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Sequential chromatin immunoprecipitation to detect SUMOylated MeCP2 in neurons

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ABSTRACT

The small ubiquitin-like modifier (SUMO) is a short peptide that can be covalently linked to proteins altering their function. SUMOylation is an essential post-translational modification (PTM). Because of its dynamic nature, low abundance levels, and technical limitations, the occupation of endogenous SUMOylated transcription factors at genomic loci is challenging to detect. The chromatin regulator Methyl CpG binding protein 2 (MeCP2) is subjected to PTMs including SUMO. Mutations in MeCP2 lead to Rett syndrome, a severe neurodevelopmental disorder. Here, we present an efficient method to perform sequential chromatin immunoprecipitation (Seq-ChIP) for detecting SUMOylated MeCP2 in neurons. This Seq-ChIP technique is a useful tool to determine the occupancy of SUMOylated transcription and chromatin factors at specific genomic regions.

1. Introduction

The covalent addition of Small Ubiquitin Modifier, SUMOylation, is an essential post-translational modification (PTM) that regulates diverse cellular processes including gene transcription, chromatin remodeling, replication, chromosome segregation, and DNA repair [1–4]. In mammals, the conjugation of SUMO to a lysine residue in a target protein is mediated by three paralogs: SUMO1–3 [1]. Most substrate proteins are rapidly deSUMOylated by one of six Sentrin/SUMO-specific proteases (SENPs) [5]. Defects in SUMOylation are associated with multiple diseases such as cancer, heart failure, and neurodegeneration [6–8].

In the nervous system, SUMOylation plays a crucial function with roles in synapse formation, axonal transport, and neuronal excitability [9–12]. One way that neurons employ a rapid modulation of their transcription program in response to neuronal activity is by regulating the PTMs of transcription factors. Transcription factors can be modified by multiple PTMs, such as phosphorylation, acetylation, ubiquitination, and SUMOylation [13]. These PTMs modulate either the target factors binding ability to DNA or interaction with protein co-factors [2].

One neuronal factor that is regulated by PTMs is the Methyl CpG binding protein 2(MeCP2) [8]. Although it was first described in the early 1990s as a transcriptional repressor binding methylated DNA, it is now appreciated that MeCP2 can also function as an activator and chromatin modifier [8,14,15]. The importance of MeCP2 is underscored as its mutations lead to a severe neurodevelopmental disorder known as Rett syndrome (RTT) [16,8]. SUMOylation modifies MeCP2, modulates its transcriptional activity, and plays a crucial role in synaptic formation [17]. However, our knowledge of the occupancy of endogenous SUMOylated MeCP2 at specific neuronal regulatory regions is lacking. The identification of neuronal promoters harboring SUMOylated MeCP2 will define its biological function during normal development. But detection of endogenous SUMOylated chromatin and transcription factors at genomic regions is problematic. Many SUMOylated proteins such as MeCP2 and other chromatin and transcription factors are low in abundance [18]. SUMOylation is very dynamic; therefore, identification of factors occupying specific genomic regions is difficult [18,19]. Furthermore, no antibodies exist that specifically recognize a SUMOylated protein at particular genomic sites. Here, we describe an efficient sequential chromatin immunoprecipitation (Seq-ChIP) method to identify SUMOylated MeCP2 at a given neuronal genomic region *in vivo*.

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2. Materials and methods

2.1. Neuronal cell culture

Mouse hippocampal neuronal cells (HT22) were maintained in DMEM (Life Technologies) supplemented with 10% fetal calf serum, 2 mM L-Glutamine, and 1 × Penicillin/Streptomycin.

2.2. Chromatin immunoprecipitation (ChIP) antibodies and primer selection

ChIP-grade antibodies were used for the Seq-ChIP. If an antibody has not been tested for ChIP, the efficiency of the antibody can be tested by co-immunoprecipitation of the target protein as previously described [20]. Western blotting can be performed following the immunoprecipitation of the protein-DNA complexes to check whether the SUMO PTM is enriched within the immunoprecipitated complex. This co-immunoprecipitation test should be utilized to test other SUMOylated transcription factors or chromatin modulators prior to the sequential ChIP. To design primers for quantitative PCR, we selected an amplicons with a size range of 75–200 base pair (bp) flanking the regulatory regions in genes of interest. We designed several sets of PCR primers located upstream and downstream of each target genomic site and compared the ChIP signals from these primer sets to observe protein enrichment at a particular locus. PCR primers were designed with the aid of several complimentary software applications such as OLIGO Primer Analysis Software (http://www.oligo.net/), Primer 3 (www.bioinfo.ut.ee/primer3/), or Primer Premier (http://www.premierbiosoft.com/primerdesign/). Prior to oligo synthesis, we checked the specificity of the primers using the National Center for Biotechnology (NCBI) Basic Local Alignment Search Tool (BLAST). In addition, we obtained a melt curve analysis following the real-time PCR analysis, to determine the specificity of the PCR primers. A suitable set of PCR primers exhibits a single peak in the melt curve. An agarose gel analysis of the PCR reaction product can be also used to test the adequacy of the primers with the demonstration of a single band.

