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Dephosphorylated but not phosphorylated microtubule associated protein MAP1B binds to microfilaments

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Abstract **We have reported that purified native MAP1B interacts with microtubules but not with microfilaments [Pedrotti and Islam, Cell Motil. Cytoskel. (1995) 30, 301–309]. However, MAP1B can be phosphorylated at multiple sites by casein kinase II (CKII) and proline-directed protein kinases (PDPK) and immunoblotting studies show that purified native MAP1B is phosphorylated at least at two CKII sites and at one PDPK site [Pedrotti et al., Biochemistry (1996) 35, 3016–3023]. We now show that phosphorylation affects the in vitro binding of MAP1B with microfilaments. Native MAP1B does not bind to microfilaments but after treatment with alkaline phosphatase the dephosphorylated MAP1B binds and cosediments with microfilaments. Dephosphorylation kinetics suggest that the PDPK site, but not CKII sites, may negatively regulate the interaction with F-actin. The ability of dephosphorylated MAP1B to crosslink microfilaments was also examined and showed that MAP1B exhibits only a weak crosslinking of F-actin when compared with MAP2.**

Key words: High-molecular weight MAPs; MAP1B; Phosphorylation; Microfilaments; Cytoskeleton

1. Introduction

Organisation and functions of the cytoskeleton may be modulated by the reciprocal interactions of its filamentous components: microfilaments, microtubules and intermediate filaments [3]. In this context an important role may be played by high molecular weight MAPs [4], e.g. MAP1A (350 kDa) and MAP2 (200 kDa), which have been shown to interact with microtubules and actin filaments [5–8]; MAP2 has also been shown to crossbridge microtubules and microfilaments in vitro [6,7]. Moreover, both MAP2 and MAP1A can also crosslink actin filaments suggesting the presence of two or more actin binding sites [5–8], and at least one of the MAP2 actin binding sites has been proposed to reside in the C-terminus microtubule binding domain [9]. This domain is known to be multiply phosphorylated [10,11] and although MAP2 phosphorylation does not affect its ability to bind to actin it attenuates its ability to crosslink microfilaments [5].

We have recently shown that purified MAP1B, which shares a significant sequence similarity with MAP1A [12], interacts with microtubules but not with microfilaments [1]. However, MAP1B is known to be phosphorylated by several proline-directed protein kinases (PDPK) and casein kinase II (CKII; [13,14]). MAP1B phosphorylation is also regulated during development: PDPK phosphorylation is abundant in axons at early neuronal development stages and restricted to growth cones at later stages [14] while CKII phosphorylation is present in both axons and dendrites even in late stages of neuronal maturation [14,15], suggesting a different function in neurogenesis for these two phosphorylation modes.

We have recently characterised the phosphorylation state of purified MAP1B and shown that phosphorylation at specific residues can modulate the affinity of MAP1B for microtubules: phosphorylation at the PDPK sites weakens its interaction with microtubules while the phosphorylation at the casein kinase II sites does not apparently alter MAP1B:microtubule interaction [2]. Consequently, in this report we have further examined the affect of phosphorylation on the interaction of MAP1B with actin. Our data suggests that dephosphorylated MAP1B can bind to actin filaments and that its interaction is modulated by the state of phosphorylation.

2. Material and methods

The following buffers were used: MES buffer (MES 0.1 M, EGTA 2.5 mM, $MgCl_2$ 0.5 mM, EDTA 0.1 mM, pH 6.4 with NaOH) and Tris buffer (Tris-HCl 2 mM, DTT 0.5 mM, ATP 0.2 mM, $CaCl_2$ 0.2 mM, pH 7.5).

2.1. Protein purification and composition

Twice cycled calf brain microtubule protein was prepared as described previously [7]. MAP2 and MAP1B were purified as described in [1]. Purified MAP1B was dephosphorylated by alkaline phosphatase (AP) treatment as described by Ulloa et al. [16].

Rabbit skeletal muscle actin was prepared as described previously [8]. For binding studies actin was extensively dialysed for 48 h at 4°C against Tris buffer and centrifuged 60 min at 100 000 × *g* to remove any aggregates.

