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Relationship Between MeCP2 And NF κ B Signaling During Neural Differentiation Of P19 Cells

Cliona O'Driscoll^{1,2}, Walter Kaufmann^{1,3}, Joseph Bressler^{1,2,4}

¹Hugo Moser Institute at the Kennedy Krieger

²Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University

³Departments of Pathology, Neurology, Pediatrics, Psychiatry and Behavioral Sciences, and Radiology and Radiological Science, Johns Hopkins University School of Medicine Baltimore, MD 21205

Abstract

The pluripotent P19 embryo carcinoma cell line was studied to determine a signaling pathway regulating MeCP2 expression. P19 cells were induced to differentiate into neurons by RA and express β -III tubulin at one day after induction and synaptophysin by 7 days. MeCP2 was first observed after β -III tubulin expression was detected and continued to rise over the course of differentiation. Both *Mecp2 e1* and *e2* mRNA forms progressively increased in differentiating cells. MeCP2 expression was increased by tumor necrosis factor (TNF) in early differentiating cells, which was blocked by NF κ B inhibitors. TNF did not increase MeCP2 expression in naïve cells. Moreover, TNF did not increase NF κ B reporter gene activity in naïve cells even though increases were observed in early differentiating cells. The protein kinase C activator phorbol 12-myristate 13-acetate (PMA) increased MeCP2 expression in naïve P19 cells, which was also blocked by NF κ B inhibitors. Interestingly, PMA increased NF κ B reporter gene activity in naïve cells. Finally, PMA, but not TNF, induced I κ B α degradation in naïve P19 cells. Taken together, our data indicates that MeCP2 expression is regulated in part by signaling pathways involving NF κ B.

Keywords

methyl DNA binding protein 2; P19 embryonic carcinoma cells; NF κ B; tumor necrosis factor; development

⁴Corresponding author; bressler@kennedykrieger.org, 443-923-2695 (fax), 443-923-2677 (tel).

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1 Introduction

Methyl-CpG-binding protein 2 (MECP2) is an X-linked gene initially identified as a transcriptional repressor (Nan et al., 1997). While MeCP2 is expressed in many tissues, it is highly expressed in the brain and its ontogeny is temporal and regional specific (Kaufmann et al., 2005; Mullaney et al., 2004). Intense interest in MeCP2 arose from the discovery that mutations in the gene underlie the majority of Rett syndrome cases (Amir et al., 1999). Rett syndrome is a neurodevelopmental disorder characterized by normal development for about one year that is then followed by regression affecting speech, movement, growth and often accompanied by the development of seizures and autistic type behavior. The mutations are generally sporadic point mutations and can arise across all regions of the gene, while over 200 mutations have been identified, 8 are most commonly found in Rett syndrome patients (Miltenberger-Miltenyi and Laccone, 2003). Interestingly, some of the mutations are associated with specific clinical pathologies and disease severities (Carter et al., 2010). The involvement of MeCP2 in the more generalized autism spectrum disorders (ASD) is supported by the observation that autistic and ASD brain samples display lower levels of MeCP2 than non-autistic samples (Nagarajan et al., 2006) and it has also been suggested that the presence of functional MECP2 variants containing microsatellite polymorphisms increases the risk of autism (Loat et al., 2008).

MeCP2 is found mainly localized in the nucleus and has a predicted molecular weight of 53 kDa, though runs as an 81 kDa protein on SDS-PAGE. Nonetheless, other molecular weight forms have been noted (Jarrar et al., 2003) and the presence of MeCP2 outside of the nucleus has been observed (Aber et al., 2003). MeCP2 has a number of molecular functions including chromatin organization (Hite et al., 2009), RNA splicing (Young et al., 2005), and transcription regulation (Nan et al., 1997; Yasui et al., 2007). MeCP2 likely performs these functions by partnering with different proteins including the chromatin modifying ATPases mSin3a (Nan et al., 1997) and ATRX (Nan et al., 2007), DNA methyltransferase, and histone deacetylase (Nan et al., 1998).

