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

Takeshi Niki,1,3 Kazuko Takahashi-Niki,1,3 Takahiro Taira,1,3 Sanae M.M. Iguchi-Ariga,2,3 and Hiroyoshi Ariga1,3

1Graduate School of Pharmaceutical Sciences and 2College of Medical Technology, Hokkaido University, Kita-ku, Sapporo, Japan; and 3CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama, Japan

Abstract

DJ-1 was identified by us as a novel oncogene that transforms mouse NIH3T3 cells in cooperation with ras. We later identified PIAS (protein inhibitor of activated STAT)xα as a DJ-1-binding protein, and found that DJ-1 restored androgen receptor (AR) transcription activity that was repressed by PIASxα. To further characterize the function of DJ-1, we cloned cDNA encoding a novel DJ-1-binding protein, DJBP, by a yeast two-hybrid system. DJBP mRNA was found to be specifically expressed in the testis. In addition to the binding of DJBP to the COOH-terminal region of DJ-1, DJBP was also found to bind in vitro and in vivo to the DNA-binding domain of the AR in a testosterone-dependent manner and to be colocalized with DJ-1 or AR in the nucleus. Furthermore, a co-immunoprecipitation assay showed that the formation of a ternary complex between DJ-1, DJBP, and AR occurred in cells in which DJ-1 bound to the AR via DJBP. It was found that DJBP repressed a testosterone-dependent AR transactivation activity in monkey Cos1 cells by recruiting histone deacetylase (HDAC) complex, including HDAC1 and mSin3, and that DJ-1 partially restored its repressed activity by abrogating DJBP-HDAC complex. These results suggest that AR is positively regulated by DJ-1, which antagonizes the function of negative regulators, including DJBP.

Introduction

DJ-1 was identified as a protein that transforms mouse NIH3T3 cells in cooperation with ras (1). The human DJ-1 gene, which is comprised of seven exons, is mapped to 1p36.2–36.3, where many chromosome aberrations in cancer have been reported (2). DJ-1 was found to be more strongly expressed in the testis than other tissues and also in sperm, suggesting that DJ-1 has at least two functions, one function in somatic cells, especially in germ cells, and one function in sperm.

With regard to the function of DJ-1 in somatic cells, we have identified PIAS (protein inhibitor of activated STAT)xα/ARIP3 (androgen receptor-interacting protein 3) as a DJ-1-binding protein (3). PIASxα/ARIP3 belongs to the PIAS family of proteins, including PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy/g, and was characterized at first as a testis-specific AR coregulator (4, 5). We have shown that PIASxα inhibits the transcription activity of the AR by binding to the DNA-binding domain of AR and that DJ-1 antagonizes this inhibition by sequestering PIASxα from the AR in CV-1, Cos1, and TM4 Sertoli cells, indicating that DJ-1 is a positive regulator of the AR (3). Although PIAS family proteins have been shown to play roles in SUMO-1 conjugation (6–11) and in the regulations of STAT (12, 13), nuclear receptors (4, 5, 14–17), and K channels (18), the contribution of DJ-1 to such functions is not clear.

RS, another name for human DJ-1, has been reported by another group as a protein of an RNA-binding protein regulatory subunit (19). It has recently been reported that DJ-1/RS was strongly expressed and secreted from cells to serum in about half of breast cancer patients, some of whom possessed an antigen against DJ-1/RS, indicating that DJ-1/RS is a biomarker for breast cancer (20). It has also been reported that pl of DJ-1 changed from 6.2 to 5.8 by treatment of cells with H2O2, paraquat, or lipopolysaccharide that resulted in the production of reductive oxygen species, suggesting that DJ-1 might be a sensor to oxidative stresses (21, 22).

With regard to the function of DJ-1 in sperm, a rat homologue of human DJ-1 has been identified by other laboratories as a key protein related to infertility of male rats that had been exposed to sperm toxicants such as ornidazole and epichlorohydrin (23–26). DJ-1/RS/CAPI has been shown to be expressed in the sperm head and to be translocated to the cytoplasm of sperm after treatment of rat sperm with the above toxicants (27, 28). Furthermore, we and others have reported that an anti-SP22 or anti-DJ-1 antisera inhibited fertility in vitro, suggesting that DJ-1 plays a role in...
fertilization (29, 30). The results suggest that DJ-1 affects both the production of sperm by activation of the AR to express male-specific genes and the fertilization function of mature sperm.

The AR is a member of the nuclear receptor superfamily and plays a role as a ligand-dependent transcription factor. After a ligand binds to the AR, the AR is translocated into the nucleus and binds to the androgen-responsive element, ARE, on the androgen-activating gene that affects development, growth, and regulation of male reproductive functions (31–34). The AR, like other nuclear receptor family proteins, possesses domains for transactivation, DNA-binding domain (DBD), and ligand-binding domain (LBD) at the NH2-terminal, central, and COOH-terminal regions, respectively. Transcriptional activity of the AR is known to be regulated by various coregulators that bind to the respective domain of AR. More than 30 proteins of such coregulators have been reported (Ref. 35, see homepage at http://ww2.mcgill.ca/androgendb).

