α-シヌクレインとDJ-1 cDNAを293T、D2細胞にtransfection後、抗パエル受容体抗体でその分解を検討した。

4．DJ-1 の薬理活性

２つ存在するラット中脳黒質の内、左側の黒質に 6-ヒドロキシドパミン(6-OHDA)及び大腸菌だ発現後精製した GST-DJ-1, GST-DJ-1(L166P), GST を 6-OHDA と同時、あるいは後で注射した。数日後、脳切片を作成し、チロシンヒドロキシラーゼ(TH)抗体でドパミン作動性ニューロンを染色した。また、メタンフェタミン等の薬剤を投与し、ラットの回転運動を測定した。

また、ドパミン代謝物 DOPAC を HPLC で測定した。

5．DJ-1 結合化合物の単離と薬理活性

富士通 BioServer を使い、DJ-1 の機能調節領域と考えられる複数の領域に結合する化合物を科学技術教育協会から購入した化合物ライブラリーよりバーチャルスクリーニングした。ターゲット領域に対して得られた上位化合物をそれぞれ４－５化合物を購入し、6-OHDA あるいは過酸化水素処理した SHSY-5Y 細胞に対するこれらの化合物の効果を MTT アッセイで検討した。

6．DJ-1 によって転写調節される遺伝子の同定

マウス NIH3T3 細胞に野生型及びパーキンソン病に見られる各種変異体 L166P DJ-1 を定常的に発現している細胞株、DJ-1 ノックダウン細胞株 D2 で発現変動している遺伝子をアフィメトリックス GeneChip を用いてマイクロアレイ解析を行った。得られて遺伝子の発現を RT-PCR で解析した。

C. 研究結果

1．抗酸化ストレス能

酸化ストレスはパーキンソン病発症と深く関わっていることが以前から報告されている。この酸化ストレスの原因として神経毒を含む環境物質の存在が示唆されている。の中で、環境化学物質であるビスフェノール A (BPB)は胎児期マウスに投与すると成獣マウスになってパーキンソン病症状を示し、また成獣マウス/ラット脳に投与するとドーパミン神経細胞死が起こることが知られている。BPA 処理により細胞は活性酸素を発生し、ミトコンドリア complex 1 活性が低下した。DJ-1 はこれに反応し発現上昇しミトコンドリアに移行し complex 1 活性の維持を図るが、BPA 濃度上昇とともに酸化型 DJ-1 が増加し機能が低下した(Ooe et al. Toxicol. Sci, 2005)。更に、DJ-1 活性を調節する C106 が酸化された DJ-1 のみを認識する抗体を作成した。これは、パーキンソン病の診断に使用可能である。

2．DJ-1のSUMO-1化と機能調節

ヒトH1299細胞のDJ-1をイムノブロット法で解析したところ、DJ-1はSUMO-1修飾されていることが明らかとなった。DJ-1には１６個のリジンが存在するのでこれら全てをアルギニンに変化した変異DJ-1を作成しH1299細胞でのSUMO-1化の有無を解析したところ、種間で高度に保存されている130番目のリジン(K130)がSUMO-1化サイトであることが明らかとなった。そこでこれらの変異体を使いDJ-1の抗酸化、細胞増殖、細胞癌化能に対する機能を解析したところ、K130へのSUMO-1化が全ての機能に必須であった。更に、L166P変異体はすべてのリジンが、あるいはK130が多重にSUMO-1化されており、これによりミトコンドリアへの局在変動、不溶化が起こり、抗アポトーシス機能の消失が見られた(Shinbo et al. Cee Death. Diff. 2006)。ハンチントン病の封入体構成タンパク質もSUMO-1化されることが最近報告されており、神経変性疾患関連タンパク質のSUMO-1化は今後重要な課題となるかもしれない。

３．DJ-1のプロテアーゼ活性

昨年度にDJ-1のプロテアーゼ基質としてパエル受容体を報告した。更に、後述のtransthyretinに加えて、レヴィー小体の主要構成成分であるα-シヌクレインもDJ-1によってin vitro, in vivoで分解された。