Although levels of MeCP2 affect neurodevelopment, little had been known concerning the regulation of MeCP2 expression. Accordingly, the objective of our study is to understand the signaling pathways regulating the expression of MeCP2 in differentiating neurons. We focused on the NF κ B transcription factor family because it mediates responses evoked by infectious agents (Shih et al., 2011) and studies have shown dysfunctional neurodevelopment in developing organisms exposed to mediators of inflammation (Deverman and Patterson, 2009; Pardo et al., 2005; Patterson, 2011). To accomplish this objective, we studied the pluripotent P19 embryonic carcinoma cell line because they undergo progressive differentiation to neurons after induction with retinoic acid (RA). We will show that, similar to other neuronal proteins, MeCP2 expression progressively increases as neurons mature. Furthermore, the expression of MeCP2 is regulated by the NF κ B signaling.

2 Results

2.1 Temporal expression of neuronal markers after treatment with RA

The pluripotent P19 embryo carcinoma cell line was chosen as the model system to study regulation of MeCP2. The advantage of the cell line is that it allows us to study very early events, even before the commitment to neural stem cell lineage. P19 cells display the capacity to differentiate to the three embryonic cell layers and express the pluripotent transcription factor Oct-4 (Bain et al., 1994; Krawetz et al.; van der Heyden and Defize, 2003). They can be induced to undergo neural differentiation by overexpressing proneural basic helix-loop-helix transcription factors (Farah et al., 2000; Huang et al.; Huang et al., 2010) and undergo very similar patterns of neuronal differentiation as mouse embryonic stem cells (Finley et al., 1996). P19 cells have previously been shown to form functional synapses as early as 3 days after RA induction and continue to mature for up to 4 weeks (McBurney et al., 1988) (MacPherson et al., 1997). In the study presented here, neuronal differentiation was selected by culturing the RA treated cells in media supplement with B27.

On Western blots, naïve cells did not display any marker of neuronal differentiation (figure 1). However, strong expression of the pluripotent transcription factor Oct-4 was observed. On the first day following RA induction, expression of Oct-4 was lost but the expression of the cytoskeletal protein β -III tubulin was detected; β -III tubulin is considered to be one of the earliest neuronal markers demonstrating commitment to the neuronal lineage (Katsetos et al., 2003). On the third day, the dendritic protein MAP2c was observed along with MeCP2. Although very little MeCP2 was detected at the 75 kDa band (the most frequent form of MeCP2 reported in the literature) in naïve cells, three distinct bands at 150 kDa, 105 kDa, and 50 kDa were detected in naïve cells with antibody against MeCP2 in the cytosol (supplementary figure 1). In this study, only reactivity at the 75 kDa band will be considered MeCP2. Both the *Mecp2 e1* and *e2* mRNA isoforms were elevated in differentiating cells, an increase that coincided with that of the respective protein (figure 1).

A progressive increase was observed in proteins associated with later stages of neuronal differentiation in P19 cells. The nuclear protein NeuN and synaptic vesicle protein synaptophysin were observed on days 5 and 7, respectively after RA induction. Levels of MeCP2 continue to rise in differentiating cells though levels dropped in the more mature cells. This could have been due to higher levels of actin due to increases in neurite outgrowth. Immunocytochemical analysis was used to verify the Western blot analysis. Neurites expressing β -III tubulin were observed as early as day 3. Double staining with antibodies against MAP2c and neurofilament revealed dendrite-like and axonal-like processes, respectively (supplementary figure 2). As expected, cells expressing glial fibrillary acid protein were not detected due to the B27 neuronal selective media (not shown).

2.2 Tumor necrosis factor (TNF) increases MeCP2 levels through a NF κ B pathway

Because inflammatory cytokines have profound effects on neurodevelopment, the influence of TNF on MeCP2 expression was examined. TNF induced an increase of 1.8-fold in the level of MeCP2 in cells at 3 days after RA induction (figure 2A). TNF failed to increase

levels of MeCP2 protein in naïve cells and at 6 days after differentiation. The induction of MeCP2 protein induction was rapid, an increase of 3.5 fold was observed at 2 hours after treatment with TNF (figure 2B). In several experiments, the range in MeCP2 protein induction was from 1.8- to 3.5 -fold in cultures at 3 days after RA treatment.