To further characterize the function of DJ-1, we cloned cDNA encoding a novel DJ-1-binding protein, DJBP, by a yeast two-hybrid system using a human testis library in this study. DJ-1, DJBP, and AR were found to form a ternary complex, in which DJ-1 bound to AR via DJBP. DJBP repressed testosterone-dependent AR transactivation and DJ-1 restored its repressed activity, suggesting that DJBP and DJ-1 have mutually antagonizing functions with respect to the AR.

Results
Cloning of cDNA Encoding DJBP From a Human Testis cDNA Library
To identify proteins that bind to DJ-1, a yeast two-hybrid screening was carried out using a human testis cDNA library with full-sized DJ-1 as a bait, and cDNA encoding a novel DJ-1-binding protein, named DJBP, was cloned. DJBP cDNA comprises 2119 bp and encodes 570 amino acids (Fig. 1). DJBP contains three LXXLL-like motifs, LXXLL, YLXXL, and ILXXL, located at amino acid numbers 74, 92, and 235, respectively. The LXXLL motif is known to be the sequence that is necessary for coactivators to interact with nuclear receptors. To examine the expression of DJBP mRNA in human tissues, Northern blotting was carried out using a premade tissue blot with labeled DJBP cDNA as a probe (Fig. 2). Two distinct bands, of 1.9 and 7 kb in length, the former of which corresponds to the length of the DJBP cDNA obtained, were detected only in the testsis (Fig. 2, lane 12). DJ-1 was also confirmed to be strongly and moderately expressed in the testsis and other tissues, respectively, as described previously (1).

Interaction of DJBP With DJ-1
To assess the association of DJBP with DJ-1 in vivo, human 293T cells were transfected with expression vectors for Flag-tagged DJ-1 (F-DJ-1) with or without HA-tagged DJBP (HA-DJBP). Forty-eight hours after transfection, cell extracts were prepared and subjected to an immunoprecipitation reaction with an anti-Flag antibody. The precipitates were then blotted with an anti-HA or -Flag antibody (Fig. 3A). The results showed that HA-DJBP was coprecipitated with F-DJ-1 in cells cotransfected with F-DJ-1 and HA-DJBP but not in cells transfected with HA-DJBP alone (Fig. 3A, lanes 1 and 2, respectively), indicating that DJBP binds to DJ-1 in 293T cells. To examine the binding of DJP to DJ-1 in a physiological condition, the proteins in the extracts prepared from bovine testes were first immunoprecipitated with an anti-DJ-1 antibody or non-specific IgG and the precipitates were immunoblotted against an anti-DJBP antibody (Fig. 3B). The anti-DJ-1 antibody did precipitate DJ-1 (Fig. 3B, lane 2 in lower panel). DJBP, on the other hand, was detected in the immunoprecipitate with the anti-DJ-1 antibody but not with IgG (Fig. 3B, lanes 1 and 2, respectively). These results clearly indicate that DJBP binds to DJ-1 in cells.

To examine the interaction of DJBP with DJ-1 in vitro, F-DJ-1 and DJBP were expressed in E. coli as glutathione S-transferase (GST)-fusion proteins and purified after releasing GST by digestion of GST-fusion proteins with PreScission protease. Purified DJBP was mixed with or without F-DJ-1 and subjected to immunoprecipitation reaction with the anti-Flag antibody followed by the detection of proteins in the precipitates with an anti-DJBP antibody (Fig. 3C). The results showed that DJBP was coprecipitated with F-DI-1 in the mixture containing F-DJ-1 and DJBP but not in the mixture containing DJBP alone (Fig. 3C, lanes 1 and 2, respectively), indicating that DJBP directly binds to DJ-1.

To determine the DJ-1-binding region in DJBP, a yeast two-hybrid assay was carried out using various deletion mutants of DJBP fused to the GAL4-activating domain and DJ-1 fused to the LexA DNA-binding domain as a bait (Fig. 3D). DJ-1 was found to bind to COOH-terminal regions of DJBP spanning amino acid numbers 193–570, 250–570, and 372–570 but not to NH2-terminal regions, indicating that amino acids 372–570 of DJBP are bound by DJ-1. This was also confirmed by the results of an in vitro pull-down assay using GST or GST-fusion proteins of DJBP containing amino acids 1–570 (full length) and 371–570, in which DJBP and its deletion mutant but not GST were bound by DJ-1 (Fig. 3E).

Colocalization of DJBP and DJ-1
To determine the localization of DJ-1 and DJBP, an expression vector for HA-DJBP was transfected into monkey Cos1 cells, and the cells were stained with anti-HA and anti-DJ-1 antibodies to show the introduced HA-DJBP and endogenous DJ-1, respectively, and visualized with FITC and rhodamine-conjugated secondary antibodies under a confocal laser microscope (Fig. 4). The results showed that endogenous DJ-1 and HA-DJBP were localized mainly in the nucleus and both proteins were colocalized in the nucleus (Fig. 4, A–C, respectively).