４．DJ-1の転写調節遺伝子の同定

siRNAにより定常的にDJ-1発現がノックダウンされているNIH3T3細胞株作成し、これらの細胞、及びL166P導入NIH3T3細胞株を使用してDNAマイクロアレイ解析を行い親株と比較した。DJ-1ノックダウン細胞では極めて大きな遺伝子発現変動が起こり、特に、ストレス誘導、炎症、更にtau, synphilinといった神経変性疾患関連遺伝子、脳細胞のアポトーシス関連遺伝子が多数含まれていた。この中でtau遺伝子のプロモーター解析を行い、DJ-1はtau遺伝子の転写抑制を行っていることを明らかにした(Nishinaga et al. Neuscience Lett. 2005)。

５．酸化ストレスとDJ-1

DJ-1はHsp70, CHIPのシャペロンと結合し、酸化ストレスによりその結合が増加した。更に、酸化ストレスによりDJ-1はミトコンドリアに一部移行し、ミトコンドリアに局在するシャペロンであるmtHsp70/Grp75と結合、共局在した (Li et al. Free Radic. Res. 2005)。

またDJ-1はHIPK1に結合しHIPK1を分解することで、酸化ストレス誘導細胞死を抑制することを明らかにした(Sekito et al. Free Radic. Res. 2006)。

６．ミトコンドリアcomplex 1の制御因子としてのDJ-1

酸化ストレスと同時に、ミトコンドリアcomplex 1の機能低下はパーキンソン病発症の本体の１つと考えられている。DJ-1はミトコンドリアcomplex 1サブユニットNDUFA4と結合し、DJ-1ノックダウン細胞では著しくComplex 1の酵素活性が低下し、逆にDJ-1ノックダウン細胞にDJ-1を発現させるとComplex 1の酵素活性が復帰することから**complex 1の正の制御因子**であることを明らかとした。また、免疫電子顕微鏡観察よりDJ-1はミトコンドリア外膜、マトリックスに加えて、Complex 1が存在する内膜にも存在することが示された。

７．パーキンソン病の創薬ターゲットとしてのDJ-1

6-OHDA投与パーキンソン病モデルラットの中脳黒質にDJ-1タンパク質を直接注入すると、黒室と線条体の**ドーパミンニューロン死とが阻止され、ドーパミンが線条体に運搬されることにより、行動異常が劇的に阻止**されることを明らかにした。パーキンソン病患者に見られる変異DJ-1であるL166Pにはこの活性がない。線条体では低下していたドーパミン、その代謝物、ドーパミントランスポーター濃度も回復していた。これは6-OHDA投与による活性酸素を注入DJ-1が細胞内に移行し消去していると考えられた。これにより、DJ-1によるパーキンソン病の創薬の可能性が示された(Inden et al. submitted)。

更にDJ-1活性は106番目のシステイン(C106)の酸化状態で制御されるが、このC106領域に結合する低分子化合物をin silicoで同定した。これらのDJ-1結合化合物はDJ-1 C106の酸化を押さえることにより、活性型DJ-1を維持することで、酸化ストレス誘導神経細胞死を抑制した。ことから、DJ-1とDJ-1結合化合物はパーキンソン病および他の神経変性疾患の創薬ターゲットおよび治療薬になることが示された。

８．ドーパミン合成のキー酵素であるチロシンヒドロキシラーゼとDJ-1が結合することが明らかとなり、ドーパミン合成にDJ-1が関与する可能性が考えられた。

９．DJ-1と他の神経変性疾患

DJ-1ノックダウン細胞ではDJ-1プロテアーゼ基質の発現上昇が見られるはずである。これにより、家族性アミロイドニューロパシー(FAP)の原因タンパク質transthyretin (TTR)を同定した。DJ-1は(TTRとともに細胞外に分泌されTTRを分解した。しかしながら、酸化ストレス付加細胞ではDJ-1は分泌されない。FAP患者では不活性型DJ-1が血清中に分泌され、健常人、FAP発症前carrierでは分泌されないことより、DJ-1がTTR分解をさせないことがFAP発症原因と１つと考えられた。

脳虚血による脳梗塞モデルラットにDJ-1タンパク質を注入すると、神経細胞死と行動機能低下が60%以上軽減された。脳梗塞後、DJ-1は脳精髄液に分泌される。

以上より、DJ-1はパーキンソン病発症の全てのステップに関与し、更に酸化ストレスが関与する他の神経変性疾患発症にも関与することが示唆された。DJ-1結合化合物は申請細胞死を抑制する根本的な治療薬の可能性が高い。

