Identification of DJ-1-Associated Regions on Human Genes from SH-SY5Y Cells using Chromatin Immunoprecipitation Sequence Technique

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Abstract

DJ-1, a cancer- and Parkinson's disease-associated protein, works as a coactivator to various transcription factors. In this study, DNA sequences that bind to DJ-1 complexes were obtained by a chromatin immunoprecipitation sequencing technique with an anti-human DJ-1 antibody using chromatin from SH-SY5Y cells. We identified 60 different sequences as potential DJ-1 complex-binding sites in genes. Of sequences identified, expression levels of DJ-1-associated site-containing genes for DNA polymerase N, estrogen receptor α and S-adenosylhomocysteine hydrolase like-2 were decreased in DJ-1-knockdown cells and in 6-OHDA-treated cells. These studies suggest that DJ-1 regulates the expression of versatile genes at the transcriptional level and that some of the genes are regulated by DJ-1 in an oxidative status-dependent manner.

Keywords: ChIP sequences; DJ-1; Transcriptional regulation; Oxidative stress; Genome-wide analysis; Cell growth

Abbreviations: ChIP: Chromatin Immunoprecipitation; RT-PCR: Reverse Transcription-PCR; 6-OHDA: 6-Hydroxydopamine

Introduction

DJ-1 was identified by us as a novel oncogene [1] and was later identified as a causative gene (park7) for a familial form of Parkinson's disease [2]. DJ-1 has multiple functions, including transcriptional regulation, anti-oxidative stress function, functions as a chaperone and protease and mitochondrial regulation [3-5]. For transcriptional regulation, DJ-1 acts as a coactivator that binds to various transcription factors, including inhibitors of the androgen receptor [6-8], p53 [9-11], poly(ADP-ribose) polymerase-1, and gene expression studies of DJ-1 [14,15,26-28]. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO2.

Chromatin immunoprecipitation (ChIP) and sequence analysis

5x10⁶ SH-SY5Y cells were treated with 50 μM 6-OHDA for 48 hrs and cross-linked with formaldehyde. DNA-protein complexes were then prepared from SH-SY5Y cells and from 6-OHDA-treated SH-SY5Y cells as described previously [6]. ChIP assays were carried out with a rabbit anti-human DJ-1 polyclonal antibody or with non-specific IgG using a ChIP assay kit (Upstate) according to the manufacturer's protocol. The rabbit anti-human DJ-1 polyclonal antibody described previously [1] was affinity-purified using a DJ-1-coupled sepharose resin. For ChIP sequences, adaptors (Illumina) were ligated to immunoprecipitated DNAs and their sequences were determined using Genome Analyzer II (Illumina, USA). Of total 7,702,242 and 5,814,987

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short reads that had been ChIP-sequenced in samples from SH-SHYS cells and 6-OHDA-treated SH-SHYS cells, 1,849,642 and 2,227,434 reads, respectively, were mapped to the human genome (UCSC hg 18, excluding haplotype sequences) by using ELAND from Illumina data analysis software, which maps sequences within 2 mismatches.

ChIP assays using cultured SH-SHYS cells were performed according to the protocol of the ChIP Assay Kit (Millipore, Billerica, MA, USA). Briefly, after proteins had been cross-linked with DNA, cell pellets were resuspended in an SDS-lysis buffer and sonicated on ice using a sonicator (UR-20R, TOMY, Tokyo, Japan) 3 times for 20 sec each time. Genomic DNA was then sheared to 300 to 1200 base pairs of length. Chromatin solution from 1×10⁶ cells/dish was preincubated with salmon sperm DNA and Protein A-agarose and incubated with antibody to DJ-1 as a coactivator by binding to various transcription factors that possess DNA-binding activity, it is thought that DJ-1 or DJ-1 complex recognizes specific sequences in respective genes. In this study, we tentatively call these sites "DJ-1-associated sites" for convenience.

We found 60 potential DJ-1-binding sites with different sequences in human genome and that their mapping numbers on chromosomes 18, 19, 7 and 4 were 3024, 80, 73 and 71, respectively. For instance, two peaks corresponding to DJ-1-associated sites, peaks a and b that are located in regions 16,767,578-16,767,618 and 17,387,379-17,387,415 on chromosome 18, were detected, and their mapping number was 62 and 3024, respectively (Table S1). Regions of peaks a and b were then found to be located downstream and in introns of genes encoding Rho-associated Colleld-coil Containing protein Kinase 1 (ROCK1) and Establishment of Cohesion 1 (ESCO1), respectively. CLUSTAL W (1.83) Multiple Sequence Alignments were then used to alien sequences obtained. Aligned sequences were, however, poly A stretch and AG repeat but not specific sequences. Since DJ-1 binds to DNA via other DNA-binding transcription factors, it is reasonable to have identified variety of different sequences as DJ-1-binding sequences. Nucleotide sequences identified in this study have been deposited to the NCBI database, and its accession number is DRA000365.