Association of DJBP With the Androgen Receptor
To determine whether DJBP associates with AR in vivo, 293T cells cultured in the presence of 100 nM testosterone were transfected with expression vectors for HA-DJBP with or without F-AR, and 48 h after transfection, cell extracts were immunoprecipitated with an anti-Flag antibody and the precipitated protein was detected with an anti-HA or the anti-Flag antibody (Fig. 5A). The results showed that HA-DJBP was coprecipitated with F-AR in cells cotransfected with both F-AR...
Nucleotide and amino acid sequences of DJBP. The nucleotide sequence of DJBP cDNA was determined by a dideoxy method using Long Reader 4200 (LI-Cor, Lincoln, Nebraska) and Gene Rapid (Amersham BioScience, Bucks, UK) autosequencers, and the deduced amino acid sequence is shown. LXXLL motifs within the amino acid sequence are underlined.

**Figure 1.** Nucleotide and amino acid sequences of DJBP. The nucleotide sequence of DJBP cDNA was determined by a dideoxy method using Long Reader 4200 (LI-Cor, Lincoln, Nebraska) and Gene Rapid (Amersham BioScience, Bucks, UK) autosequencers, and the deduced amino acid sequence is shown. LXXLL motifs within the amino acid sequence are underlined.
and HA-DJBP but not in cells transfected with HA-DJBP alone (Fig. 5A, lanes 1 and 2), respectively), indicating that DJBP binds to AR in 293T cells. To assess the direct interaction between DJBP and AR, a GST pull-down assay was carried out using purified GST-DJBP and GST. Purified proteins were reacted in the presence of 100 nM testosterone with 35S-AR synthesized in vitro in a reticulocyte lysate, and the bound proteins were visualized by fluorography (Fig. 5B). The results showed that labeled AR bound to DJBP but not to GST, indicating that DJBP directly interacts with the AR (Fig. 5B, lanes 1 and 2), respectively). Because it is known that some coregulators for the AR bind to the AR in a ligand-dependent manner, testosterone (a ligand of the AR)-dependent binding activity of DJBP to AR was examined by a liquid β-galactosidase assay in yeast. Because DJBP binds to the COOH-terminal half of the AR, as shown in Fig. 6B, this truncated form of the AR, ARDN, was used in this assay (Fig. 5C). The results showed that β-galactosidase activity obtained between DJBP and ARDN increased with increase in the dose of testosterone. To further confirm the testosterone-

FIGURE 2. Northern blot analysis of DJBP in various human tissues. Human Northern RNA blot (12 major tissues, Origene, Rockville, MD) was hybridized with labeled cDNAs of DJBP (upper panel), DJ-1 (middle panel), and β-actin (lower panel) as probes. Positions of size markers are shown on the right in the upper panel.

FIGURE 3. Interaction of DJBP with DJ-1. A, 293T cells were transfected with pcDNA3-F-DJ-1 and pcDNA3-HA-DJBP. At 48 h after transfection, cell extracts were prepared and subjected to an immunoprecipitation reaction using an anti-Flag antibody (M2, Roche Applied Science, Rotkreuz, Switzerland). The precipitates were blotted with an anti-HA or the anti-Flag antibody. B, The proteins in the extract from bovine testes were first immunoprecipitated with an anti-DJ-1 antibody or non-specific IgG. The proteins in the precipitates were separated in a 12.5% polyacrylamide gel and blotted with an anti-DJBP antibody or the anti-DJ-1 antibody. Lane 1, the extract used for the binding reaction was applied to the same gel (Input). C, F-DJ-1 and DJBP were expressed in Escherichia coli as GST-fusion proteins and purified after releasing GST by digestion of GST-fusion proteins with PreScission protease (Amersham BioScience) as described in “Materials and Methods.” One microgram of each purified protein was mixed and subjected to an immunoprecipitation reaction using an anti-Flag antibody. The precipitates were blotted with an anti-DJBP antibody. Lane 3, the DJBP used for the reactions (Input) was run in parallel. D, Yeast L40 cells were transformed with expression vectors for LexA, LexA-DJ-1, and various deletion mutants of DJBP fused to the GAL4-activation domain. The β-galactosidase activity of each colony was measured. E, DJBP(1–570), DJBP(372–570), and DJ-1 were expressed in E. coli as GST-fusion proteins and purified. GST-free DJ-1 was then prepared after releasing GST from GST-DJ-1 by digestion with PreScission protease. DJ-1 and GST-DJBP were mixed together and subjected to a pull-down assay as described in “Materials and Methods.” The proteins bound to GST-DJBP were blotted with an anti-DJ-1 antibody. Lane 4, the DJ-1 used for the reactions (Input) was run in parallel.
dependent binding activity of DJBP to the AR in vivo, 293T cells transfected with the various amounts of testosterone were transfected with expression vectors for Flag-DJBP with or without the AR, and 48 h after transfection, cell extracts were immunoprecipitated with the anti-Flag antibody and the precipitated protein was detected with the anti-Flag antibody or an AR antibody (Fig. 5D). The results showed that AR was coprecipitated in a testosterone-dependent manner with F-DJBP in cells cotransfected with both AR and F-DJBP but not in cells transfected with the AR alone (Fig. 5D, lanes 1, 2, 4, 5, 7, 8, 10, and 11), indicating that DJBP binds to the ligand-binding form of the AR in 293T cells.