2.3. Sequential ChIP and quantitative Polymerase chain reaction (PCR)

To detect SUMOylated MeCP2 at neuronal chromatin, we established a Sequential-Chromatin immunoprecipitation (Seq-ChIP) method:

(1) 2.5×10^8 HT22 cells were crosslinked with 1% formaldehyde for 10 min at room temperature with gentle agitation then quenched by 125 mM glycine with agitation for an additional 10 min. The crosslinked cells were washed twice each with ice cold phosphate buffered saline (PBS). The cells were pelleted by centrifugation (Eppendorf 5424 centrifuge) for 5 min at 2000 rpm and resuspended in Lysis Buffer (1% SDS, 5 mM EDTA, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl) supplemented with protease inhibitors (phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche Diagnostics) prior to use. The chromatin fraction was sheared using a microtip sonicator (Branson) on an output 7 setting for 22 cycles of 10 s sonication followed by rest on ice for 50 s. A small aliquot of the chromatin was removed and checked for sonication efficiency (< 500 bp) by agarose gel electrophoresis. The sheared chromatin was then centrifuged for 10 min at 4 °C maximum speed. The supernatant containing the chromatin was diluted 1:10 with ChIP Dilution Buffer (20 mM Tris (pH 8.0), 1% Triton-X 100, 150 mM NaCl, 2 mM EDTA. Supplemented with protease inhibitor before use). Next, the chromatin was pre-cleared by adding 100 µl agarose A/G beads (Santa Cruz) at 4 °C for 2 h with constant rotation. The mixture was centrifuged at 4 °C 2000 rpm for 5 min. The supernatant was removed, retaining 1% as an input. The remainder of the diluted chromatin was then aliquoted into 1.5 ml tubes with an aliquot per ChIP antibody.

(2) To perform the first ChIP, 6 µg of antibody (IgG control (Santa Cruz sc-2027), anti-SUMO1 (Santa Cruz sc-9060), and anti-MeCP2 (Millipore 07013)) was added to each tube of the diluted chromatin and rocked overnight at 4 °C. Following the overnight incubation, agarose A/G beads (150 µl) were added to each sample and rotated for an additional 2–4 h at 4 °C.

(3) The beads were pelleted and washed once each with Buffer 1 (0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.0), 150 mM NaCl), Buffer 2 (0.1%SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.0), 500 mM NaCl), Buffer 3 (1% NP40, 1% Deoxycholic acid, 1 mM EDTA, 10 mM Tris–HCl (pH 8.0), 250 mM LiCl), and Buffer 4 (2 mM EDTA, 10 mM Tris–HCl (pH 8.0)). After the final wash, the DNA-protein complexes were eluted twice with 100 µl 10 mM DTT at 37 °C for 30 min with gentle shaking twice and combined. The beads were pelleted, the supernatant was saved, and the wash steps with Buffers 1–4 (iii) were repeated once as described above. The supernatants were combined from both steps. One tenth of the eluate was saved as a template for the primary ChIP for qPCR analysis. The remaining supernatant was diluted with 20 times ChIP dilution buffer and the secondary ChIP was performed beginning with the addition of the second ChIP antibody at the above step (ii).

(4) After the final wash, as listed above in step (iii), the DNA was eluted from the beads with 250 µl Elution Buffer (1 mM EDTA, 10 mM Tris–HCl (pH 7.5), 1% SDS) twice for 30 min at room temperature with constant rotation. Proteinase K (final concentration 10 µg/ul) and NaCl (final concentration 200 mM) were added to the eluted chromatin from the primary ChIP, secondary ChIP, and input. The crosslinks were reversed at 65 °C for 4 h to overnight. The DNA was purified using phenol/chloroform extraction. The purified DNA was resuspended in 60 µl double-distilled H_20 (ddH_20).

2.4. Seq-ChIP quantitative PCR analysis

PCR reactions were set up in triplicate using the primer sets shown in Table 1. Each PCR was assembled as described in Table 2 using SYBR® Green PCR Master Mix (Life Technologies). The analysis was performed using the real-time PCR ABI7500 system (Applied Biosystem). For data analysis $2^{-(Ct_{Ab} - Ct_{input})}$. Ct = cycle threshold. Statistical analysis was done using the Student's *t*-test. A positive result showed a significant enrichment of the MeCP2 protein at genomic regions compared with either IgG or the negative control regions.

3. Results

3.1. Scheme of sequential ChIP to detect SUMOylated MeCP2 in neurons

MeCP2 is an important regulator in the brain [8]. However, the precise mechanism of action for MeCP2 is unknown. Although MeCP2 is subjected to numerous PTMs, it is not known where along the genome SUMOylated MeCP2 binds. SUMO modifications can alter its substrate protein–protein interactions, nuclear-cytoplasmic location, ubiquitination status, and transcriptional activity [7]. Thus, the identification of regulatory regions harboring SUMOylated MeCP2 is crucial for understanding the role for this PTM