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 fragments that bind to DJ-1 complexes were obtained by a chromatin immunoprecipitation sequencing 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 also 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], polypyrImidine tract-binding Protein-associated Splicing Factor (PSF) [12], Keap1, an inhibitor of nuclear factor erythroid-2 related factor 2 [13], sterol regulatory element binding protein (SREBP) [14] and RREB1 [15], and regulates their transcriptional activity, resulting in various effects on signaling pathways, cell cycle movement, oxidative stress reaction and dopamine synthesis. It is therefore thought that loss of and excess activation of DJ-1 lead to the onset of neurodegenerative diseases such as Parkinson's disease and cancer [16-21], respectively. Only a few genes regulated by DJ-1, however, have been identified.

Chromatin immunoprecipitation (ChIP) assays are used to identify a transcription factor that binds to specific regions in genes of interest. For genome-wide screening of transcription factors and for identification of their recognition sequences on genomes, the ChIP technique has been applied to next-generation DNA sequencers and this technique is named ChIP sequencing [22-24].

In this study, we screened DJ-1 complex-binding sites in the genome of human SH-SY5Y cells by the ChIP sequencing and obtained 60 different sequences, including sequences upstream of the POLN gene and in introns of ESR1 and AHCYL2 genes. We also found that the expression levels of POLN, ESR1 and AHCYL2 genes were decreased in DJ-1-knockdown cells and that the expression levels and a number of DJ-1-associated sites were decreased in cells under oxidative conditions. These results suggest that DJ-1 regulates expression of versatile genes at the transcriptional level and that some of the genes are regulated by DJ-1 in a DJ-1 oxidative status-dependent manner.

Materials and Methods

Cell culture

Human SH-SY5Y and mouse NIH3T3 cells were purchased from American Type Culture Collection. DJ-1-knockdown SH-SY5Y cells (about 50% knockdown of DJ-1 expression) [25] and DJ-1-knockdown NIH3T3 cells (D2 cells) (about 40% knockdown of DJ-1 expression) [26] were established previously. DJ-1-knockdown NIH3T3 cells (D2 cells) were well-characterized and used in transcriptional regulation 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 (GAI1, Illumina). Of total 7,702,242 and 5,814,987

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short reads that had been ChIP-sequenced in samples from SH-SHYSY5Y cells and 6-OHDA-treated SH-SHYSY5Y 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-SHYSY5Y 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-lysing 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 species-matched IgG or with specific antibodies overnight at 4°C. DNA fragments immunoprecipitated were then used as templates for PCR with Ex taq (TaKaRa Bio, Kyoto, Japan) and reacted for 32-40 cycles of 30 sec at 96°C, 30 sec at 60°C and 30 sec at 72°C, and 5 min at 72°C. Reverse transcription polymerase chain reaction (RT-PCR) was carried out using Superscript III (Invitrogen). Nucleotide sequences of forward and reverse primers used for RT-PCR and real-time PCR are as follows: ESCO1ChIP-F: 5'-GCCCTCAGCTGTCATGTCCTT-3', AHCYL2ChIP-R: 5'-GTCCAGAGGATTGCTTGAGG-3', AHCYL2ChIP-F: 5'-TTCCTAGGCACCAGCAATCT-3', POLNChIP-F: 5'-AAAAGACTGGGTGGGAGGAGT-3', POLNChIP-R: 5'-CCCCCTCAGCTTGTGTTGTTT-3', ESR1ChIP-R: 5'-ATGGAAGTGTTTGGTATTGTTT-3', ESCO1ChIP-F: 5'-GCTAAGATGACACCGCAACA-3', ESCO1ChIP-R: 5'-GGGCACTCTTGAATGAGTGT-3'. DNA fragments immunoprecipitated were then used as templates for PCR with Ex taq (TaKaRa Bio, Kyoto, Japan) and reacted for 3 min at 94°C, 39 cycles of 30 sec at 94°C and 30 sec at 60°C.

Statistical analyses

Statistical analyses were carried out using analysis of variance (one-way ANOVA) followed by unpaired Student's t-test, and data are expressed as means ± S.D.