TNF mediated gene expression is often through activation of the NF κ B signaling pathway. Increases in both forms of *Mecp2* mRNA were observed, suggesting that TNF increases *Mecp2* transcription (figure 3 A). The effect of TNF on mRNA levels was attenuated with the NF κ B transcriptional inhibitor 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline (Act) (Tobe et al., 2003), but not with the translocation inhibitor JSH23 (Shin et al., 2004). When MeCP2 protein was examined, both inhibitors blocked the effects of TNF though the effects of JSH23 were less than that of Act (figure 3 B). The level of MeCP2 protein and mRNA did not change in the presence of inhibitor alone (data not shown).

2.3 NF κ B signaling in naïve and differentiating P19 cells

To further understand why naïve P19 cells did not display increases in MeCP2 levels after treatment with TNF, NF κ B signaling was examined using a reporter assay in naïve cells and at 4 days after induction with RA. TNF stimulated NF κ B signaling in the 4 induced cells but not in naïve cells (figure 4 A). A regulatory junction in NF κ B signaling is I κ B α , which anchors the NF κ B transcription factor in the cytoplasm. Upon phosphorylation, I κ B undergoes ubiquitination allowing NF κ B to translocate to the nucleus (Hoffmann et al., 2006). On Western blots, naïve cells displayed lower levels of the NF κ B family member p65 and higher levels of I κ B α compared to cells induced to differentiate with RA (figure 4 B).

2.4 PMA induces MeCP2 levels in naïve cells and activates NF κ B signaling

If the NF κ B signaling pathway is impaired in naïve cells, then other activators of NF κ B should also fail to augment MeCP2 levels. No increase in NF κ B reporter activity was observed in naïve P19 cells stimulated with lipopolysaccharide (LPS) and TNF, which both require cell surface receptors. In contrast, the protein kinase C activator PMA, which activates NF κ B signaling in different types of cells (Bomsztyk et al., 1991; Daniel et al., 1995), stimulated NF κ B reporter activity in naïve P19 cells (figure 5 A). Moreover, PMA also stimulated a 2.8-fold increase in levels of levels of MeCP2 protein in naïve P19 cells (figure 5 B). Protein kinase C activation evokes several signal transduction pathways. Similar to the induction of MeCP2 protein by TNF in the 3-day old differentiated cultures, the NF κ B inhibitors Act and JSH blocked the induction of MeCP2 protein in PMA treated cells.

I κ B α degradation is an early event in NF κ B signaling and is catalyzed by I κ B kinase (IKK). In TNF treated naïve cells, A decrease in levels of I κ B α was observed (figure 6 A) at 30 minutes in PMA treated naïve cells but I κ B α levels increased after TNF treatment (figure 6 B). The higher levels of I κ B α levels might explain the absence of NF κ B signaling in naïve cells treated with TNF.

3.0 Discussion

This study demonstrates that the expression of MeCP2 occurs after P19 cells undergo commitment to a neuronal lineage. Commitment was confirmed by the disappearance of the pluripotency marker Oct-4, and the appearance of neuronal proteins. Neuronal commitment was verified by the expression of the β -III tubulin, which was observed prior to the appearance of MeCP2. MeCP2 and the dendritic protein MAP2c, a marker of established neuronal differentiation and dendritic development, are detected at approximately the same stage. Levels of MeCP2 continue to rise throughout the maturation process. As cells begin to express synaptophysin, levels of MeCP2 reached their highest level. As previously observed MeCP2 is likely not involved in the initial stages of neuronal differentiation but the later stages of maintenance and refinement. Our observations are very similar to those made in the developing rodent brain and in neural stem cells *in vitro* (Kaufmann et al., 2005; Mullaney et al., 2004). MeCP2 is expressed early but the highest levels are observed during development when neurons are establishing synapses.