D. 考察

DJ-1の基本的な４つの機能―転写調節、抗酸化ストレス、プロテアーゼ、ミトコンドリア complex 1の制御因―が存在することを明らかにした。転写調節因子としては以前からのアンドロゲン受容体の正の転写調節因子であることを明らかにしてきたが、脳で発現する、また脳変性疾患に関する遺伝子との関連は不明である。DJ-1 ノックダウン細胞を使用しての DNA マイクロアレイ解析より、tau, synphilin、transthyretin が候補となったので今後解析の必要がある。

２番目の抗酸化ストレス因子としての機能はパーキンソン病との関連で特に注目される。弧発性パーキンソン病が酸化ストレスによって生ずるという考え方は古くから支持されていたが、それを担う遺伝子、タンパク質の同定は行われていなかった。本研究においてDJ-1がその遺伝子の候補であり、実験的に証明した事は極めて意義が高い。弧発性パーキンソン病では実際に非還元型で活性型と考えられるDJ-1の消失と同時に、野生型DJ-1に見られる酸化状態と異なった DJ-1 が見られることはパーキンソン病発症過程での DJ-1 の抗酸化ストレス機能の重要性が示唆される。

３番目のプロテアーゼ機能もまたパーキンソン病発症と直接関わる現象である。家族性パーキンソン病原因遺伝子として同定された Parkin, UCH-L1 はユビキチンリガーゼ、脱ユビキチン化酵素である。パーキンソン病では tau, α-synuclein、Pael-receptor などのレヴィー体、あるいは小胞体への凝集が起こり、これらが細胞死を誘導することが知られており、ユビキチン化されプロテアソーム系で分解される。一方、DJ-1 はシステインプロテアーゼであり、プロテアソーム系と独立して Pael-receptor, α-synuclein を分解した。このように、生体にとって有害なタンパク質は複数の系により分解される事を示す最初の例である。DJ-1 のプロテアーゼ基質は Pael-receptor に加えて、家族性アミロードシスの原因タンパク質トランスサイレチン(TTR)を同定し、細胞内での DJ-1 による分解を示した。家族性アミロードシス患者では、TTR による凝集体と DJ-1 の局在はミラーイメージになっており、パーキンソン病患者のα-synuclein のケースと似ている。現在、α-シニュクレイントランスジェニックマウスと野生型 DJ-1 トランスジェニックマウスを掛け合わせており、今後解析が期待される。

パーキンソン病患者に見られる DJ-1 変異体は程度の差はあれ、すべて抗酸化ストレス機能が低下していた。最初に見つかった L166P は極めて異常であり、タンパク質としての構造がとれず、過剰に SUMO-1 化され不溶化されていた。DJ-1 の全ての機能に１３０番目のリジンへの SUMO-1 化は必須である。パーキンソン病患者では健常人と比較して不溶化 DJ-1 の存在が報告されており、これらの DJ-1 が過剰に SUMO-1 化されている可能性が存在し、今後臨床サイドと提携してこの可能性を検討したい。

パーキンソン病発症の１つとしてミトコンドリア complex 1 の機能低下がある。DJ-1 の４番目の機能として、complex 1 の正の制御因子であることが明らかとなった。実際、酸化ストレスを与える薬剤、またビスフェノール A などを細胞に投与するとミトコンドリア、complex 1 の酵素活性が低下し、同時に DJ-1 はミトコンドリアに移動しその機能維持に関与した。DJ-1 は DNA に損傷を与える紫外線などの照射によるストレス時には核に、また酸化ストレスではミトコンドリアとそのストレスを受けた場所に移動しストレス除去にあたるこ

とが明らかといなった。パーキンソン病患者では既にDJ-1自身の機能低下のため、このストレス防御ができなくなっていると考えられる。

今回、6-OHDAを中脳黒質に投与したパーキンソン病モデルラットにDJ-1タンパク質を直接投与すると黒質と線条体のドーパミンニューロン死の阻害とそれに伴う行動異常が劇的に回復した。投与DJ-1は速やかに細胞内に移行し活性酸素除去にあたったことより、DJ-1が創薬ターゲットになりうることを示したことは大きな成果であった。

現在パーキンソン病の治療薬としてはドパミン神経細胞死によって不足するドパミンの対症療法—ドパミン前駆体、ドパミン分解系の阻害薬の投与—が行われているが、この治療中も神経細胞死は進行する。従って、根本的に酸化ストレス誘導神経細胞死を抑制する薬剤の開発が必須である。今回、DJ-1の活性中心であるC106に結合し、酸化ストレス誘導神経細胞死を抑制する複数の化合物を同定した。現在、パーキンソン病モデルラットで薬理活性を検討しているが、細胞レベルに加え、動物レベルでも効果があれば画期的な治療薬の開発につながる。