Protein concentration was determined using Bio-Rad (Rockville Centre, NY) protein reagent and bovine serum albumin used as standard. SDS-PAGE was performed using the 'Phast System' (Pharmacia, Piscataway, NJ) and gels were stained with Coomassie blue R-250 [10] and, where described, scanned at a wavelength of 595 nm using a LKB gel Scanner equipped with a peak integrator.

2.2. Assay procedures

Preliminary experiments at several different initial MAP1B:actin molar ratios showed that maximal MAP1B binding was achieved at MAP1B:actin ratios of about 1:30. For sedimentation assays, F-actin was polymerised in MES buffer at 37°C for 25 min and at the end of the incubation period 50 μg of F-actin were incubated at 37°C for a further 15 min with either 10 μg of native (1B-N) or dephosphorylated (1B-AP) MAP1B. After incubation the samples were centrifuged in a TL 100 ultracentrifuge (Beckman Instruments, Ltd., Toronto, Ontario) for 25 min at 100 000 × *g*, 37°C. Pellets were resuspended in Laemmli buffer and analysed by SDS-PAGE. Gels were densitometrically scanned and integrated peak areas determined as described previously [1].

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Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ATP, adenosine 5'-triphosphate; MAPs, microtubule-associated proteins; MES, 2-(*N*-morpholino) ethanesulphonic acid; Tris, Trizma base; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; PMSF, phenylmetane sulphonyl fluoride; CKII, casein kinase II; PDPK, proline-directed protein kinase; AP, alkaline phosphatase

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For crosslinking experiments the sedimentation assay was performed as described previously [7]. Aliquots containing F-actin and MAP1B were layered on a sucrose cushion (30% w/v in MES buffer) and centrifuged at 37°C for 40 min at 30000×*g*. After resuspension, pellets were fractionated by SDS-PAGE. Before sedimentation aliquots were fixed with glutaraldehyde (0.1% v/v) and negatively stained for electron microscopy [17].

3. Results

We previously reported that purified MAP1B does not bind to F-actin [1]. Using specific monoclonal and polyclonal antibodies which recognise the phosphorylation state of two CKII sites (Ab125, Ab842) and two PDPK sites (Ab531, Ab150) we found that the purified protein was phosphorylated at both CKII sites but only at one PDPK site [2]. The sites can be dephosphorylated by alkaline phosphatase treatment and complete dephosphorylation of the PDPK site requires less than 1 h while that for the CKII sites requires between 3 and 4 h [2]. We therefore investigated whether phosphorylation hampered a possible interaction by comparing the actin binding properties of native and alkaline phosphatase (AP) treated MAP1B.

MAP1B was dephosphorylated for various times with AP and the effect of AP treatment on MAP1B:F-actin interaction was examined by sedimentation. Pure G-actin was polymerised in MES buffer at 37°C for 25 min before incubation with either native MAP1B or AP-treated MAP1B for a further 15 min. At the end of the incubation period F-actin was sedimented by centrifugation and the pellets and supernatants analysed by SDS-PAGE. As shown in Fig. 1, AP-treated MAP1B co-sedimented with F-actin and was detected

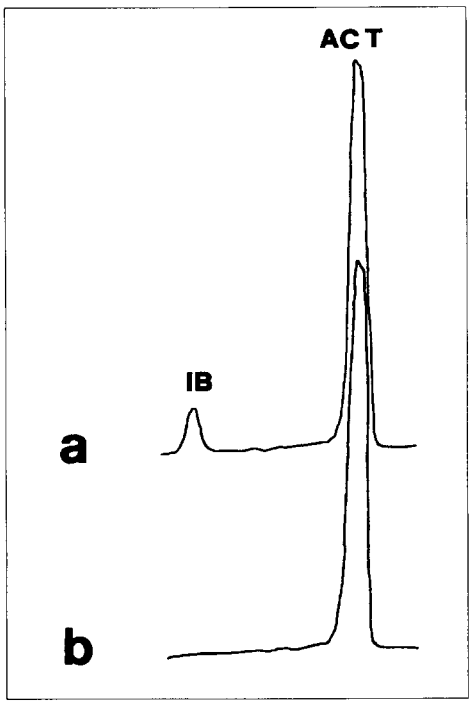

Fig. 1. Sedimentation assay. F-actin (0.9 mg/ml) was incubated in the presence of 0.15 mg/ml of either MAP1B treated for 4 h with alkaline phosphatase (a) or native MAP1B (b). F-actin was pelleted by centrifugation and the pellets were fractionated by SDS-PAGE on a 4–15% acrylamide gel, stained with Coomassie brilliant blue, and densitometrically scanned. The positions of MAP1B (1B) and actin (ACT) are indicated.

