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Phosphorylation of methyl-CpG binding protein 2 (MeCP2) regulates the intracellular localization during neuronal cell differentiation

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Abstract

Methyl-CpG binding protein 2 (MeCP2) is a transcriptional repressor which recognizes methylated CpG dinucleotides. Mutations in the MeCP2 gene is known to cause human autistic disease Rett syndrome, but its molecular mechanisms remain to be elucidated. Since MeCP2 is a DNA-binding protein, it has been believed that MeCP2 functions only in the nucleus. We herein show that MeCP2 is localized in the cytosol as well as in the nucleus of neuronal cells. Through the use of immunofluorescence and Western blot analyses, MeCP2 was found to be localized both in the nucleus and cytosol of rat PC-12 and mouse Neuro2a cells before neuronal differentiation, and it was translocated into the nucleus during differentiation. In primary cultured neurons from mouse cortex, MeCP2 was expressed in whole cell bodies on the first day of culture while after 7 days of culture, MeCP2 was localized mainly in the nucleus. Furthermore, MeCP2 was re-localized in the nucleus and cytosol after 14 days of culture. To study the molecular mechanisms of translocation, we analyzed the post-translational modification of MeCP2. The cytosolic MeCP2 was Ser/Thr-phosphorylated, while the nuclear MeCP2 was not. Both the cytosolic and nuclear MeCP2 were SUMOylated, which has been reported to be a nuclear transport signal. Our data suggests that the nuclear translocation of neuronal MeCP2 was induced during differentiation and/or maturation, and that Ser/Thr-phosphorylation regulates its translocation.

Keywords: MeCP2; Intracellular localization; Neuronal differentiation; Phosphorylation; Post-translational modification; SUMOylation

1. Introduction

Methyl-CpG binding protein 2 (MeCP2), a member of the methyl-DNA-binding protein (MBD) family, is a key factor in epigenetic transcriptional regulation. MeCP2 recognizes methylated CpG dinucleotides in the mammalian genome, and constitutes a transcriptional repressor protein complex by recruiting Sin3a and histone deacetylases (HDACs) (Nan et al., 1998; Jones et al., 1998). Mutations of the MeCP2 gene are found in a majority of patients with Rett syndrome (RTT) (Amir et al., 1999). RTT is a genetic neurodevelopmental disease in females. The patients usually develop normally from birth to 6–18 months of age and then exhibit neurological symptoms such as psychomotor dysfunction, epilepsy, and autism thereafter (Hagberg, 1985).

Since it has been recognized that MeCP2 represses the transcription of target genes, such as BDNF and DLX 5 in the mammalian genome, MeCP2 has been believed to only be expressed in the nucleus (Shahbazian et al., 2002; Kishi and Macklis, 2004; Mullaney et al., 2004). However, MeCP2 has been reported to be localized not only in the nucleus but also outside of the nucleus (Mnatzakanian et al., 2004) and in the post-synaptic compartment (Aber et al., 2003). These reports suggest that MeCP2 may have a role outside of the nucleus including synaptic regulation (Aber et al., 2003; Nelson et al., 2006). However, there is little understanding of the cytosolic function of MeCP2.

It has recently been reported that two MeCP2 isoforms are being expressed in several tissues including the brain (Mnatzakanian et al., 2004; Kriaucionis and Bird, 2004). The expression of newly discovered MeCP2B appears to predominate over MeCP2A (Kriaucionis and Bird, 2004). Although MeCP2B may have some important functional

Abbreviations: DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HS, horse serum; IEF, isoelectric focusing; MeCP2, methyl-CpG binding protein 2; O-GlcNAc, O-linked *N*-acetylglucosamine; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SUMO, small ubiquitin-related modifier

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domains on its N-terminus that resemble extracellular signal related kinase-1, it remains unclear whether MeCP2B functions differently from MeCP2A.

Although the theoretically calculated molecular weight of MeCP2 based on its amino acid sequence is approximately 53 kDa, the actual molecular weight of MeCP2 by a Western blot analysis that has been reported thus far is various such as, approximately 75 kDa in several cell lines (Lorincz et al., 2001) and in rodent brain tissues and cells (Jones et al., 1998; Klose and Bird, 2004; Jugloff et al., 2005) and 100 kDa in human cerebral cortex and lymphoid cells (Jarrar et al., 2003). This molecular weight discrepancy may thus indicate the presence of post-translational modification in MeCP2. Recently, MeCP2 has been reported to be phosphorylated and such phosphorylation is associated with the inhibition of DNA-methylation-based transcriptional repression of the *BDNF* gene (Chen et al., 2003; Dennis and Levitt, 2005). However, the detailed mechanisms of the Ser/Thr-phosphorylation on transcriptional control remain to be elucidated.

In this study, to better understand the functional regulation mechanisms of MeCP2, we examined intracellular localization and post-translational modifications of MeCP2 during the neuronal differentiation of rat PC-12 and mouse Neuro2a cells and on the maturation of primary cultured mouse cortical neurons.

2. Materials and methods

2.1. Cell culture

PC-12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% horse serum (HS; Sigma) and 5% fetal bovine serum (FBS; Sigma). Neuro2a cells were maintained in DMEM containing 10% FBS. All cells were supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin and grown in a 37 °C humidified incubator. To neuronally differentiate PC12 cells, the cells were plated onto a poly-lysine-coated dish or cover glass and treated with 50 ng/ml of nerve growth factor (Chemicon, Temecula, CA, USA) every other day. To neuronally differentiate Neuro2a cells, the cells were treated once with 30 μM of retinoic acid (WAKO; Osaka, Japan) in DMEM medium containing 2% FBS.