E. 結論

本計画はDJ-1の基本的な機能解析を行い、その機能変動がいかにパーキンソン病発症の原因となるかを解明することを第1の目的とし、それらを踏まえて創薬応用を第2の目的とした。

前者においては、DJ-1は転写調節因子、抗酸化ストレス、プロテアーゼ、ミトコンドリアcomplex 1の機能調節の異なる4つの機能を有する事が明らかとなり、これらの機能消失がパーキンソン病の発症原因になると考えられた事は極めて重要である。DJ-1の機能消失が家族性パーキンソン病PARK7に加えて弧発性パーキンソン病の発症に関連する可能性がある。更に、パーキンソン病以外に複数の脳神経変性疾患患者でDJ-1の発現変動、不溶化などが報告され、また、Parkin, tau, α-synucleinとDJ-1との複合体形成も報告され始めたことから、広く脳神経変性疾患患者にDJ-1が関与する可能性がある。事実、FAPとの関連を検討し、DJ-1によるTTR分解と発症が密接に関与していることを示した。

また、パーキンソン病モデルラット脳にDJ-1タンパク質を直接投与するとドーパミンニューロン死とそれに伴う行動異常が劇的に阻止されることより、DJ-1による創薬が考えられた。また、同様に、虚血性脳卒中ラットもDJ-1注入により、大幅な病態改善が見られた。更に、DJ-1結合低分子化合物を複数同定し、DJ-1タンパク質と同様に酸化ストレス誘導神経細胞死を抑制したことは、今後の治療薬への発展を期待させるものであり大きな成果であった。

F. 健康危険情報

なし

G. 研究発表（本研究に関連するもののみを記す）

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The Takeda Science Foundation Symposium on PharmaSciences（武田科学振興財団薬科学シンポジウム） 12/5/05 東京

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Functional analysis of DJ-1 in Familial amyloidotic polyneuropathy
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抗酸化ストレスタンパク質 DJ-1 とパーキンソン病
第２７回日本薬学会年会シンポジウム　3/29/06 仙台

8) 大江洋正、平敬宏、有賀早苗、有賀寛芳
ビスフェノールAによる活性酸素種の増加と細胞傷害に対するDJ-1の防御機構
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多機能タンパク質 DJ-1 の家族性アミロードシスにおける機能解析
第２８回日分子生物学会年会　12/9/05 福岡

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第 28 回日分子生物学会年会　12/9/05 福岡

11) 宮崎　真、北浦広剛、松本健一、有賀早苗、有賀寛芳
DJ-1 に結合する低分子化合物の同定とその酸化ストレスによる神経細胞死抑制能
第２８回日分子生物学会年会　12/9/05 福岡

12) 北村佳久、平　敬宏、位田雅俊、高田和幸、谷口隆之、有賀早苗、有賀寛芳
パーキンソン病モデルラットにおけるドパミン神

経細胞死および行動異常の DJ-1 タンパク質による抑制効果
第２８回日分子生物学会年会　12/9/05 福岡

13) 平　敬宏、関戸亜弥、西永裕美、仁木剛史、仁木加寿子、有賀早苗、有賀寛芳
DJ-1 の HIPK1 への結合による細胞死制御と DJ-1 によって変動する遺伝子の解析
第２８回日分子生物学会年会　12/9/05 福岡

