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# Microtubule-binding sites of the CH domain of EB1 and its autoinhibition revealed by NMR

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ABSTRACT

End-binding protein 1 (EB1) is one of the best studied plus-end tracking proteins. It is known that EB1 specifically binds the plus ends of microtubules (MTs) and promotes MT growth. EB1 activity is thought to be autoinhibited by an intramolecular interaction. Recent cryo-EM analyses showed that the CH domain of Mal3p (*Schizosaccharomyces pombe* EB1 homolog) binds to GMPCPP-MT (Sandblad, L. Cell 127 (2006) 1415-24), and strongly binds GTPγS-MT which is proposed to mimic MT plus ends better than GMPCPP-MT (Maurer S.P. et al. Cell 149 (2012) 371–82). Here, we report on the MT binding sites of the CH domain of EB1 as revealed by NMR using the transferred cross-saturation method. In this study, we used GMPCPP-MT and found that the MT binding sites are very similar to the binding site for GTPγS-MT as suggested by cryo-EM (Maurer S.P. et al. Cell 149 (2012) 371–82). Notably, the N-terminal tip of helix α6 of the CH domain did not make contact with GMPCPP-MT, in contrast to the cryo-EM study which showed that it is closely located to a putative switch region of β-tubulin in GTPγS-MT (Maurer S.P. et al. Cell 149 (2012) 371-82). Further, we found that the intramolecular interaction site of EB1 overlaps the MT binding sites, indicating that the MT binding sites are masked by interaction with the C-terminal domain. We propose a structural view of autoinhibition and its release mechanism through competition binding with binding partners such as adenomatous polyposis coli protein.



## 1. Introduction

Microtubules (MTs) are dynamic hollow tubes comprising α, β-tubulin dimers that disassemble and reassemble at two ends, the slow-growing (minus) and fast-growing (plus) ends. Recently, plus-end tracking proteins (+TIPs) have emerged as regulators of MT dynamics. The plus end explores the cell periphery and shows dynamic instability, switching rapidly between the two phases of growth and shrinkage [1].

Genetic and biochemical studies have shown that +TIPs interact with each other and form protein complexes [1]. End-binding protein 1 (EB1) promotes MT polymerization and interacts directly with many other +TIPs and cytoskeletal proteins such as cytoplasmic linker protein 170 (CLIP-170) and the dynactin large subunit p150$^{Glued}$, which contain the CAP-Gly domain, and mitotic centromere-associated kinesin, microtubule-actin crosslinking factor and adenomatous polyposis coli (APC), which contain the SxIP motif [2,3]. Thus, EB1 has been proposed to form the core of the microtubule plus-end complex and acts as a hub in interactions with +TIPs [4,5].

EB1 consists of a CH domain and the C-terminal dimerization domain [3]. The CH domain is responsible for MT-binding, and consists of six helices [6]. Cryo-EM analysis has shown that the CH domain of the *Schizosaccharomyces pombe* homolog of EB1, Mal3p, predominantly recognizes the MT lattice seam using GMPCPP-MT [7]. A modeled structure based on EM observations showed that the CH domain binds inter-protofilaments and both α and β tubulins. However, the resolution of the EM observations was insufficient to account for the MT binding sites of the CH domain. Mutagenesis studies of the CH domain showed that residues indispensable for MT-binding or the promotion of MT polymerization were dispersed widely across the whole CH domain molecular surface [6,8,9]. Recently, cryo-EM analysis of GTPγS-MT showed that the CH domain of Mal3p binds the B-lattice but not the seam of MTs [10]. Maurer S.P. et al. showed that EB1 predominantly recognizes GTPγS-MT which mimics MT plus ends better than GMPCPP-MT [11]. Thus, the MT binding site of the CH domain and binding mechanism should be clarified.

