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Regulation of cell proliferation by nucleocytoplasmic dynamics of postnatal and embryonic exon-II-containing MBP isoforms

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ABSTRACT

The only known structural protein required for formation of myelin, produced by oligodendrocytes in the central nervous system, is myelin basic protein (MBP). This peripheral membrane protein has different developmentally-regulated isoforms, generated by alternative splicing. The isoforms are targeted to distinct subcellular locations, which is governed by the presence or absence of exon-II, although their functional expression is often less clear. Here, we investigated the role of exon-II-containing MBP isoforms and their link with cell proliferation. Live-cell imaging and FRAP analysis revealed a dynamic nucleocytoplasmic translocation of the exon-II-containing postnatal 21.5-kDa MBP isoform upon mitogenic modulation. Its nuclear export was blocked upon treatment with leptomycin B, an inhibitor of nuclear protein export. Next to the postnatal MBP isoforms, embryonic exon-II-containing MBP (e-MBP) is expressed in primary (immature) oligodendrocytes. The e-MBP isoform is exclusively present in OLN-93 cells, a rat-derived oligodendrocyte progenitor cell line, and interestingly, also in several non-CNS cell lines. As seen for postnatal MBPs, a similar nucleocytoplasmic translocation upon mitogenic modulation was observed for e-MBP. Thus, upon serum deprivation, e-MBP was excluded from the nucleus, whereas re-addition of serum re-established its nuclear localization, with a concomitant increase in proliferation. Knockdown of MBP by shRNA confirmed a role for e-MBP in OLN-93 proliferation, whereas the absence of e-MBP similarly reduced the proliferative capacity of non-CNS cell lines. Thus, exon-II-containing MBP isoforms may regulate cell proliferation via a mechanism that relies on their dynamic nuclear import and export, which is not restricted to the oligodendrocyte lineage.

1. Introduction

Oligodendrocytes are the myelinating cells of the central nervous system (CNS) with a distinct and carefully regulated proliferation and differentiation timeline. During their maturation, they synthesize myelin sheaths, which enwrap axons to provide fast nerve conduction and to ensure axonal integrity [1]. Myelin membranes contain various myelin specific proteins of which myelin basic protein (MBP) is the second most abundant one and, most importantly, the only known structural protein that is indispensable for CNS myelin formation [2–5].

Several MBP isoforms are present in oligodendrocytes and it has been well established that the MBP family is generated from a large, 11 exon-containing gene complex called Golli (gene in the oligodendrocyte lineage). Given the presence of two primary transcription starting sites, two subfamilies can be distinguished, i.e., classical MBPs, expressed by myelinating oligodendrocytes, and golli MBPs that are also expressed by many other cells [6–9]. From this large gene complex, the alternative splicing of the seven most downstream exons (denoted by Roman numerals I–VII) gives rise to a single MBP mRNA transcript [10], which is transcribed into different classical MBP isoforms, four of which, i.e., 21.5-, 18.5-, 17-, and 14-kDa, are predominantly expressed in rats. The 14- and 18.5-kDa isoforms lack exon-II and localize to compact myelin, whereas the exon-II-containing 17- and 21.5-kDa MBP isoforms localize predominantly to the nucleus, but also appear in the cytoplasm [11–13]. These classical postnatal MBP isoforms are synthesized in a developmentally-regulated manner. Thus, exon-II-positive MBP isoforms are expressed during early stages of myelination, whereas the expression of exon-II-negative MBP isoforms peaks in late myelination [14–16]. Interestingly, in addition to the classical MBP postnatal isoforms, embryonic isoforms also exist [17–18], including an exon-II-containing 16-kDa MBP isoform, the expression of which

Abbreviations: BSA, bovine serum albumin; BrdU, 5-Bromo-2′-deoxy-uridine; CDK, cyclin-dependent kinase; CNS, central nervous system; CRM1, chromosome region maintenance 1; C/N, cytoplasm / nucleus; FCS, fetal calf serum; FGF, fibroblast growth factor; FRAP, fluorescence recovery after photobleaching; e-MBP, embryonic myelin basic protein; GFP, green fluorescent protein; Golli, gene in the oligodendrocyte lineage; HD, high density; LD, low density; LMB, leptomycin B; mAb, monoclonal antibody; MBP, myelin basic protein; NES, nuclear export signal; N/C, nucleus / cytoplasm; N/M, nucleus / membrane OPC, oligodendrocyte progenitor cell; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PFA, paraformaldehyde; PLL, poly-L-lysine; RFP, red fluorescent protein; RT, room temperature; SF, serum-free

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persists during the early postnatal phase in mice and rats [16–18], and which expression is not limited to the oligodendrocyte lineage [17].