H. 知的財産権の出願・登録状況

1.特許取得

名称:神経変性疾患治療薬
出願番号: 特願 2005－339011
出願人: 北海道大学、
発明者: 有賀寛芳
特許出願日：平成１７年１１月２４日

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sekito, A., Taira, T., Niki, T., Iguchi-Ariga, S.M.M. & Ariga, H.	Stimulation of transforming activity of DJ-1 by Abstrakt, a DJ-1-binding protein	Int. J. Oncol.	26	685-690	2005
Shinbo, Y., Taira, T., Niki, T., Iguchi-Ariga, S.M.M. & Ariga, H.	DJ-1 restores p53 transcription activity inhibited by Topors/p53BP3	Int. J. Oncol.	26	641-648	2005
Li, H.M., Niki, T., Taira, T., Iguchi-Ariga, S.M.M. & Ariga, H.	Association of DJ-1 with chaperones and enhanced association and colocalization with mitochondrial Hsp70 by oxidative stress	Free Radic. Res.	39	1091-1099	2005
Nishinaga, H., Takahashi-Niki, K., Taira, T., Andreadis, A., Iguchi-Ariga, S.M.M. & Ariga, H	Expression profiles of genes in DJ-1-knockdown and L166P DJ-1 mutant cells	Neuroscience Lett.	390	54-59	2005
Ooe, H., Taira, T. , Iguchi-Ariga, S.M.M. & Ariga, H.	Induction of reactive oxygen species by bisphenol A and abrogation of bisphenol A-induced cell injury by DJ-1	Toxicol. Sci.	88	114-126	2005
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Sekito, A., Koide-Yoshida, S., Niki, T., Taira, T., Iguchi-Ariga, S.M.M. & Ariga, H.	DJ-1 interacts with HIPK1 and affects H2O2-induced cell death	Free Radic. Res.	40	155-165	2006
Ito, G., Ariga, H., Nakagawa, Y. & Iwatsubo, T.	Roles of distinct cysteine residues in *S*-nitrosylation and dimerization of DJ-1	Biochem. Biophys. Res. Commun.	339	667-672	2006

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

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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.

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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.

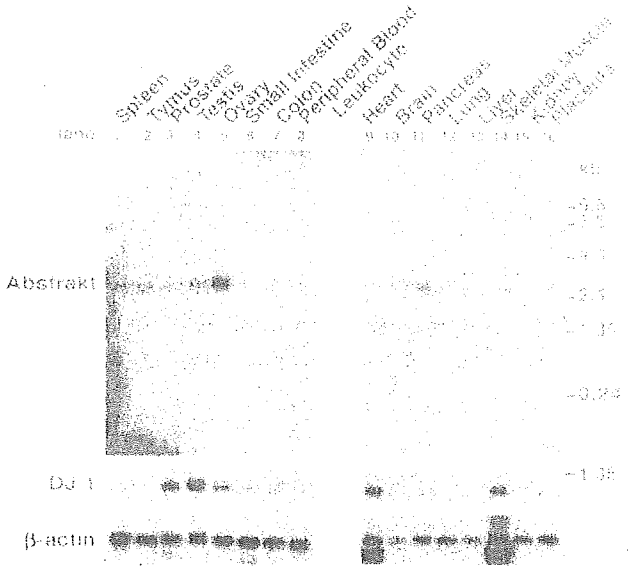

Figure 1. Expressions of Abstrakt mRNA in human tissues. Northern blot analysis was carried out using multiple Northern blot sheets of human tissues (Clontech) with labeled cDNAs of Abstrakt, DJ-1 and ß-actin.

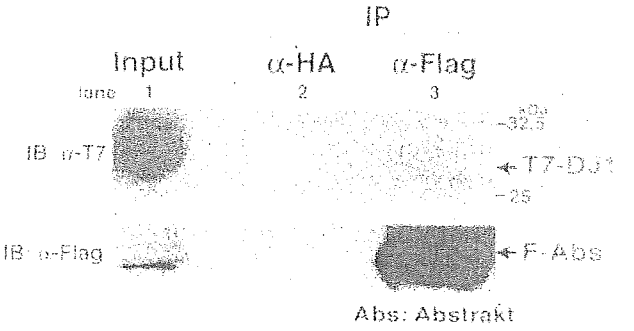

Figure 2. Association of DJ-1 with Abstrakt. DJ-1 and Abstrakt were tagged with either T7 or FLAG, and their expression vectors were transfected into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) 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