To determine the AR-binding region of DJBP, 293T cells were cotransfected with the AR and various Flag-tagged deletion mutants of DJBP, and the AR that co-immunoprecipitated with DJBP in cells were detected with an anti-AR antibody after the immunoprecipitation of cell extracts with an anti-Flag antibody (Fig. 6A). In addition to the full-sized DJBP, DJBP deletion mutants of amino acids 372–570 and 476–570 also immunoprecipitated the AR, whereas DJBP deletion mutants of amino acids 1–371 and 372–475 did not (Fig. 6A, lanes 1, 3, 5, 2, and 4, respectively), indicating that the AR binds to amino acids 476–570 of DJBP which do not contain LXXLL motifs. To determine the DJBP-binding region of the AR, on the other hand, Cos1 cells were cotransfected with the HA-tagged DJBP and various Flag-tagged deletion mutants of the AR, and DJBP that co-immunoprecipitated with ARs in cells was detected with an anti-HA antibody after the immunoprecipitation of cell extracts with the anti-Flag antibody (Fig. 6B). Deletion constructs of the AR are schematically shown in Fig. 6C. The results showed that while two mutants of the AR, ΔC and LBD, were not bound by DJBP, the deletion mutants of the AR, ΔN and DBD, were, like the wild-type AR, bound by DJBP (Fig. 6B, lanes 2, 5, 3, 4, and 1, respectively). These results indicate that DJBP binds to the DNA-binding domain of the AR and that the LXXLL motif is not necessary for DJBP to interact with the AR. To examine the colocalization of DJBP with the AR in cells, Cos1 cells were cotransfected with F-DJ-1 and HA-DJBP in the presence of 100 nM testosterone, and transfected cells were stained with anti-AR and anti-HA antibodies at 48 h after transfection. HA-DJBP was found to be colocalized with the AR in the nucleus (data not shown).

Repression of AR Transactivation Activity by DJBP and Restoration of Its Repression by DJ-1

Because we showed in this study that DJBP binds to both DJ-1 and AR and in a previous study that DJ-1 does not directly bind to the AR (3), we then examined the relationships among DJBP, DJ-1, and AR. 293T cells cultured in the presence of 100 nM testosterone were transfected with various combinations of F-AR, HA-DJBP, and DJ-1-HA, the cell extracts were immunoprecipitated with an anti-Flag antibody, and the precipitants were blotted with anti-HA and Flag antibodies (Fig. 7A, lanes 1–8). HA-DJBP was co-immunoprecipitated with F-AR as expected (Fig. 7A, lanes 2 and 3). DJ-1-HA, on the other hand, was co-immunoprecipitated with F-AR in cells transfected with both HA-DJBP and F-AR but not in cells transfected with F-AR alone (Fig. 7A, lanes 3 and 4), respectively. Expression levels of the respective proteins of F-AR, HA-DJBP, and DJ-1-HA were comparable in transfected cells (Fig. 7A, see Input). Furthermore, the amounts of DJ-1 and HA-DJBP co-immunoprecipitated with F-AR were found to be stimulated in the presence of 100 nM testosterone (Fig. 7A, lanes 9 and 10). Thus, these results clearly indicate that DJ-1, DJBP, and AR form a ternary complex, in which DJ-1 associates with the AR via DJBP.

To then assess the relationship between the biological activities of DJ-1, DJBP, and AR, monkey Cos1 cells were transfected with pARE2-TATA-Luc as a reporter and with or without pcDNA3-F-AR together with various amounts of pcDNA3-HA-DJBP or pEGFP-N1 in the presence or absence of 100 nM testosterone. The plasmid, pCMV-β-gal, was also cotransfected into cells to normalize the transfection efficiency. Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured (Fig. 7B). A testosterone-dependent transcription activity of the AR was first confirmed in the transfected cells without pcDNA3-HA-DJBP (data not shown). It was found that neither DJBP nor EGFP responded to pARE2-TATA-Luc reporter in the absence of the AR. The results then showed that while introduction of EGFP that did not bind to the AR, pEGFP-N1, did not affect the AR activity, DJBP repressed the testosterone-dependent transcription activity of the AR in a dose-dependent manner and about 70% of the AR activity was repressed after 1 µg of pcDNA3-HA-DJBP was transfected into cells (Fig. 7B), suggesting that DJBP acts as a negative regulator of the AR. DJ-1-HA or EGFP was then cotransfected into Cos1 cells together with 1 µg of pcDNA3-HA-DJBP and pARE2-TATA-Luc, and the luciferase activities were determined (Fig. 7C). The results showed that while introduction of vector, pEGFP-N1, did not affect the AR activity, DJ-1 partially restored AR transcription activity repressed by DJBP in a dose-dependent manner. Because DJ-1 directly binds to DJBP but not the AR, these results suggest that DJ-1 changes the AR from a transcriptional negative form to an active form as we reported in the case of DJ-1 and PIASxα (3).