Mecp2 expression was regulated by NF κ B signaling. In early differentiating cultures, MeCP2 was induced by TNF and blocked by NF κ B inhibitors. In naïve P19 cells, PMA induced MeCP2 expression, which was also blocked by the NF κ B inhibitors. Interestingly, PMA induced NF κ B signaling in naïve cells but TNF did not. It is not clear whether NF κ B signaling directly regulates MeCP2 expression, or whether intermediate signaling pathways are required. In any event, the involvement of NF κ B in MeCP2 expression in early differentiating neurons supports the growing literature describing the involvement of NF κ B in the maturing brain (Romano et al., 2006). NF κ B is constitutively active in excitatory synapses (Kaltschmidt and Kaltschmidt, 2009) and regulates glutamate decarboxylase 6 expression (O'Mahony et al., 2006). A recent study reported the involvement of NF κ B signaling pathway in initiating and forming new synapses through the regulation of PSD 95 (Boersma et al., 2011). The unresponsiveness NF κ B in naïve cells could be due to the differences between how TNF and phorbol esters activate NF κ B. TNF activation requires receptor and adaptor proteins whereas protein kinase C activation by phorbol esters phosphorylates I κ B α , which undergoes degradation (Hughes et al., 2001). The protein kinase C signal is more direct and stronger. The NF κ B signaling is also likely harder to activate in naïve P19 cells. We found very high levels of I κ B α in naïve cells which decrease during neuronal maturation.

It has been proposed that TNF is one of the mediators by which glial cells regulate synapse number (Turrigiano, 2006). A problem might arise, however, when there is inflammation during development resulting in increased levels of TNF as well as other inflammatory cytokines (Kawai and Akira, 2005). Higher levels of inflammatory cytokines were shown in cerebral spinal fluid in individuals with autism (Vargas et al., 2005). Moreover, mice born from infected dams or dams challenged with inflammatory inducers, display distinct neurobehavioral problems (Deverman and Patterson, 2009). If developing neurons express receptors for these inflammatory cytokines, increased expression of *Mecp2* and other genes regulated by NF κ B would also increase and might, in part, be responsible for the impaired neurodevelopment.

4 Experimental procedures

4.1 Cell culture

P19 cells (ATCC) were grown in α -Minimum Essential Media (Invitrogen) with 7.5% calf serum and 2.5% fetal bovine serum (Hyclone). The cells grew to 70% confluency in T-175 cm² flasks, dislodged by scraping and replated at a 1:5 dilution to a new flask. Inhibitors of NF κ B were obtained from EMD Millipore, JSH-23 (JSH) was used at a final concentration of 10 μ M and NF κ B Activation Inhibitor -Amino-4-(4-phenoxyphenylethylamino) quinazoline (Act) was used at a final concentration of 100 nM. Inhibitors were resuspended in DMSO and added to the cell cultures 30 minutes before adding TNF and PMA. Tumor necrosis factor (Invitrogen), PMA and LPS (Sigma-Aldrich) were used at 10 ng/mL, 50 nM, and 10 ng/mL respectively.

4.2 Immunocytochemistry

Cells were washed in PBS and fixed with 4% paraformaldehyde for 15 min. The cells were then permeabilised/blocked in 5% normal goat serum, 0.1% triton- $\times$ 100 in PBS for 2 h. The coverslips were incubated with primary antibodies (α -MeCP2 from Millipore, Mouse monoclonal antibodies against mouse neurofilament 200 kDa, β III tubulin and synaptophysin were purchased from Sigma-Aldrich, all others from Abcam) overnight at 4 °C. After washing the Coverslips were incubated with secondary antibody (goat anti-rabbit IgG, or rabbit anti-mouse at 1:200, from Jackson labs) for 1 h at room temperature. Coverslips were mounted with Prolong gold with DAPI (Invitrogen, Carlsbad, CA). The slides were viewed on the Zeiss axioplan microscope.

4.3 RA Induction

Cells were plated at 1×10^6 cells/bacteriological 10 cm petri plate with 500 nM RA in growth media. After 3 days, floating spheres were harvested and collected by centrifugation at 300 RPM for 5 min. 200,000 viable cells were plated per poly-L-lysine (0.1%) coated glass coverslip in Neurobasal Media supplemented with B27 (Invitrogen) without RA. This day is termed day 1 of differentiation; prior to RA induction the cells are termed naïve.