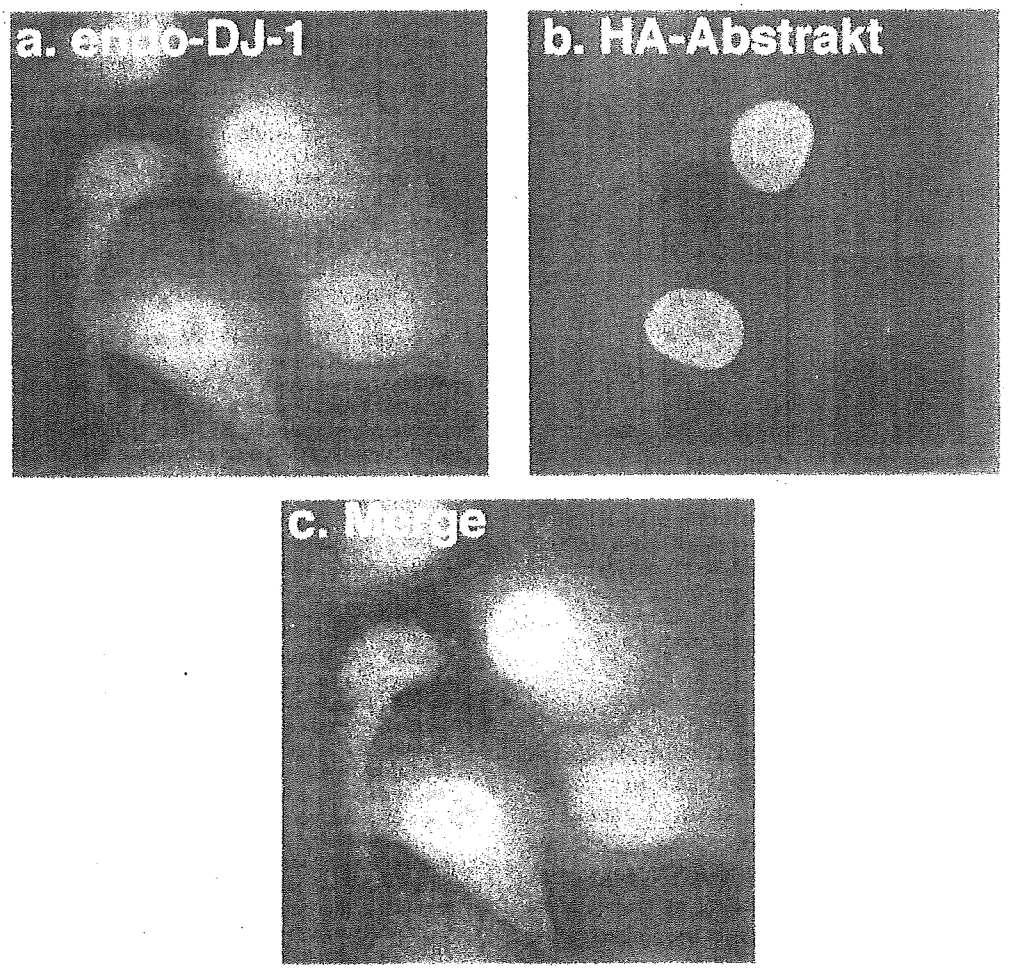

Figure 3. Colocalization of DJ-1 and Abstrakt. HeLa cells were transfected with HA-Abstrakt by the calcium phosphate precipitation technique (31). Forty-eight hours after transfection, cells were fixed, reacted with an anti-DJ-1 polyclonal antibody and an anti-HA monoclonal antibody, and visualized with 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

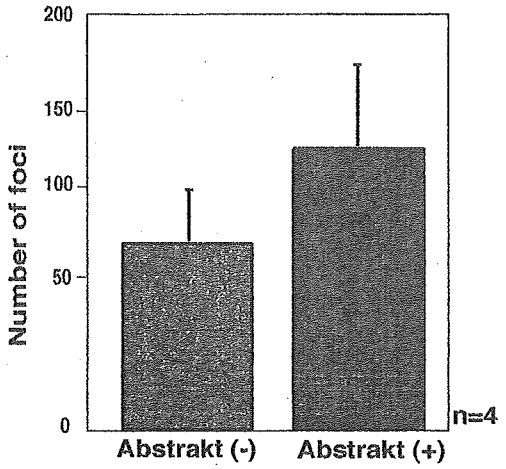

Figure 4. Effect of Abstrakt on the focus forming activity of DJ-1. Rat 3Y1 cells in a 10-cm dish were transfected with 1 μg of pH-ras and/or pcDNA3-F-DJ-1 by the calcium phosphate precipitation method (31), and the medium was changed every 3 days. Fourteen days after transfection, the cells were stained 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

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

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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.