Association of DJBP With Histone Deacetylase Complex

Because transcriptional repression pathways of genes are often connected with histone deacetylase (HDAC) or corepressor complex, it is possible that the transcriptional repression of the AR by DJBP uses the same or similar pathway connected with HDAC. To explore this possibility, an expression vector for FLAG-tagged DJBP was transfected into human 293T cells cultured in the presence of 100 nM testosterone. Forty-eight hours after transfection, the cell extract was prepared and the proteins in the extract were first immunoprecipitated with the anti-FLAG antibody or non-specific IgG. The precipitates were immunoblotted against an anti-AR antibody (Fig. 8A). It was first confirmed that the anti-FLAG antibody did not precipitate proteins in the extracts from cells into which FLAG-DJBP had not been transfected, and that the anti-FLAG antibody precipitated FLAG-DJBP (Fig. 8A, lanes 3 and 1, respectively). The endogenous AR, on the other hand, was detected in the immunoprecipitate with the anti-FLAG antibody but not with IgG (Fig. 8A, lanes 1 and 2, respectively), indicating again that DJBP was associated with the AR in 293T cells.
same blot as that shown in Fig. 8A was reprobed with anti-HDAC1 and-mSin3A antibodies (Fig. 8A). Endogenous HDAC1 and mSin3A were detected in the immunoprecipitates with the anti-FLAG antibody but not with IgG, indicating that DJBP and the AR are complexed with a corepressor complex, including HDAC1 and mSin3A. It was found that neither HDAC1 nor mSin3A directly bound to DJBP by the pull-down assay, in which GST-DJBP was reacted with $^{35}$S-HDAC1 or $^{35}$S-mSin3A synthesized in vitro in a reticulocyte lysate (data not shown), suggesting that the DJBP-bound AR or the DJBP-AR complex recruits the corepressor complex.

To confirm the existence of the HDAC complex in the transrepression pathway of the AR to DJBP, TSA, an inhibitor of HDAC1, was added to the cells transfected with the AR and various amounts of DJBP in the presence of 100 nM testosterone, and the luciferase activity was measured (Fig. 8B). The results showed that TSA abrogated the DJBP-repressed transcription activity of the AR and further increased the transcription activity of the AR to the level comparable to that obtained after introduction of the AR alone, suggesting that repression of the AR transcription activity by DJBP is carried out by the HDAC complex.

Abrogation of the DJBP-HDAC Complex by DJ-1

To determine the mechanism underlying the abrogation of the DJBP-repressed AR transcription activity by DJ-1, FLAG-tagged DJBP and various amounts of HA-tagged DJ-1 or EGFP were transfected into human 293T cells cultured in the presence of 100 nM testosterone. Forty-eight hours after transfection, the cell extract was prepared and the proteins in the extract were first immunoprecipitated with an anti-FLAG antibody. The precipitates were immunoblotted against anti-HA, anti-HDAC1, anti-GFP, and anti-FLAG antibodies as described in Fig. 8A (Fig. 9). It was first confirmed that the constant amounts of endogenous HDAC1 were expressed and that DJ-1-HA and EGFP were expressed in a dose-dependent manner in transfected cells (Input, Fig. 9, lanes 9–16). It was also confirmed that FLAG-DJBP was precipitated with the anti-FLAG antibody (Fig. 9, lanes 1–3, 5, and 6). While EGFP was not detected in the precipitates, DJ-1-HA was also detected with the anti-HA antibody in the immunoprecipitates from cells that had been transfected with FLAG-DJBP (Fig. 9, lanes 2 and 3), indicating that DJBP was associated with DJ-1 in ectopic expressed 293T cells. It was found that endogenous HDAC1 was, however, first detected in the immunoprecipitates from cells that had been transfected with FLAG-DJBP alone and then the amounts of immunoprecipitated HDAC1 decreased in a dose-dependent manner (Fig. 9, lanes 1–3), indicating that DJ-1 abrogated the DJBP-HDAC complex, thereby leading to the restoration of the AR transcription activity repressed by DJBP.

Discussion

In this study, we identified a novel DJ-1-binding protein, named DJBP, by a yeast two-hybrid system using a human testis cDNA library. DJBP contains three LXXLL-like motifs that are known to be necessary for coactivators to interact with nuclear receptors. Expressions of DJBP mRNAs of 2 and 7 kb in length were found to be limited to the testis of human tissues,
and the cDNA cloned in this study appears to correspond to the 2-kb mRNA. Although the cDNA corresponding to the 7-kb mRNA is not available at present, it might be of an alternative splicing form of DJBP mRNAs.

From the results of various binding experiments in vitro and in vivo, it was found that DJBP directly binds to both DJ-1 and AR in a testosterone-dependent manner and that DJ-1 associates with AR via DJBP in a ternary complex in cells.