Furthermore, although many efforts have been made, a consensus regarding the regulation of EB1 has yet to be reached. It was reported

*Abbreviations:* HSQC, heteronuclear single quantum correlation spectroscopy; TROSY, Transverse relaxation optimized spectroscopy; CSP, chemical shift perturbation; TCS, transferred cross-saturation; +TIPs, plus-end tracking proteins; MT, microtubule; EB1, end-binding protein 1; CH domain, calponin homology domain; CAP-Gly domain, cytoskeleton-associated protein glycine-rich domain; CLIP, cytoplasmic linker protein; APC, adenomatous polyposis coli

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that the $K_D$ of the EB1-MT interaction is 0.44 μM [12]. However, other research implied that EB1 and its homologs interact with MTs with lower affinities [7,13,14]. Some studies have reported that EB1 can induce the polymerization of low concentrations of tubulin [15,16], whereas intact EB1 by itself has no effect on MT polymerization. It should be noted that some reports have suggested that EB1 exerts autoinhibition. It is thought that MT-binding activity manifests following removal of the purported EB1 autoinhibitory tail (a.a. 249–268, numbers in human EB1) or activation by proteins that bind EB1 [8,16–18]. Hayashi et al. showed direct interaction between the CH and C-terminal domains of EB1 by NMR, although the NMR signals were not assigned and its binding surface was not determined [16].

The C-terminal domain of EB1 undergoes dimerization, folding into a four-helix bundled structure comprising a long helix (residues 191–230), a short loop, a short helix (residues 237–246) running anti-parallel to the long helix, and a C-terminal tail [19,20]. Interestingly, the C-terminal domain also serves as a platform for protein-protein interactions engaged by other + TIPs and cytoskeletal proteins. Crystallographic studies have revealed that the SxIP motif of counterparts is recognized by a hydrophobic pocket of the C-terminal domain of EB1 [2]. A conserved FYF sequence of the C-terminal domain contributes toward formation of the hydrophobic pocket. The CAP-Gly domain of $p150^{Glued}$, which lacks the SxIP motif, also interacts with an almost identical site [3]. Thus, the hydrophobic pocket of the C-terminal domain may represent an EB1 hot spot region associated with multiple interactions.

Notwithstanding continued efforts to reveal the nature of the multiple interactions of EB1, two important issues remain to be solved, one being identification of the MT binding site of the CH domain, and the other being delineation of the intramolecular interaction sites of full-length EB1 responsible for its autoinhibition. In this study, we report on three binding surfaces of EB1, as determined from direct spectroscopic evidence using NMR, comprising (i) the MT binding site of the CH domain, (ii) the C-terminal domain binding site of the CH domain, and (iii) the CH domain binding site of the C-terminal domain. We performed a transferred cross-saturation (TCS) experiment to detect the MT binding surface of the CH domain, an approach which can detect weak interactions in large molecular weight and fast exchange systems [21–25]. Furthermore, we discuss the autoinhibition mechanism as deduced from mapping of the NMR results.