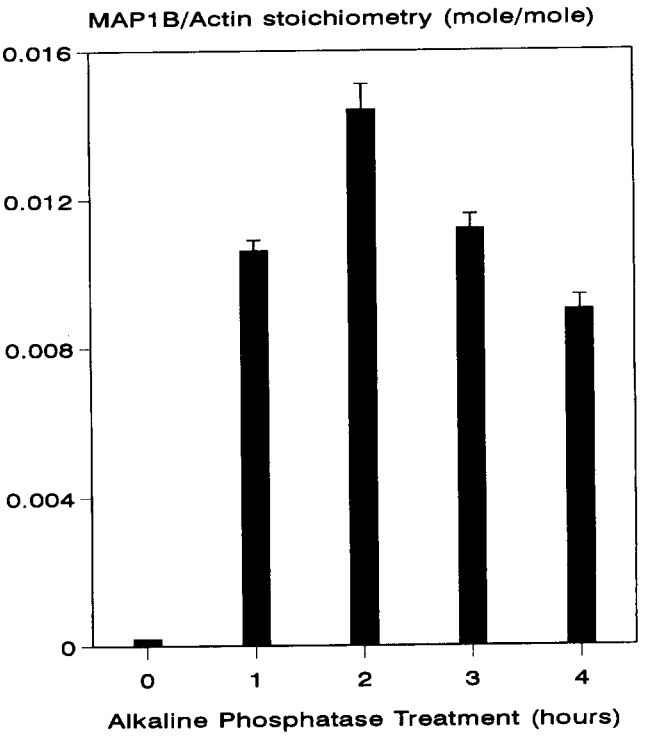

Fig. 2. AP treatment of MAP1B and binding to F-actin. MAP1B was treated with alkaline phosphatase for various times (0 to 4 h) and then incubated with F-actin. At the end of the incubation time the proteins were sedimented by centrifugation and the pellets, after resuspension, analysed by SDS-PAGE. The gels, after staining with Coomassie blue and destaining, were densitometrically scanned and integrated peak areas used to calculate the MAP1B:actin binding stoichiometries. Each reported value is an average of 5–6 determinations.

in the pellets while native MAP1B failed to pellet with F-actin (also see below). MAP1B alone, in the absence of F-actin, did not sediment under these conditions.

Gels were also densitometrically scanned and integrated peak areas used to calculate MAP1B:F-actin binding stoichiometry. The molecular mass of MAP1B (244 kDa) and actin (43 kDa) were used to calculate the molar ratio. The stoichiometry of MAP1B:G-actin was calculated and plotted as a function of the time of AP treatment (Fig. 2). Native MAP1B failed to bind to F-actin but after 1 h of AP treatment a significant amount of MAP1B bound to actin. Maximal MAP1B:actin interaction was achieved after 2 h of AP treatment and subsequently exhibited a slight decrease with increasing time of dephosphorylation.

We also determined whether AP-treated MAP1B could, in addition to binding, also cause gelling of F-actin. AP-treated MAP1B or MAP2 (used as a control) were therefore incubated with F-actin at 37°C for 15 min. At the end of the incubation period aliquots were removed and negatively stained for electron microscopy. As shown in Fig. 3, large gelled networks of microfilaments were observed in the presence of MAP2 (Fig. 3B) but in the presence of MAP1B these structures were very much less abundant (Fig. 3C). In the absence of either MAP2 or MAP1B only filamentous actin was observed (Fig. 3A). Sedimentation experiments confirmed that in the presence of MAP2 large amounts of gelled actin were recovered in the pellets but only minimal amounts of