4.4 Western blotting

Whole cell lysates were prepared in 60 μ l of RIPA (PBS, 1% Igepal, 0.5% w/v Deoxycholic acid, 0.1% SDS) buffer with protease inhibitor cocktail set I and phosphatase inhibitor cocktail set II (Calbiochem, La Jolla, CA). Pellets were sonicated to complete lysis. Protein concentration was determined by Bradford assay using Bovine Serum Albumin (BSA) as standard. Cell lysates were boiled for 4 min in sample buffer containing 0.31 M Tris-HCl pH 6.8, 1% SDS, 5% glycerol, 3.6 M β -mercaptoethanol, 5 mM PMSF, and 0.05% bromophenol blue. 25 μ g of protein were separated on 4-20% gradient gels (Lonza, Walkersville, MD) at 120 V. The proteins were transferred to nitrocellulose membranes at 100 V for 90 min. Nitrocellulose membranes were blocked with Odyssey blocking buffer (Licor, Lincoln, NE) and incubated with appropriate primary antibody overnight. Secondary antibody (Licor) that was conjugated with IRDye was diluted 1:10000 in 1:1 Odyssey buffer: PBS and 0.1% Tween and incubated with membrane for 1 h and washed. Blots were

visualized on the Licor Odyssey system, which allowed us to simultaneously measure MeCP2 and neuronal markers with the loading control β -actin (to normalize MeCP2 levels) or glyceraldehyde phosphate dehydrogenase (GAPDH), which was used to normalize $\text{I}\kappa\text{B}$ on the same blot. Saturation in the loading control was not achieved. In addition to the antibodies described above, a monoclonal antibody against NeuN was purchased from Chemicon and rabbit antibodies against Oct 4 and β -actin were purchased from Abcam and Sigma, respectively.

4.5 Reporter assay

P19 cells were plated at a density of 20,000/cm² in a 48 well plate and allowed to attach overnight. The following morning cells were transfected with Lipofectamine 2000 (Invitrogen) (as per manufacturer's instructions) and pGL4.32[*Luc2*P/NF κ B-RE/Hygro] (Promega) and CMV- β -Galactosidase. After 18 h the cells were treated for the indicated times. Cells were lysed and assayed for luciferase and β -galactosidase activity. Luciferase assay was performed using the Promega Luciferase assay system, the same cell lysates were used to calculate β -Galactosidase activity by monitoring the formation of ONP (yellow) from ONPG (colorless) at 420 nm, in a reaction catalyzed by β -Galactosidase. Results are expressed as Relative Luminescent Units/ β -Galactosidase.

4.6 Quantitative RT-PCR

mRNA was isolated as per manufacturer's instructions using Trizol reagent (Invitrogen). 2 μ g of RNA was converted to cDNA using superscript III (Invitrogen) as per the manufacturer's instructions. Real Time PCR was performed on the cDNA using primers for *Mecp2*_{e1} and *e2* specific primer sets using the IQ SYBR green real time PCR super mix (Biorad). Primer sets were *Mecp2*_{e1}F 5'-AGGAGAGACTGGAGGAAAAGT-3', *Mecp2*_{e2}F 5'-CTCCATAAAAATACAGACTCACCAGT-3', *Mecp2*_{bothR} 5'-CTTAAACTTCAGTGGCTTGTCT-3'. β -actin was used to normalize the results.

4.7 Statistical Analysis

Statistical analysis was performed using the Prism 5 package. All data were presented as mean $\pm$ SEM. One way ANOVA followed by Tukey post hoc test was performed on sample sets. P<0.05 *, P<0.01 ** and P<0.001 *** were considered statistically significant. Experiments were repeated 3-4 times as indicated in the legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The ontogeny of neuronal markers in differentiating P19 cells

(A) 30 μ g of whole cell lysates were prepared from RA induced P19 cells on the days indicated and subjected to SDS-PAGE on a 4-20% gradient gel. Membranes were probed with the indicated antibodies overnight and were visualized using the Licor Odyssey system (A) The Western blot is from a representative experiment and was performed three times. Quantification of MeCP2 is shown below. *Mecp2* mRNA levels were determined by real time PCR (B) and results were normalized to GAPDH expression levels. Experiments had 2-3 replicates per assay. Results shown are the combined data from all three experiments and standard error of the mean was determined. Significance was determined by one way ANOVA with Tukey's posthoc test and all values were compared to naïve cells, which were not treated with RA (0). NS, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Figure 2. TNF increases expression of MeCP2 in early differentiating P19 cells