Thus far, MBP-related research has largely focused on exon-II-negative MBP isoforms, whereas knowledge of the function of nuclear exon-II-positive MBP isoforms is rather scanty. Specifically, exon-II-negative isoforms play a role in myelin compaction, and also serve as 'molecular sieves' by selectively allowing access of only proteins with short cytoplasmic tails into myelin membranes [2,19–21]. In addition, these isoforms act in signaling, cytoskeleton polymerization and stabilization, and calcium–calmodulin binding [2,22–25]. In contrast, the exon-II-containing isoforms localize to the nucleus and cytoplasm when expressed in HeLa cells and oligodendrocytes [12,13], but the functional consequences of this distinct subcellular localization are still unresolved. However, as observed for other nuclear proteins, the localization of exon-II-containing MBP is affected by cell–cell contact [12], whereas nuclear translocation appears to be an active process, which is time, energy, and temperature dependent [12,26]. In terms of functioning, it is known that the 21.5-kDa MBP exon-II-containing isoform plays a role in calcium homeostasis in oligodendrocytes [23]. In addition, via the induction of a diffusible factor, 21.5-kDa MBP promotes proliferation of the immortalized N19-oligodendrocyte cell line, and enhances neurite outgrowth [27].

Here, we have investigated the role of the subcellular localization of postnatal and embryonic exon-II-positive MBP isoforms and their link with proliferation. Photobleaching (FRAP) and live-cell imaging techniques demonstrated a dynamic nucleocytoplasmic translocation of postnatal exon-II-containing 21.5-kDa MBP–RFP upon mitogenic modulation. Our findings further revealed that a 16-kDa exon-II-containing embryonic MBP isoform (e-MBP) is expressed in many cell lines, including non-myelinating cell lines, and like the postnatal 21.5-kDa MBP isoform, is involved in cell proliferation. Therefore, exon-II-containing MBP isoforms play a specific regulatory role not only in the early regulation of the myelin machinery, but in a more general context, also in (embryonic) cell proliferation.

2. Materials and methods

2.1. Cell culture

2.1.1. Primary oligodendrocytes

Primary oligodendrocyte cultures were generated from 1 to 3 day old Wistar rats as described [28]. Briefly, rats were decapitated, forebrains were collected, and a single cell suspension was obtained by mechanical and enzymatic (papain) digestion. Cells were cultured in DMEM (Gibco, Invitrogen, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS, not heat-inactivated, Bodinco, Alkmaar, The Netherlands), L-glutamine (Invitrogen) and penicillin/streptomycin (P/S, Invitrogen) for 10–14 days on poly-L-lysine (PLL, 5 μg/mL, Sigma, St. Louis, MO)-coated tissue culture flasks (Nunc, Roskilde, Denmark). Oligodendrocyte progenitor cells (OPCs), growing on top of an astrocyte monolayer, were then isolated by a shake-off procedure, followed by differential adhesion. After shaking, OPCs were collected from the medium and for Western blot analysis plated on PLL-coated 10-cm dishes (Nunc, Naperville, IL) at a cell density of 10^6 cells/dish. The OPCs were cultured in SATO medium [28], containing PDGF-AA (10 ng/mL, Peprotech, Rocky Hill, NJ) and FGF-2 (10 ng/mL, Peprotech) for 2 days. Differentiation was induced by growth factor withdrawal and cells were allowed to differentiate for 3, 7, and 10 days in SATO medium supplemented with 0.5% FCS. Medium was refreshed twice a week.

