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Myelin basic protein is a glial microtubule-associated protein – Characterization of binding domains, kinetics of polymerization, and regulation by phosphorylation and a lipidic environment

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ABSTRACT

The 18.5-kDa splice isoform of myelin basic protein (MBP) predominates in the adult brain, adhering the cytoplasmic leaflets of the oligodendrocyte membrane together, but also assembling the cytoskeleton at leading edges of membrane processes. Here, we characterized MBP's role as a microtubule-assembly protein (MAP). Using light scattering and sedimentation assays we found that pseudo-phosphorylation of Ser54 (murine 18.5-kDa sequence) significantly enhanced the rate but not the final degree of polymerization. This residue lies within a short KPGSG motif identical to one in tau, a ubiquitous MAP important in neuronal microtubule assembly. Using polypeptide constructs, each comprising one of three major amphipathic α-helical molecular recognition fragments of 18.5-kDa MBP, we identified the N-terminal α1-peptide as sufficient to cause microtubule polymerization, the rate of which was significantly enhanced in the presence of dodecylphosphocholine (DPC) micelles to mimic a lipidic environment.

1. Introduction

Myelin basic protein (MBP) is essential for the formation of myelin of the central nervous system of higher vertebrates [1–3]. The predominant 18.5-kDa classic splice isoform holds the cytoplasmic leaflets of the oligodendrocyte membrane in close apposition, but also assembles microfilaments and microtubules at the leading edges of membrane processes being extended to form the sheath [3,4]. The protein is intrinsically disordered and interacts with various partners via short molecular recognition fragments (MoRFs) that undergo local disorder-to-order transitions, with modulation by myriad post-translational modifications [4–6]. The 18.5-kDa MBP has also been identified as a microtubule-associated protein (MAP) in bovine brain and in cultured oligodendrocytes by co-immuno-precipitation and co-localization [7–12]. Phosphorylation of MBP at unknown sites has been reported to strengthen the protein's ability to polymerise and bundle microtubules *in vitro* [13], and to increase its interaction with microtubules in oligodendrocytes [14]. However, phosphorylation specifically at Thr94 and Thr97 (of the bovine isoform, corresponding to Thr92 and Thr95 in the murine 18.5-kDa sequence numbering, Fig. 1A) had little effect on its ability to polymerise and bundle microtubules, but decreased its ability to bind them to the membrane surface *in vitro* [15]. Charge reduction of the protein by pseudo-deimination at 6 sites throughout the sequence also decreased its ability to tether microtubules to membranes [15]. Furthermore, MBP and these modified forms were able to bind microtubules and microfilaments to each other [15]. Thus, many studies support the idea that, at the tips of the extending oligodendrocyte membrane processes that will form the compact regions of myelin, 18.5-kDa MBP may also tether the cytoskeleton to the membrane, bundle microfilaments and microtubules and bind them to each other, and help regulate process extension, retraction, and axonal ensheathment [3,4]. The effect of seryl-specific phosphorylation of MBP by protein kinase C has not yet been investigated, but there is a linear motif found in both MBP and in tau (a primarily neuronal MAP) that comprises a phosphorylated Ser54 (murine 18.5-kDa MBP sequence numbering, Fig. 1A) [16]. Our goals here were to map the segments of MBP that interact with tubulin, and to investigate the modulatory roles of site-specific seryl pseudo-phosphorylation.

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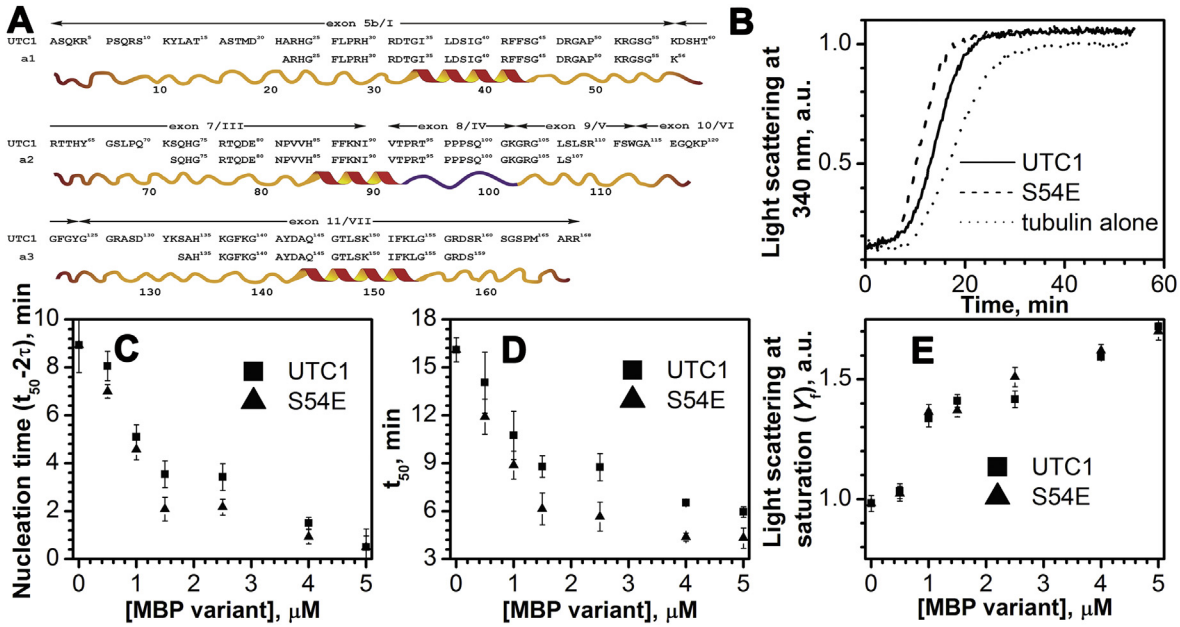