(A) P19 cells were treated with TNF for 4 hrs on the indicated day of differentiation (Naïve, 3 and 6 days after treatment with RA). MeCP2 levels were analyzed by Western blotting and normalized to β -actin. (B) MeCP2 expression was assessed by Western blotting from lysates from cultures at 3 after treatment with RA and induced with TNF for the indicated lengths of time. Western blots (top) are from a representative experiment and quantification (bottom) is from 4 experiments. Significance was determined by one way ANOVA with the Tukey's posthoc test. In panel A * = $p < 0.05$ and NS = not significant when cultures treated with TNF were compared to untreated cultures on the same day. In panel B, * = $p < 0.05$ and *** = $p < 0.001$ compared to naïve (0) cultures.

Figure 3. NF κ B inhibitors block TNF-dependent increases in *Mecp2* mRNA and protein
 The NF κ B inhibitors Act (100 nM) and JSH (10 μ M) were added to cultures 30 minutes prior to adding TNF in cultures at 3 d after treatment with RA. After 4 hours of TNF treatment, RNA was isolated using Trizol and *Mecp2* mRNA was measured by real time PCR (A). Similarly, 30 μ g of whole cell lysates were prepared and subjected to Western blotting, membranes were probed for MeCP2 and β -actin (B). Western blot (top panel) is from a representative experiment and quantifications (bottom) are from 4 experiments (bottom panel). Significance was determined by one way ANOVA with the Tukey's posthoc test. * = $P < 0.05$. ** = $p < 0.01$, *** = $p < 0.001$

Figure 4. NFκB signaling is attenuated in naïve P19 cells

(A) Naïve cells (not treated with RA) and at 4 days post RA treatment were transfected with the NFκB luc- reporter expression vector and a CMV- β-Galactosidase expression vector using lipofectamine 2000 as per the manufacturer's instructions. Lysates were prepared from cells that were stimulated with TNF for 18 hrs. Luminescence was normalized to β-Galactosidase expression in the same cell lysate and expressed as relative luminescence (RLU). (B) Level of NFκB family member p65 and IκBα were measured in naïve cells and in cells at 3 and 6 d after treatment with RA. Western blotting was carried out on 30 μg of whole cell lysate and the membranes were probed with the indicated antibodies, blots were quantified on the Odyssey Licor system. Western blot is from a representative experiment and (B) quantifications (of p65 and IκB are from 4 experiments (right panels). Significance was determined by one way ANOVA with Tukey's posthoc test. *= $p < 0.05$ and *** = $p < 0.001$, $n=3$

Figure 5. PMA activates NFκB signaling and increases MeCP2 levels in naïve P19 cells
 (A) NFκB -Luc activity was measured in transfected naïve P19 cells stimulated with PMA, TNF, and LPS for 6 h. Luminescence was normalized to β-Galactosidase expression and expressed as relative luminescence units (RLU). (B) MeCP2 protein was measured by Western blots in 30 μg of whole cell lysates prepared from naïve P19 cells (C), naïve cells treated with 100 nM PMA for 6 hrs (no inhib), and with PMA in the presence of the inhibitors Act or JSH. Western blot is from a representative experiment (top panel) and quantifications are from 4 experiments (bottom panel). A representative blot is shown below the bar graph. Significance was determined by one way ANOVA with Tukey's posthoc test. *= $p < 0.05$ and *** = $p < 0.001$

Figure 6. PMA but not TNF treatment influences IκBα degradation in naïve P19 cells
 30 µg of whole cell lysate was prepared from naïve P19 cells treated with PMA (A) or TNF (B) for different lengths of time. The samples were analyzed by western blotting and expression of IκBα was normalized to GAPDH expression. Quantification is from 4 experiments (top panel) and a representative Western blot (bottom panel) is shown. Significance was determined by one way ANOVA with the Tukey's posthoc test. **= P<0.01.