Results and Discussion

Identification of DJ-1-targeting genes in SH-SHYSY5Y cells

ChIP-sequencing was then carried out to identify potential DJ-1 binding/recognition sites in cells using GAII, and mapping of DJ-1 associated/recognition sites in genes was carried out using UCSC Genome Browser on Human Mar. 2006 (NCBI36/hg18 Assembly). Mapping peaks on human genome were detected using Illumina Genome studio ChIP sequence module ver.1.0, and DNA sequences that had been immunoprecipitated with the anti-DJ-1 antibody but not with IgG were mapped. Since it is not clear whether DJ-1 directly binds to DNA and since it has been reported that DJ-1 acts 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 intron of genes encoding Rho-associated Coiled-coil Containing protein Kinase 1 (ROCK1) and Establishment of Cohesion 1 (ESCO1), respectively. CLUSTAL W (1.83) Multiple Sequence Alignments were then used to align sequences obtained. Aliened 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

Of the genes identified, the highest hit of DJ-1-binding sites in the gene sequence 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 ESCO1 gene, ChIP assays were carried out using chromatin from SH-SHYSY5Y 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 ESCO1 gene spanning +27109 to +27367. To examine the relationship between the ESCO1 gene and DJ-1, total RNA was extracted from parental and knockdown cells of human SH-SHYSY5Y and mouse NIH3T3 cells, and the expression levels of ESCO1, 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 C), expression levels of ESCO1 mRNA in DJ-1 knockdown cells of NIH3T3 and in SH-SHYSY5Y cells were reduced to about 80% and 40%, respectively, of those in parental NIH3T3 cells and in SH-SHYSY5Y cells.

| Human | Mouse |
|-------|-------|
| Primer name | Sequence (5'---3') | Primer name | Sequence (5'---3') |
| ESCO1 3191F | cctggctgctcactaatca | ESCO1 2109F | tgcctcaatcggcttct |
| ESCO1 3396F | tgtggtgcatcaacgtcttc | ESCO1 2332R | gacactcgtgaggtcattt |
| GPHN2114R | ccagctgtgattcacaaggct | GPHN1955F | ccttgcaagttgagtcata |
| GPHN3147R | agtactggactagccat | GPHN2114R | gatgttggtcttaaacat |
| POLN 1559F | aagctggctgaggtcacttt | POLN 115F | tgcctcaatcggcttct |
| POLN 1767R | aagctggctgaggtcacttt | POLN 294R | tgcctcaatcggcttct |
| ESCR 1824R | agarccactcggtcggtcagga | ESCR 2891F | aagcggagcgcatcaggaac |
| ESCR 1577R | gtagctgtggagctgcata | ESCR 3045R | ggagtctgctgcacccat |
| ACHEL2Y56R | gtagctgtggagctgcata | ACHEL2Y56R | gtagctgtggagctgcata |
| RELB 1924F | ttcctcagcctggtcattc | RELB 1405F | ttcctcagcctggtcattc |
| RELB 2038R | acgctgtggagctgcata | RELB 1565R | acgctgtggagctgcata |
| DJ-1 299F | tgcctcaatcggcttct | DJ-1 649F | tgcctcaatcggcttct |
| DJ-1 1511R | ttcctcagcctggtcattc | DJ-1 809R | ttcctcagcctggtcattc |
| ACTB 875F | cttcctggtggtgcata | ACTB 412F | cttcctggtggtgcata |
| ACTB 952R | gtagctgtggagctgcata | ACTB 520R | gtagctgtggagctgcata |

Table 1: Nucleotide sequences of primers used for RT-PCR and real-time PCR.
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 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-SY5Y 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-SY5Y 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-SY5Y cells that had been treated with 6-OHDA compared to those in non-treated DJ-1-knockdown SH-SY5Y 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-SY5Y cells were reduced to about 40-50% and 78-60%, respectively, of that in untreated SH-SY5Y 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-SY5Y 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-SY5Y 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 cells of human SH-SY5Y and mouse NIH3T3 cells, suggesting that the ESR1 gene is regulated by DJ-1 at the transcriptional level under a non-stressed condition. ESR1 is required for proper sister chromatid cohesion. Although there is no evidence at present, DJ-1 might control the segregation of sister chromatids.
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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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