Because AR binds to the COOH-terminal region spanning amino acids 476–570 of DJBP, in which three LXXLL-like motifs are not present, it is thought that these motifs in DJBP are not necessary for binding to the AR, like several AR-binding proteins, including BRCA1 (36), Cyclin E (37), RAF (38), TFIH (39), CAK (39), RB (40), Ubc9 (41), c-Jun (42), and Cyclin D1 (43). DJBP, on the other hand, binds to the DNA-binding domain of the AR (AR-DBD). Because the

FIGURE 6. Determination of regions of binding between DJBP and AR. A. 293T cells cultured in the presence of 100 nM testosterone were transfected with various deletion mutants of Flag-tagged DJBP and AR. Forty-eight hours after transfection, the proteins in the cell extract were precipitated with an anti-Flag antibody and blotted with an anti-AR antibody. Lane 7, the cell extract used for the reactions (Input) was run in parallel. B. Cost-1 cells cultured in the presence of 100 nM testosterone were transfected with various deletion mutants of Flag-tagged AR and HA-tagged DJBP. Forty-eight hours after transfection, the proteins in the cell extract were precipitated with an anti-Flag antibody and blotted with an anti-HA antibody. Lane 6, the cell extract used for the reactions (Input) was run in parallel. C. The AR and its deletion mutants used for the reactions are schematically shown, and the DJBP-binding activities are shown on the right.
FIGURE 7. Effect of transcription activity of the AR by DJBP and DJ-1. A, 293T cells cultured in the presence of 100 nM testosterone were transfected with various combinations of pcDNA3-F-AR, pcDNA3-HA-DJBP, and pEF-DJ-1-HA (lanes 1–8). Lanes 9 and 10, plasmids used in lane 3 were transfected into 293T cells in the absence or presence of 100 nM testosterone. Forty-eight hours after transfection, the proteins in the cell extract were precipitated with an anti-Flag antibody and blotted with anti-Flag and anti-HA antibodies. In the lower two panels (Input), the cell extract used for the reactions was run in parallel and blotted with the anti-HA antibody. B. Cos1 cells cultured in the presence of 100 nM testosterone were transfected with 0.12 μg of pCMV-h-gal, 0.03 μg of pcDNA3-F-AR, and 1.2 μg of pARE2-TATA-Luc together with various amounts of pcDNA3-HA-DJBP or pEGFP-N1 (HA-DJBP + AR or EGFP + AR, respectively). Cos1 cells were also transfected with the same DNAs as those above except for omitting pcDNA3-F-AR (HA-DJBP or EGFP, respectively). Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured. C. Cos1 cells cultured in the presence of 100 nM testosterone were transfected with 0.12 μg of pCMV-h-gal, 0.03 μg of pcDNA3-F-AR, 1.2 μg of pARE2-TATA-Luc, and 1 μg of pcDNA3-HA-DJBP together with various amounts of pEF-DJ-1-HA or pEGFP-N1. Forty-eight hours after transfection, cell lysates were prepared and their luciferase activities were measured.
amino acid sequences of DBD among nuclear receptor family proteins were well conserved, DJBP might also bind to other nuclear receptors. Of the proteins that bind to AR-DBD, SNURF (44), Ubc9 (41), and ARIP3/PIASXα (4) have been reported to associate with multiple nuclear receptors, including the AR.

DJBP was found to repress the transcription activity of AR to 30% of that without DJBP. The mechanism underlying this repression of AR activity by DJBP was assessed by the findings that DJBP recruited the corepressor complex, including HDAC1 and mSin3A. These results suggest that an active form of the AR changes into an inactive form by translocation to an inactive structure within the chromatin. Repression activity of DJBP toward the AR was sensitive to TSA, a specific inhibitor of HDAC, indicating that HDAC is involved in the transrepression pathway of the AR. The introduction of DJ-1 relieved the repressed the AR transcription activity by abrogation of DJBP-HDAC complex. It was reported that TSA augments dihydrotestosterone induction of AR levels (45). Although the results in this report suggested that the androgen-induced chromatin remodeling and transcription occur in the AR-responsive genes, the results in our study here is a first report to identify the AR-HDAC complex bridged by DJBP.

FIGURE 8. Binding of DJBP to the AR in the HDAC complex in vivo and effect of trichostatin A (TSA) on the repression activity of DJBP toward the AR. A, 293T cells cultured in the presence of 100 nM testosterone were transfected with an expression vector for FLAG-DJBP by the calcium phosphate precipitation technique, and the cell extract was prepared 48 h after transfection. Proteins in the extract were first precipitated with an anti-FLAG mouse monoclonal antibody or non-specific IgG, and the precipitates were immunoblotted with an anti-AR rabbit polyclonal antibody (N-20, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins in the extract from non-transfected cells were similarly treated with antibodies. The same blot as that described above was reprobed with an anti-HDAC1 antibody (H-51, Santa Cruz Biotechnology) and an anti-mSin3A antibody (AK-11, Santa Cruz Biotechnology). Lanes 4 and 5, the proteins used for the reactions (Input) were run in parallel. B, Cos1 cells cultured in the presence of 100 nM testosterone were transfected with 0.12 μg of pCMV-β-gal, 0.03 μg of pcDNA3-F-AR, and 1.2 μg of pARE2-TATA-Luc together with various amounts of pcDNA3-HA-DJBP. Two-hundred nanomolars of TSA was added to the culture 40 h after transfection. After 48 h, cell lysates were prepared, and the luciferase activities in the lysates were measured.
Functions of the AR are regulated by various complex formations. PIASxα, a member of the PIAS family of proteins, was characterized at first as a testis-specific AR coregulator, ARIP3 (4, 5). We have shown that PIASxα inhibits the transcription activity of the AR by binding to the DNA-binding domain of the AR and that DJ-1 antagonizes this inhibition by sequestering PIASXα from the AR in CV-1, Cos1, and TM4 Sertoli cells, indicating that DJ-1 is a positive regulator of the AR (3). DJBP was found to bind to the AR, leading to formation of a ternary complex between DJ-1, DJBP, and the AR. Different to the mode of action of PIASxα on the AR, DJBP was found to inhibit AR transcription activity by recruiting histone-deacetylase complexes, including HDAC1 and mSin3, and DJ-1 was found to restore the repressed activity of the AR. These findings suggest, as schematically shown in Fig. 10, that DJ-1 restores AR activity that has been repressed by different mechanisms.