For primary cortical cultures, E18 mouse embryonic brains were collected from C57BL/6 mice by Caesarian section. The cortices were dissected out in ice-cold Hanks' balanced salt solution (Sigma), cut into pieces, placed in Neurobasal™ medium (Gibco, Grand Island, USA) supplemented with L-glutamine and B27 (Gibco) and followed by trituration using a pipette. The cells were plated at a density of 2×10^4 cm^{-2} onto a poly-lysine-coated cover glass. All animal experiments were approved by University of Yamanashi Animal Care and Use Committee.

2.2. Immunofluorescent cell staining

The cultured cells were washed three times with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 30 min, and then washed three additional times with PBS and were incubated in 0.1% TritonX-100/PBS for 30 min. The cells were blocked with 1% skimmed milk in PBS for 1 h and incubated with rabbit polyclonal anti-MeCP2 antibody (1:500 dilution; Upstate Biotechnology, Lake Placid, USA) and monoclonal anti-β-tublin isotype III-antibody (1:250 dilution; Sigma) at 4 °C overnight. After washing, the cells were incubated with an Alexa Fluor 594 conjugated anti-rabbit IgG antibody and/or Alexa 488 conjugated anti-mouse IgG antibody (1:5000 dilution; Molecular Probes Inc., Eugene, OR, USA) in PBS at room temperature for 1 h. The nuclei were stained with Hoechst 33342 (Dojindo Lab., Kumamoto, Japan). The prepared cover slips were mounted onto slides with Vectashield mounting medium (Vector Lab., Burlingame, USA). The stained cells were observed under fluorescent microscopy (Olympus, Tokyo, Japan).

2.3. Transfection of the Mecp2 expression vector

Two micrograms of the *Mecp2* expression vector, kindly provided by Dr. Kudo (Kudo, 1998), were transfected into Neuro2a cells in a 100 mm dish using Lipofectamine 2000 according to the manufacture's instructions (Invitrogen, Carlsbad, CA, USA), and incubated at room temperature for 20 min. Retinoic acid treatment was performed 3 days after transfection of the *Mecp2* vector.

2.4. Western blotting

Neuro2a cells were fractionated to nuclear and cytosolic fractions by using the Nuclear Extract Kit (Active Motif, Carlsbad, USA). The protein concentration of each sample was determined by a protein quantification kit (Dojindo). Thirty micrograms of each protein sample were electrophoresed on an 8% polyacrylamide gel, and transferred onto a polyvinylidene fluoride (PVDF) membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 1 h. The membranes were blocked with 1% skimmed milk or 1% bovine serum albumin (BSA) in PBS for 1 h, and incubated with primary rabbit polyclonal anti-MeCP2 antibody, anti-phosphor-Ser/Thr mouse IgG (Qiagen Inc., Valencia, CA, USA), anti-O-GlcNAc mouse IgM (Covance Inc., NJ, USA) or anti-SUMO-1 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 °C overnight. After washing, either biotin conjugated either goat anti-rabbit antibody (1:5000 dilution; WAKO) or biotin conjugated anti-mouse IgG + IgM antibody (1:5000 dilution; Nacalai Tesque Inc., Kyoto, Japan) was added to the membranes and incubated at room temperature for 1 h, followed by incubation with a streptavidin biotin complex peroxidase kit (Nacalai Tesque) at room temperature for 30 min. Immunoreactivity was visualized using a peroxidase detection kit for immunostaining (Nacalai Tesque) or Western blotting detection ECL reagents (Amersham Biosciences Inc., Piscataway, USA). The quantification of the results was accomplished using the computerized imaging program Quantity One (Bio-Rad, Hercules, USA).

2.5. Immunoprecipitation

For immunoprecipitation, nuclear and cytosolic fractions were incubated for 1 h at 4 °C with anti-phospho-Ser/Thr antibody. Fifty microliters of Protein G Sepharose beads (Amersham) were then added and incubated for 1 h at 4 °C. Immunocomplexes were collected by centrifugation (12,000 × *g*, 20 s) and washed three times with lysis buffer from the Nuclear Extraction Kit. These final pellets were suspended in sample buffer (1% SDS, 100 mM DTT, 50 mM Tris) and dissociated from the beads by heating to 95 °C for 3 min.

2.6. Two-dimensional electrophoresis (2DE)

Two-dimensional electrophoresis was performed with a Mini-PROTEAN 2D apparatus (BIO-RAD). In running of the first dimension isoelectric focusing (IEF) (pH 3–10), 100 mM sodium hydroxide and 10 mM phosphoric acid were used as a cathode buffer and an anode buffer, respectively. The protein sample was loaded into a tube gel and was run with 500 V for 10 min, followed by 750 V for 2.5 h. In running of the second dimension IEF, the gel was extruded from the tubes and electrophoresed on 8% SDS-PAGE gels.

3. Results

3.1. Cytosolic localization of Mecp2 and its translocation of Mecp2 during neuronal differentiation

Intracellular localization of Mecp2 in PC-12 cells (Fig. 1A day 0) and Neuro2a cells (Fig. 1B day 0) was investigated using immunocytochemical staining with anti-Mecp2 antibody. Prior