## 2. Materials and methods

### 2.1. Expression and purification of CH domain and C-terminal domain of EB1

CH domain (1–130) and C-terminal domain (189–268) of human EB1 cDNAs were amplified from Human HeLa QUICK-Clone™ cDNA (Clontech). The cDNA of the C-terminal domain was cloned into expression vector pGEX-6P-3 (GE Healthcare) as a fusion protein with GST. cDNAs of the CH domain were cloned into pET47-b (Novagen) as His-tagged fusion proteins. Expression vector for K59E was prepared by an extension reaction using PrimeSTAR Max (TaKaRa) with the wild-type CH domain cloned into pET47-b as a template, and followed by DpnI digestion. Primers K59E_F (GGCTCC ATTGCCTTGGAGAAAGTGAAATTCCAA) and K59E_R (TTGGAATTTCAC TTTCTCCAAGGCAATGGAGCC) were used. The DNA sequence of each construct was confirmed by DNA sequencing. These were then transformed into *E. coli* strain BL21Star™ (DE3) (Invitrogen) for protein expression. Bacteria were cultured overnight in 20 mL LB medium containing 50 μg/mL ampicillin or 25 μg/mL kanamycin at 37 °C, and then transferred into 400 mL LB medium for further growth. After the $OD_{660}$ of the culture reached 0.5, cells were collected by centrifugation at 6000 rpm for 15 min at 20 °C, and then re-suspended in 2 L of M9 minimal medium containing antibiotics and 0.5 g/L $^{15}NH_4Cl$, with 4 g/L glucose or 1 g/L [$^{13}C_6$]-glucose for $^{15}N$- or $^{13}C/^{15}N$-labeling, respectively. Uniformly $^{2}H/^{15}N$-labeled protein was prepared using 99% $D_2O$-buffered M9 minimal medium containing 0.5 g/L $^{15}NH_4Cl$ and 1 g/L [$D_7$]-D-glucose. 50 mL of Spectra 9 (D, 97%+; $^{15}N$, 98%+, Spectra Stable Isotopes) was added to 1 L medium for $^{2}H/^{15}N$ labeling. After the $OD_{660}$ of the culture reached 0.5, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1.0 mM to induce protein expression. Induction was performed overnight at 20 °C ($^{15}N$- or $^{13}C/^{15}N$-labeling) or 30 °C ($^{2}H/^{15}N$-labeling). Following overnight induction, cells were harvested by centrifugation. Cells were then lysed by sonication and the cell lysate was centrifuged. The supernatant containing the GST fusion protein was loaded onto a DEAE Sepharose Fast Flow (GE Healthcare) column and Glutathione Sepharose 4B (GE Healthcare) column, and then eluted with buffer containing 30 mM reduced glutathione, 50 mM Tris–HCl (pH 8.0), 400 mM KCl, 0.1 mM EDTA and 1 mM dithiothreitol (DTT). The N-terminal GST-tag was cleaved using HRV3C protease. The target protein was purified by gel filtration (HiLoad 26/60 Superdex 75 pg, GE Healthcare) with buffer containing 50 mM K-phosphate (pH 7.0), 100 mM KCl, 1 mM DTT and 0.1 mM EDTA. His-tagged fusion proteins were loaded onto Ni-NTA Agarose (Qiagen) with buffer containing 50 mM Tris–HCl (pH 8.0), 400 mM KCl and 0.5 mM DTT, and eluted stepwise using imidazole. The histidine tag was cleaved using HRV3C protease. Eluted proteins were then further purified by gel filtration(HiLoad 26/60 Superdex 75 pg). The His-tagged CH domain was also purified in the same manner but without tag cleavage.

### 2.2. Resonance assignments

NMR experiments for resonance assignments were performed at 303 K on a Bruker AVANCE 600 instrument equipped with a TXI cryogenic probe. NMR samples included 50 mM K-phosphate buffer (pH 7.0), 100 mM KCl, 1 mM DTT and 0.1 mM EDTA in 93% $H_2O$ and 7% $D_2O$. The final concentration of protein in each sample was ca. 1 mM in a final volume of 250 μl. All NMR spectra were processed using the software package NMRPipe [26] and analyzed using Sparky [27]. $^{1}H$, $^{13}C$ and $^{15}N$ assignments were mainly obtained from standard multidimensional NMR methods using HNCACB, HN(CO)CACB, CBCA(CO)NH, HN(CA)CO and HNCO for main chain assignments of CH and C-terminal domains [28]. $^{1}H$, $^{13}C$ and $^{15}N$ backbone resonance assignments of CH domain and C-terminal domain were deposited in the BMRB under accession number 18348 and 18371, respectively.

### 2.3. Binding analyses of CH domain with MT

The effect of salt on binding of the CH domain to GMPCPP-MT and GTPγS-MT was investigated. Polymerized tubulin in the presence of GMPCPP or GTPγS was initially prepared. To this end, 0.15 mM tubulin was polymerized in buffer containing 20 mM PIPES (pH 6.9), 1 mM $MgCl_2$, 1 mM EGTA and 150 mM KCl supplemented with 1.5 mM GMPCPP or GTPγS at 37 °C for 20 min. Following polymerization, 100 μM taxol was added to suppress depolymerization. Polymerized tubulins were then mixed with $^{15}N$-labeled wild-type CH domain with or without the His-tag, or K59E (final concentration 0.15 mM, CH domain:tubulin = 1:1) and HSQC spectra were measured at 303 K. At low salt concentration, CH domain NMR signals were almost absent, but gradually appeared with increasing salt concentration (180, 210, 240, 270 and 300 mM KCl). Signal recoveries by salt were estimated using the average signal intensity of all peaks. NMR spectra were analyzed by Sparky [27].

### 2.4. Transferred cross-saturation (TCS) experiment

Transferred cross-saturation (TCS) experiments were performed at 303 K on a Bruker AVANCE III instrument equipped with a TCI cryoprobe. In the experiment, the molar ratio of $^{2}H/^{15}N$-labeled K59E to MT was set to 1:1. NMR samples were dissolved in 20 mM PIPES