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';

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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,

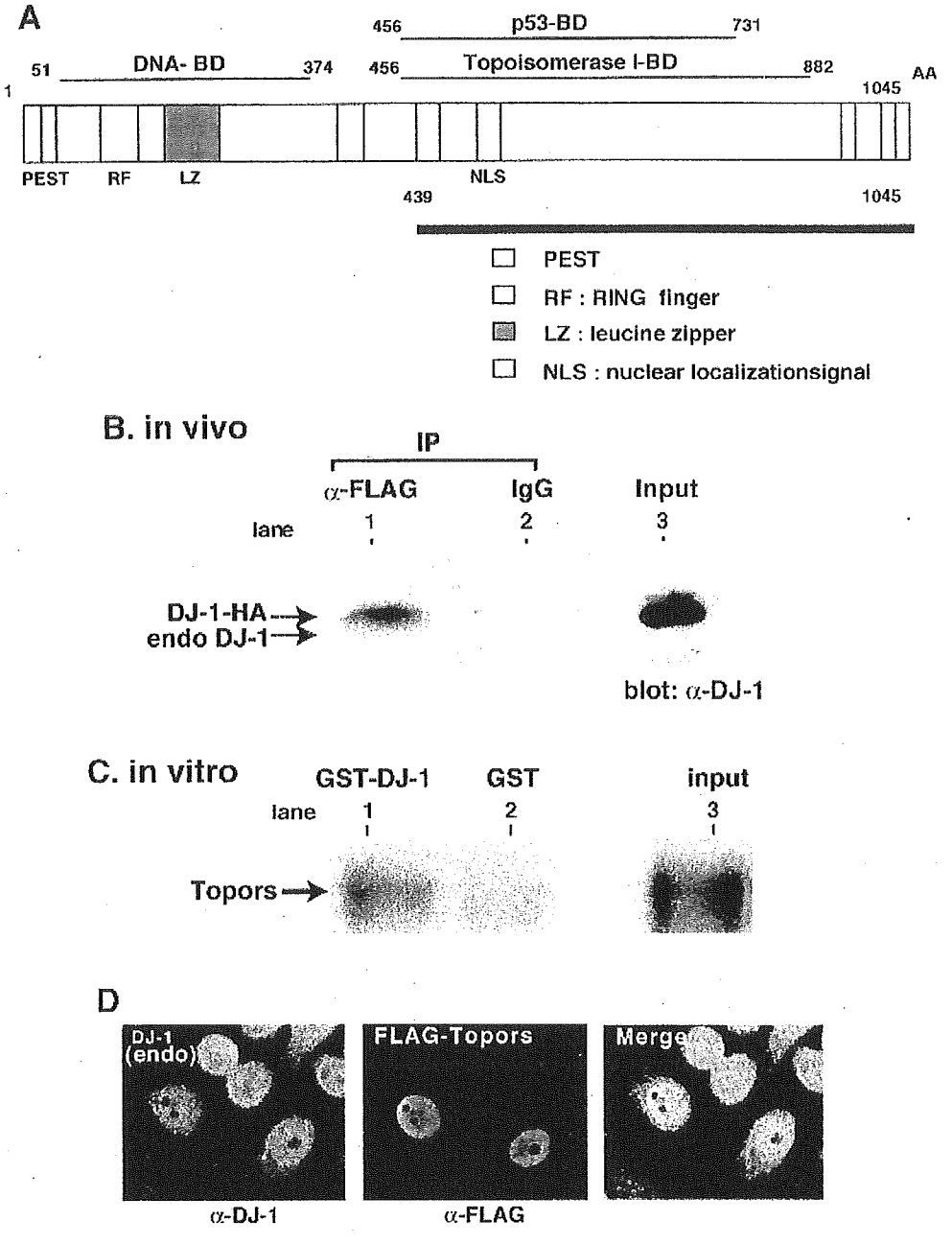

Figure 1. Association of DJ-1 with Topors. (A), Schematic drawings of Topors. RE, NLS and LZ indicate regions of the ring finger domain, nuclear localization signal and leucine zipper region, respectively. (B), DJ-1 and Topors were tagged with either HA or FLAG, and their expression vectors were transfected 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.