DJBP and DJ-1 were found to be expressed specifically and strongly, respectively, in the testis. Furthermore, it was found that DJ-1 was also expressed in sperm and played roles in fertilization reactions. These results suggest that DJBP also plays a role in spermatogenesis or fertilization along with DJ-1. To clarify this possibility, we are investigating the expression patterns, including location and timing, of DJBP in the testis and sperm.

**FIGURE 9.** Abrogation of recruitment of HDAC complex to DJBP by DJ-1. 293T cells cultured in the presence of 100 nM testosterone were transfected with 5 μg of pcDNA3-F-DJBP, various amounts of pEF-DJ-1-HA and pEGFP-N1 by the calcium phosphate precipitation technique, and the cell extract was prepared 48 h after transfection. Proteins in the extract were first precipitated with an anti-FLAG mouse monoclonal antibody, and the precipitates were immunoblotted with an anti-HA rabbit polyclonal antibody, an anti-HDAC1 antibody, or the anti-FLAG antibody as described in Fig. 8. The 0.25-, 1-, and 1-μg pEF-DJ-1-HA or pEGFP-N1 was transfected into cells in lanes 2, 3, and 4, respectively. Lanes 9–16, the cell extracts used for the reactions (Input) were run in parallel.

**FIGURE 10.** Schematic drawings of mechanisms by which DJ-1 antagonizes negative modulators of the AR by DJBP and PIASxα.
Materials and Methods

Cells
Human 293T and monkey Cos1 cells were cultured in DMEM supplemented with 10% calf serum.

Plasmids
Nucleotide sequences of the oligonucleotide used for PCR primers were as follows:

DJBP-ATG(Bam), 5'-GGATCCATGGGTCATTTC-3';
DJBP-ATG(Eco), 5'-GGGAATTCATGGGTCATTTC-3';
DJBP-193(Eco), 5'-GGGAATTCTATATAAATGGGAAAT-3';
DJBP-250(Eco), 5'-GGGAATTCATCACCAGGAGTTTG-3';
DJBP-372(Eco), 5'-GGGAATTCAGGACTCCACCCTTGC-3';
DJBP-372(Bam), 5'-GGGGATCCGGCACTCCACCCTTGC-3';
AR-DN(Eco), 5'-GGGAATTCAGGACTCCACCCTTGC-3';
AR-DBD(Eco), 5'-GGGAATTCAGGACTCCACCCTTGC-3';
AR-LBD(Eco), 5'-GGGAATTCAGGACTCCACCCTTGC-3';
DJBP-ATG(Eco), 5'-GGGAATTCATGGGTCATTTC-3';
DJBP-193(Eco), 5'-GGGAATTCATGGGTCATTTC-3';
DJBP-250(Eco), 5'-GGGAATTCATGGGTCATTTC-3';
DJBP-372(Eco), 5'-GGGAATTCATGGGTCATTTC-3';
DJBP-475(Xho), 5'-GGGGATCCCAGAATGCACAC-3';
DJBP-476(Bam), 5'-GGGAATTCGGACCTTATGGG-3';
DJBP-372(Bam), 5'-GGGAATTCGGACCTTATGGG-3';
DJBP-372(Xho), 5'-GGGGATCCGGCACTCCACCCTTGC-3';
DJBP-372(Eco), 5'-GGGGATCCGGCACTCCACCCTTGC-3';
DJBP-372(Bam), 5'-GGGGATCCGGCACTCCACCCTTGC-3';
DJBP-476(Bam), 5'-GGGAATTCGGACCTTATGGG-3';
DJBP-372(Bam), 5'-GGGAATTCGGACCTTATGGG-3';