Reduced expression of the establishment of cohesion 1 (ESCO1) gene in DJ-1 knockdown cells

The genes identified, the highest hit of DJ-1-binding sites in the ChIP plot was placed in intron of the Establishment of Cohesion 1 (ESCO1) gene on chromosome 18. To confirm the binding activity of DJ-1 to the EESCO1 gene, ChIP assays were carried out using chromatin from SH-SHYS cells and an anti-DJ-1 antibody or non-specific IgG. As shown in Figure 1, the anti-DJ-1 antibody but not IgG precipitated the EESCO1 gene spanning +27109 to +27367. To examine the relationship between the EESCO1 gene and DJ-1, total RNA was extracted from parental and knockdown cells of human SH-SHYS and mouse NIH3T3 cells, and the expression levels of EESCO1, DJ-1 and β-actin (ACTB) mRNA were examined by semi-quantitative RT-PCR. ACTB mRNA was used as a loading control. As shown in (Figures 2A and 2C), expression levels of EESCO1 mRNA in DJ-1 knockdown cells of NIH3T3 and in SH-SHYS cells were reduced to about 80% and 40%, respectively, of those in parental NIH3T3 cells and in SH-SHYS cells.

### Table 1: Nucleotide sequences of primers used for RT-PCR and real-time PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′-→3′)</th>
<th>Sequence (5′-→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESCO1 ChIP-F</td>
<td>5′-GCTAAGATGACACCGCAACA-3′</td>
<td>5′-GCCTCAGCTGTCATGTCCTT-3′</td>
</tr>
<tr>
<td>ESCO1 ChIP-R</td>
<td>5′-TGGGCCCTTAATCTAATGTGA-3′</td>
<td>5′-TGGGCCCTTAATCTAATGTGA-3′</td>
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<tr>
<td>ESR1 ChIP-F</td>
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<td>5′-GTCAGGCTGGTCTCGAACTC-3′</td>
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<tr>
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<td>5′-GTCAGGCTGGTCTCGAACTC-3′</td>
<td>5′-GTCAGGCTGGTCTCGAACTC-3′</td>
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<tr>
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</tr>
<tr>
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Expression levels of the *ESCO1* gene in parental and its knockdown human and mouse cells were also examined by quantitative real-time PCR. Results again showed reduced expression of the *ESCO1* gene in DJ-1-knockdown cells (Figures 2B and 2D).

Frequency of DJ-1-associated sites and expression levels of genes under an oxidative stress condition

The frequency of potential DJ-1-associated sites mapped was changed after SH-SY5Y cells had been treated with 50 µM 6-OHDA for 48 hrs. As shown in Table 2, five fragments were decreased by more than 7 fold compared to those in SH-SY5Y cells without 6-OHDA treatment. To first examine whether the expression of these genes is regulated by DJ-1 under normal conditions, total RNAs were extracted from NIH3T3 and D2 cells and the expression levels of GPHN, POLN, ESR1, AHCYL2, RELB, DJ-1 and ACTB mRNA were examined by RT-PCR. ACTB mRNA was used as a loading control. As shown in Figure 3A, expression levels of *GPHN*, *POLN*, *ESR1* and *AHCYL2* genes were significantly decreased, while expression level of the *RELB* gene was not changed in D2 cells. Expression levels of *GPHN*, *POLN*, *ESR1* and *AHCYL2* genes were further examined using DJ-1-knockdown SH-SY5Y cells. As shown in Figures 3B, expression levels of *POLN*, *ESR1* and *AHCYL2* genes were significantly decreased and expression level of the *GPHN* gene was not changed. Since expression levels of *POLN*, *ESR1* and *AHCYL2* genes were significantly reduced in DJ-1-knockdown cells of both NIH3T3 and SH-SY5Y cells, these genes were further examined by real-time PCR, and significant reduction of their expression levels in DJ-1-knockdown SH-SY5Y cells was again observed (Figures 3C).
Furthermore, binding activity of DJ-1 to POLN, ESR1 and AHCYL2 genes were confirmed by ChIP assays using chromatin from SH-SYSY cells and an anti-DJ-1 antibody (Figure 1).