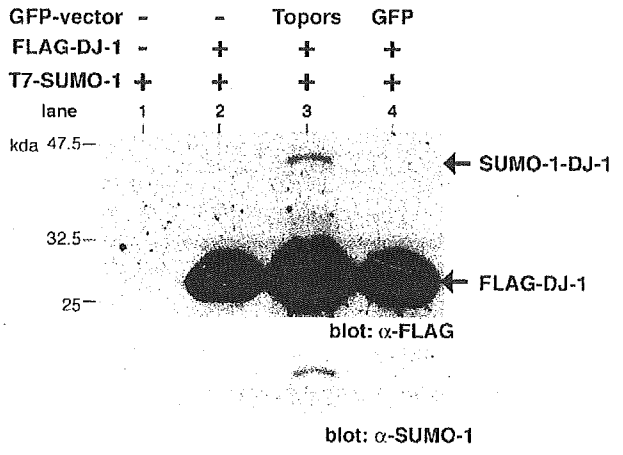

Figure 2. SUMO-1 conjugation to DJ-1 by Topors. H1299 cells were co-transfected with FLAG-DJ-1, T7-SUMO-1, and GFP-Topors or GFP alone. Forty-eight hours after transfection, proteins extracted from transfected cells were immunoprecipitated with an anti-FLAG antibody and blotted with the anti-FLAG antibody 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-

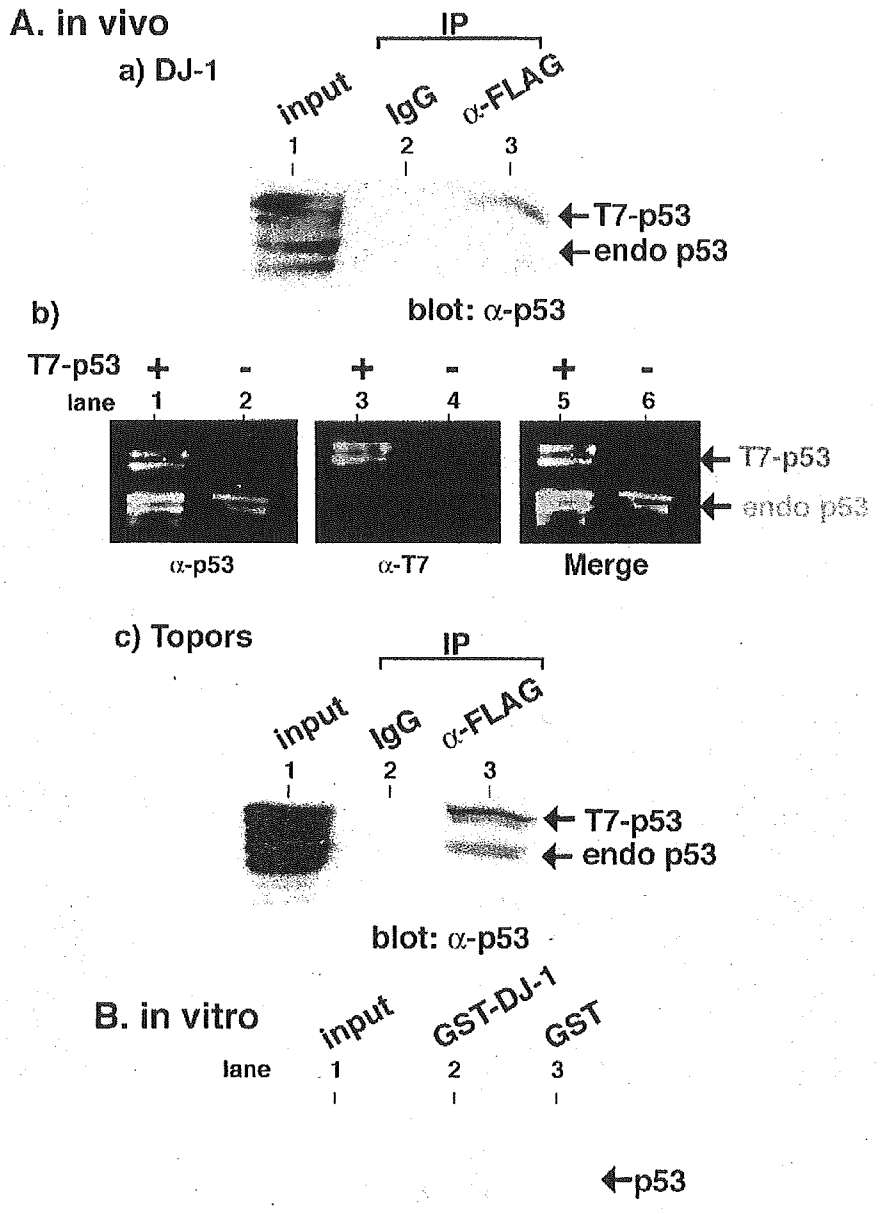

Figure 3. Association of DJ-1 with p53. (A-a), DJ-1 and p53 were tagged with either FLAG or T7, and their expression vectors were transfected into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with 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