Table 1. Plasmid Construction

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Template</th>
<th>5'-Primer</th>
<th>3'-Primer</th>
<th>Restriction Enzyme Site</th>
<th>Plasmid Inserted</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3-HA-DJBP</td>
<td>pME18S-DJBP</td>
<td>DJBP-ATG(Bam)</td>
<td>SP6</td>
<td>BamHI, Xho1</td>
<td>pcDNA3-HA</td>
</tr>
<tr>
<td>pGEX-DJBP</td>
<td>pME18S-DJBP</td>
<td>DJBP-ATG(Bam)</td>
<td>SP6</td>
<td>BamHI, Xho1</td>
<td>pGEX-6P-1</td>
</tr>
<tr>
<td>pGAD-DJBP(1–570)</td>
<td>pME18S-DJBP</td>
<td>DJBP-ATG(Eco)</td>
<td>SP6</td>
<td>EcoRI, Xho1</td>
<td>pGAD-GHX</td>
</tr>
<tr>
<td>pGEX-DJBP</td>
<td>pME18S-DJBP</td>
<td>DJBP-ATG(Eco)</td>
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</tr>
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<td>pGAD-DJBP(250–570)</td>
<td>pME18S-DJBP</td>
<td>DJBP-250(Eco)</td>
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<td>pGAD-GHX</td>
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<td>pGAD-DJBP</td>
<td>pcDNA3-HA-DJBP</td>
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<td>SP6</td>
<td>EcoRI, Xho1</td>
<td>pGAD-GHX</td>
</tr>
<tr>
<td>pGAD-DJBP</td>
<td>pcDNA3-F-AR</td>
<td>AR-DN(Eco)</td>
<td>SP6</td>
<td>EcoRI, Xho1</td>
<td>pcDNA3-F</td>
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<tr>
<td>pGAD-DJBP</td>
<td>pcDNA3-HA-DJBP</td>
<td>DJBP-ATG(Bam)</td>
<td>SP6</td>
<td>BamHI, Xho1</td>
<td>pcDNA3-F</td>
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<td>pGAD-DJBP</td>
<td>pcDNA3-HA-DJBP</td>
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</tr>
<tr>
<td>pGAD-DJBP</td>
<td>pcDNA3-F-AR</td>
<td>AR-DN(Eco)</td>
<td>SP6</td>
<td>BamHI, Xho1</td>
<td>pcDNA3-F</td>
</tr>
<tr>
<td>pGAD-DJBP</td>
<td>pcDNA3-F-LBD</td>
<td>AR-LBD(Eco)</td>
<td>SP6</td>
<td>BamHI, Xho1</td>
<td>pcDNA3-F</td>
</tr>
</tbody>
</table>

Note: Combinations of plasmids constructed, primers, templates, and restriction enzyme sites and plasmids inserted are shown.
Interaction of DJBP With DJ-1 and AR in Vivo

293T cells were transfected with pcDNA3-F-DJ-1 and pcDNA3-HA-DJBP in the presence or absence of 100 nM testosterone by the calcium phosphate method (48). Forty-eight hours after transfection, the cell extract was prepared and subjected to an immunoprecipitation reaction using an anti-FLAG antibody conjugated with agarose beads (M2, Sigma Chemical Co., St. Louis, MO). The precipitates were separated on 12.5% SDS-PAGE and blotted with an anti-HA antibody (12CA5, Roche). To examine formation of a ternary complex between DJ-1, DJBP, and AR, 5 μg of pcDNA3-F-AR, 2.5 μg of pcDNA3-HA-DJBP, and 1 μg of pEF-DJ-1-HA were transfected into 293T cells in the presence of 10 μM testosterone, and then the same immunoprecipitation assay as that described above was carried out.

Interaction of DJBP with DJ-1 and AR in Vitro

F-DJ-1 and DJBP were expressed in E. coli as GST-fusion proteins and purified after releasing GST by digestion of GST-fusion proteins with PreScission protease (Amersham BioScience) as described previously (49). Purified F-DJ-1 and DJBP were mixed and subjected to an immunoprecipitation reaction using an anti-Flag antibody followed by an anti-DJBP antibody as described above. The anti-DJBP antibody used in this reaction was of the IgG fraction prepared from rabbits immunized with purified DJBP as an immunogen. To examine the binding of DJBP with the AR, purified GST-DJBP or GST was incubated with 35S-AR synthesized in vitro using a reticulocyte lysate of a TnT-transcription-translation coupled system (Promega, Madison, WI) and subjected to a pull-down assay using an glutathione-Sepharose as described previously (49).

Immunofluorescence

Cos1 cells were transfected with 1 μg of pcDNA3-HA-DJBP or pcDNA3-F-AR by the calcium phosphate method (48). Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde, stained with anti-DJ-1 (3), anti-AR (N-20, Santa Cruz Biotechnology), and HA antibodies, and visualized under a confocal laser microscope.

β-Galactosidase Liquid Assay

L40 cells were transformed with expression vectors for DJ-1 fused to the LexA DNA-binding domain, pGLex-DJ-1, and for various deletion mutants of DJBP fused to the GAL4-activation domain, pGAD-GHX-DJBPs. Transformed cells were cultured in YPAD medium in the presence of testosterone for 4 h, and pARE2-TATA-Luc, a reporter plasmid, and 0.13 μg of pGL45P-Luciferase Assay

in YPAD medium in the presence of testosterone for 4 h, and pARE2-TATA-Luc, a reporter plasmid, and 0.13 μg of pCMV-β-gal, an expression vector for β-galactosidase, by the calcium phosphate method (48). Forty-eight hours after transfection, the luciferase activity in cells was measured after normalization of the transfection efficiency with β-galactosidase activities as described previously (49).

Acknowledgments

We thank Yoko Misawa and Kiyomi Takaya for their technical assistance.

References


Molecular Cancer Research

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

1 1 Ministry of Education, Science, Sport, Culture and Technology of Japan.

2 The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank/EBI Data Bank with accession number AB073862.

Takeshi Niki, Kazuko Takahashi-Niki, Takahiro Taira, et al.


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