Fig. 1. (A) Sequences of murine 18.5-kDa MBP variants (168 amino acids). The 1st row indicates the exons (*golli* numbering in Arabic/Sanskrit numerals, classic numbering in Roman numerals). The 2nd row indicates the "untagged" (no C-terminal LEH_6 purification tag, as in our previous studies) C1 (unmodified) charge component. The 3rd row shows the α-peptides comprising the main membrane-anchoring amphipathic α-helices. The 4th row represents the main secondary structure elements of 18.5-kDa MBP in a myelin environment. This figure has been adapted from Ref. [4]. (B) Time-based light scattering profiles of microtubule assembly (10 μM tubulin) in the presence of unmodified and pseudo-phosphorylated 18.5-kDa MBP variants (0.5 μM each). This figure is representative of at least three replicates. The following parameters are plotted as a function of MBP charge variant concentration: (C) length of nucleation phase (or lag-time, $t_{50} - 2\tau$), (D) t_{50}, and (E) light scattering at saturation (Y_f). Data are presented as means ± standard deviations calculated from analysis performed by fitting individual kinetic curves (Figure S2) using Eq. (1). Each kinetic curve was replicated at least three times and analyzed individually.

2. Materials and methods

2.1. Purification of proteins and peptides

Tubulin was obtained from fresh bovine brain using established methods [17]. The unmodified 18.5-kDa recombinant murine MBP isoform (C1 component of MBP), UTC1, was expressed in *E. coli* BL21-CodonPlus(DE3)-RP cells (Stratagene, La Jolla, CA), in untagged ("UT") form, *i.e.*, without any purification tags, and purified as described [18]. The pseudo-phosphorylated variant of UTC1 with a single S54E substitution was generated by site-directed mutagenesis [19]. The α1-, α2-, and α3-segments of murine 18.5-kDa MBP encoding residues (A22-K56), (S72-S107), and (S133-S159), respectively, were cloned into the Champion™ pET SUMO (small ubiquitin modifier) Expression System (Invitrogen Life Technologies, Burlington, ON) and purified as described with a final HPLC step [20]. Standard curves for each α-peptide were created using HPLC to ensure that consistent and accurate protein concentrations were used in all assays (Figure S1).

2.2. Kinetics of microtubule assembly induced by MBP variants

Light scattering was used to probe microtubule polymerization [13]. All components required for the assay, except tubulin and GTP, were premixed. Because of its lability, tubulin was thawed and added as the last reagent. Stocks of MBP full-length variants and MBP-α-peptides of known concentration were made by resuspending known weights of lyophilized proteins in MilliQ water. Concentrations were routinely re-verified spectrophotometrically, or by injecting 10-μL samples into the HPLC and extrapolating concentration from the calibration curves (Figure S1).

Time-based light scattering profiles of microtubule assembly were collected at different MBP (or α-peptide):tubulin molar ratios ranging from 0.05 to 0.5 (or 0.75–90), in the final reaction volume of 60 μL in GPEM buffer (80 mM PIPES-KOH, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP, pH 6.9). First, concentrations of each MBP full-length charge variant or α-peptide in the range of 0–5 μM and 0–900 μM, respectively, were prepared in PEM buffer (80 mM PIPES-KOH, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9). Then, pre-frozen stocks of bovine tubulin were thawed quickly in room temperature water and placed immediately on ice. Pre-made GTP stocks (10 mM GTP, pH 7.6 with NaOH) were added to the tubulin stock and allowed to incubate on ice for 5 min. Tubulin/GTP stocks were then added to each pre-heated (to 37° C) working reaction mixture to a final concentration of 10 μM tubulin and 1 mM GTP, and immediately read (at 37° C) using a microplate reader (Polarstar Omega, BMG Laboratory Technologies, Offenburg, Germany). In comparative experiments, the detergent dodecylphosphocholine (DPC, Avanti Polar Lipids, Alabaster, AL) was added to a final working concentration of 1.2 mM (above its critical micellar concentration of 1.0 mM) prior to the addition of the tubulin/GTP mixture. Measurements were done in time-based absorbance mode, assuming that any change in light intensity passing through the sample (*i.e.*, at 180°) was due to light scattering and not absorbance. A total of 250 measurements were taken at an interval of 13 s. At least three replicates at each molar ratio were measured and analyzed independently. Data were analyzed using ORIGIN software (version 8.0, OriginLab Corporation, Northhampton, MA).

2.3. Nucleation-dependent polymerization (NDP) analysis

The light scattering curves were fitted to the equation:

$$Y_t = (s_i t + Y_i) + \left[(s_f t + Y_f) - (s_i t + Y_i)\right] \Big/ \left[1 + \exp(-(t - t_{50})/\tau)\right] \quad (1)$$

where Y_t is the light scattering signal in arbitrary units (AU) obtained at time t, Y_f is the y-intercept of the final linear region that describes saturation, Y_i is the y-intercept of the initial linear region