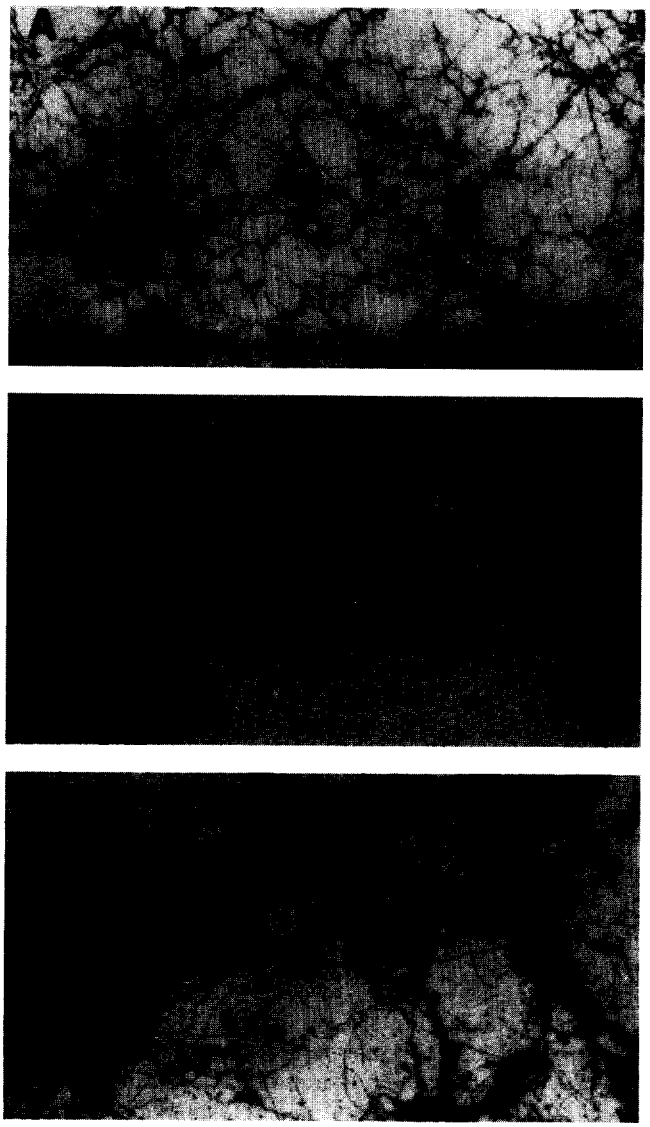

Fig. 3. Electron microscopy. Negatively stained F-actin incubated either in the absence of MAPs (A) or in the presence of AP-treated MAP1B (B) or MAP2 (C) is shown at a magnification of ×14000.

gelled actin were pelleted in the presence of MAP1B (data not shown).

4. Discussion

High molecular weight MAPs have been implicated in crosslinking microtubules and other cytoskeletal filaments or cytoplasmic organelles [4]. Both MAP2 and MAP1A have been shown to be actin-binding and actin-crosslinking proteins [5–7] and a crossbridging element between microtubules and microfilaments [6,7]. Surprisingly, MAP1B which exhibits a significant sequence homology with MAP1A [12] does not appear to interact with microfilaments [1].

However, purified native MAP1B is phosphorylated at least at two CKII sites and one PDPK site [2] and MAP phosphorylation has been shown to influence the interaction with both microtubules [2,18] and microfilaments [5]. Phosphorylation of MAP1B can modulate the interaction not only with microtubules [2] but also with microfilaments: native (phosphorylated) MAP1B does not bind while AP-treated (dephosphorylated) MAP1B binds and co-sediments with F-actin (Fig. 1). Furthermore, dephosphorylation kinetics suggest that the PDPK site (recognised by Ab150), removed after 1 h of AP treatment, may be important in negatively regulating the MAP1B:actin interaction. By contrast, removal of the CKII sites (recognised by Ab125 and Ab842), complete dephosphorylation normally requiring between 3 and 4 h of AP treatment, leads to a slight decrease in the MAP1B:actin stoichiometry suggestive of a positive enhancement of interaction.

In comparison with MAP2 and MAP1A, much less MAP1B binds to actin as suggested by the lower stoichiometry and a low ability to crosslink actin filaments. In this context, it is important to mention that MAP1B is present in extremely high concentrations in the growth cones and consequently this interaction may nevertheless be physiologically important. Furthermore, PDPK phosphorylation is mainly present at early development stages, during axonal sprouting, while CKII phosphorylation remains in late stages of development [13,16,19–21]. Dephosphorylation at the PDPK site increases microtubule binding capacity [2] and induces the interaction with microfilaments. These events coupled with the gradual replacement of MAP1B with MAP1A may be an expression of a gradual stabilisation.

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