2.1.2. Cell lines

The oligodendrocyte progenitor cell line OLN-93, a kind gift from Dr. Christiane Richter-Landsberg [29], was cultured in DMEM supplemented with L-glutamine, P/S and 10% FCS (heat-inactivated) under standard incubation conditions (humidified atmosphere, 7.5% CO_2, 37 °C). Experiments were performed at passage 25–36. For carrying out a proliferation assay and immunocytochemical analysis, the cells were cultured on 8-well chamber slides (Nunc), pre-coated with PLL at the indicated cell densities for 3 days. Cells were cultured in 10% FCS unless otherwise indicated. After 2 days in culture, cells were treated with 20 μM roscovitine (Sigma) or leptomycin B (LMB, 10 ng/mL, LC Laboratories, Woburn, MA) for 24 and 6 h, respectively. For Western blot analysis, cells were plated on PLL-coated 10-cm tissue culture dishes (Corning Costar, Lowell, MA). Cell densities were calculated according to the corresponding surface area of 8-well chambers. For fluorescence recovery after photobleaching (FRAP) and live-cell imaging, OLN-93 cells were plated on PLL-coated 2-well Labtek-II chambered coverglass (Nunc, 50,000 cells/well). After 24 h, cells were transfected with 21.5-kDa MBP–RFP [25] or RFP, using Lipofectamine™ 2000 Transfection Reagent (Invitrogen) as described in the manufacturer's instructions. Experiments were performed 24 h after transfection. HEK293, HeLa, and HepG2 cells were cultured as OLN-93 cells. For BrdU assays and e-MBP immunocytochemistry, cells were plated a day prior to the analyses on 13-mm glass cover slides in 24 well plates at a cell density of 25,000 (HeLa, HepG2) or 35,000 (HEK293) per well.

2.2. Rat brain tissue

Brain tissue of Wistar rats was homogenized, using a Wheaton homogenizer, in 1 mL of ice-cold TE buffer (10 mM Tris–HCl, 2 mM EDTA, 0.25 M sucrose) and a cocktail of protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim, Germany). Samples were stored at −80 °C until further biochemical analysis.

2.3. Constructs and lentiviral transduction

The generation of the plasmid coding for 21.5-kDa MBP–RFP has been described previously (pERFP-C1-rmMBP-21.5-UTR [23]). The plasmid coding for RFP was pERFP-C1. Lentiviral-mediated knockdown of gene expression was performed using the pHR′trip-PGK-eGFP-WPRE-H1 vector. For this purpose, the vector was modified to allow for insertion of different short hairpins. In short, the pHR′trip-PGK-eGFP-WPRE-H1 vector was amplified with two opposing primers containing either the recognition sequence for *Asc*I (5′-CGTGGCGCGCCATCTGTGGTCTC ATACAGAACTT-3′) or *Sbf*I (5′-CGTCCTGCAGGGGAAAAGCTTATGAATTC GGC-3′) and self-ligated (restriction sites underlined). The PCR amplification was performed using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). Short hairpin sequences cloned into the modified vector had the following topology: 5′-acaaGGCGCGCC(N19-23)actcgaga(N19-23c)gtttttCCTGCAGGacaa-3′ (*Asc*I and *Sbf*I restriction sites in capital letters). Ordered DNA oligonucleotides (Biolegio, Nijmegen, The Netherlands) were primed with a reverse primer (5′-TTGTGCCTGC AGGAAAAA-3′) and filled in using Phi29 DNA polymerase (New England Biolabs, Beverly, MA). Ligation was performed using 1 μL of hairpin DNA and 150 ng of digested modified pHR′trip-PGK-eGFP-WPRE-H1. The target sequence for MBP, directed against the interface of exon-III and exon-IV, was: 5′-acaaaGGCGCGCCA GCAGAGGA CCCAAGATGAAACTCGAGATTCATCTTGGGTCCTCTGCGTTTTTCCTGCA GGcacaa-3′. Lentiviral particles were produced as described [30]. Cells were exposed to two times diluted lentiviruses containing MBP shRNA, for 16 h in the presence of 4 μg/mL hexadimethrine bromide (polybrene; Sigma). Concomitant expression of GFP confirmed shRNA transduction. Analyses were performed 10–15 days after transduction.

2.4. RT-PCR

Total RNA was isolated on day 3 using the InviTrap Spin Cell RNA Mini Kit (Invitek, Stratec, Berlin, Germany). The RNA yield and purity were quantified spectrophotometrically by measuring A260 and A280 absorbances in a Nano-Drop ND-Spectrophotometer (V3.1.0, Thermo