To examine the effect of oxidative stress and DJ-1 on expression of POLN, ESR1 and AHCYL2 genes, total RNAs were extracted from SH-SYSY cells treated with or not treated with 6-OHDA, and expression levels of these mRNAs were examined by semi-quantitative RT-PCR and by quantitative real-time PCR. It was first confirmed that expression levels of POLN, ESR1 and AHCYL2 genes were reduced in DJ-1-knockdown SH-SYSY cells that had been treated with 6-OHDA compared to those in non-treated DJ-1-knockdown SH-SYSY cells (Figure 4C), indicating that treatment of 6-OHDA did not affect the positive effect of DJ-1 on the expression of these genes. As shown in Figures 4A and 4B, the expression levels of POLN and AHCYL2 mRNA in 6-OHDA-treated SH-SYSY cells were reduced to about 40-50% and 78-60%, respectively, of that in untreated SH-SYSY cells by analysis of RT-PCR and real-time PCR. The expression level of ESR1 mRNA, on the other hand, was not changed, rather increased, after cells had been treated with 6-OHDA. Since the expression levels of these genes were reduced in DJ-1-knockdown cells and since the expression levels of POLN and AHCYL2 genes but not that of the ESR1 genes were reduced in SH-SYSY cells that had been treated with 6-OHDA, these results suggest that DJ-1 regulates gene expression in an oxidative stress-dependent or independent manner.

In this study, we newly found 60 potential DJ-1-associated/recognizing sites in human genes by ChIP sequencing using a next-generation DNA sequencer. DJ-1-associated sites were found to be located upstream, in introns and downstream of coding regions of genes that cover many genes possessing versatile functions. Of the DJ-1-associated sites identified, the highest mapping score was obtained in the intron of the establishment of cohesion 1 (ESCO1) gene, and the expression level of ESCO1 mRNA was decreased in DJ-1-knockdown cells of human SH-SYSY and mouse NIH3T3 cells, suggesting that the ESCO1 gene is regulated by DJ-1 at the transcriptional level under a non-stressed condition. ESCO1 is required for proper sister chromatid cohesion. Although there is no evidence at present, DJ-1 might control the segregation of sister chromatids.

Furthermore, we found that the number of potential DJ-1-associated sites in human genome was changed after cells had been treated with 6-OHDA. DJ-1-associated sites identified are regions upstream of the DNA polymerase N (POLN) gene, downstream of the Estrogen Receptor α (ESR1) gene and in the intron of the Adenosylhomocysteine Hydrolase-like 2 (AHCYL2) gene, and expression levels of these genes were significantly decreased in DJ-1-knockdowned SH-SYSY cells before and after treatment of the cells with 6-OHDA, indicating that DJ-1 positively regulates the expression of these genes regardless of oxidative stress. While expression levels of POLN and AHCYL2 genes were also decreased in SH-SYSY cells treated with 6-OHDA compared to those in cells without 6-OHDA treatment, expression of the ESR1 gene was not changed after oxidative stress. Cysteine residues, especially cysteine at amino acid number 106 (C106), of DJ-1 are oxidized in cells treated with 6-OHDA. Since the expression levels of these genes were reduced in DJ-1-knockdown cells and since the expression levels of POLN and AHCYL2 genes but not that of the ESR1 genes were reduced in SH-SYSY cells that had been treated with 6-OHDA, this results suggest that DJ-1 regulates gene expression in an oxidative stress-dependent or independent manner.

More than 7 fold changes in DJ-1-binding sites in SH-SYSY cells treated with 6-OHDA compared to those in untreated SH-SYSY cells are shown

Table 2: Mapping number of DJ-1-binding sites after SH-SYSY cells has been treated with 6-OHDA.

<table>
<thead>
<tr>
<th>Chromosome</th>
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<th>Control/6-OHDA (-fold)</th>
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<td>4</td>
<td>a</td>
<td>11.6</td>
<td>POLN</td>
<td>upstream</td>
</tr>
<tr>
<td>6</td>
<td>g</td>
<td>7.4</td>
<td>ESR1</td>
<td>on intron</td>
</tr>
<tr>
<td>7</td>
<td>c</td>
<td>8.3</td>
<td>AHCYL2</td>
<td>on intron</td>
</tr>
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<td>14</td>
<td>a</td>
<td>16.4</td>
<td>GPHN</td>
<td>on intron</td>
</tr>
<tr>
<td>19</td>
<td>b</td>
<td>8.2</td>
<td>RELB</td>
<td>downstream</td>
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</tbody>
</table>

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Conclusions

In conclusion, expressions of ESCO1, POLN, ESR1 and AHCYL2 genes are regulated by DJ-1 to protect cells against oxidative stress-induced onset of diseases such as Parkinson’s disease. These findings revealed new target genes regulated by DJ-1. It would be interesting to further analyze the effects of DJ-1 on segregation of sister chromatids, DNA replication through the ESCO1, ROS-generated translesion synthesis through POLN and 17beta-estradiol-exerting protective action against ischemic injury through ESR1, and metabolism of homocysteine through AHCYL2.

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