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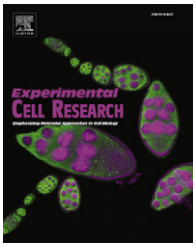

Research Article

A mutation of the fission yeast EB1 overcomes negative regulation by phosphorylation and stabilizes microtubules

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ABSTRACT

Mal3 is a fission yeast homolog of EB1, a plus-end tracking protein (+TIP). We have generated a mutation (89R) replacing glutamine with arginine in the calponin homology (CH) domain of Mal3. Analysis of the 89R mutant *in vitro* has revealed that the mutation confers a higher affinity to microtubules and enhances the intrinsic activity to promote the microtubule-assembly. The mutant Mal3 is no longer a +TIP, but binds strongly the microtubule lattice. Live cell imaging has revealed that while the wild type Mal3 proteins dissociate from the tip of the growing microtubules before the onset of shrinkage, the mutant Mal3 proteins persist on microtubules and reduces a rate of shrinkage after a longer pausing period. Consequently, the mutant Mal3 proteins cause abnormal elongation of microtubules composing the spindle and aster. Mal3 is phosphorylated at a cluster of serine/threonine residues in the linker connecting the CH and EB1-like C-terminal motif domains. The phosphorylation occurs in a microtubule-dependent manner and reduces the affinity of Mal3 to microtubules. We propose that because the 89R mutation is resistant to the effect of phosphorylation, it can associate persistently with microtubules and confers a stronger stability of microtubules likely by reinforcing the cylindrical structure.

Introduction

Microtubules are highly dynamic polymers that constantly switch between phases of growth and shrinkage [1]. Microtubule dynamics at the plus end, called dynamic instability, is important for proper cellular functions, such as cell division, differentiation and migration [2–4]. Plus-end tracking proteins (+TIPs), which accumulate selectively at growing microtubule plus ends [5–7] are considered to play an important role in regulation of stability of microtubules. EB1, originally identified as a binding partner of the adenomatous polyposis coli (APC) tumor suppressor [8], is one of the +TIPs highly conserved among eukaryotes. The subsequent studies demonstrated that most of the other +TIPs that can directly interact with microtubules are also able to bind to EB1, suggesting that EB1 may function as a central hub in the network of +TIPs [9]. EB1 associates with the microtubule filament as

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a dimer [10,11] via the calponin homology (CH) domain at the amino-terminal [12,13]. EB1 and Mal3, a fission yeast homolog of EB1, can promote microtubule assembly *in vitro* [14–16]. The effects of these proteins on microtubule dynamics vary between the experimental systems. While they stimulate growth initiation and suppress catastrophes *in vivo* [10,17], they stimulate both catastrophe and rescue in reconstituted systems [10,16,18]. Recent studies have also shown that Mal3 decorate the microtubule lattice seams [15,16,19], suggesting that binding of EB1/Mal3 stabilizes the cylindrical structure of the microtubule.

As deletion of *mal3*$^+$ gene does not lead to the cell lethality, it provides an opportunity to study the effect of loss of function of Mal3 on a number of biological processes in fission yeast. Loss of Mal3 leads to abnormal short microtubules [20]. Mal3 recruits the kinesin Tea2 onto the cytoplasmic microtubules, and promotes Tea2 motor activity [21]. Tea2 transports Tip1, the fission yeast homolog of CLIP-170 [22], toward the growing microtubule plus ends in Mal3-dependent manner [17,18,23]. Like EB1 in higher eukaryotes, Mal3 also plays an important role at the kinetochore in fission yeast. It binds Spc7, a member of the conserved Spc105/KNL-1 family of kinetochore proteins, required for the integrity of the spindle as well as for targeting of MIND complex to the kinetochore [24,25]. Deletion of *mal3*$^+$ gene results in a delay in mitosis, which is dependent on the Bub1-dependent spindle checkpoint. As simultaneous deletion of *mal3*$^+$ and *bub1*$^+$ genes causes monopolar attachment of sister centromeres, Mal3 cooperates with Bub1-dependent spindle checkpoint to promote bipolar attachment [26].

The activity of EB1/Mal3 to stabilize microtubules and promote its assembly must be regulated *in vivo*. We attempted to generate a *mal3* mutant, which could deviate from regulation. Here we report characterization of such a mutant both *in vivo* and *in vitro*.

Results

Construction of mal3-89R mutant

We aimed to investigate a mechanism to regulate Mal3 and the consequence of a failure in such regulation. A pool of the *mal3* mutants was generated by error-prone PCR and transformed into a wild type fission yeast strain after cloning into pREP81. Among approximately 1.6×10^5 transformants screened, we obtained a plasmid that caused a growth defect upon induction of the gene hereafter designated *mal3-89R* (Fig. S1A). The growth defect caused by overexpression of the *mal3-89R* gene was largely due to a delay in mitosis. Judged by chromosome condensation as a mitotic index (MI), 22.3% of cells were in mitosis when the *mal3-89R* was overexpressed. The wild type *mal3*$^+$ gene did not affect growth (Fig. S1A) or cause a delay in mitosis (MI: 2.0%) when overexpressed from pREP81. Overexpression of *mal3-89R* in mutants defective in the spindle checkpoint function caused a much severer growth defect (Fig. S1C), indicating that it activated the checkpoint.

The *mal3-89R* mutant contained a single point mutation replacing glutamine at the position 89 with arginine (Q89R) within the CH domain important for microtubule-binding [12,13] (Fig. 1A). As shown in Fig. 1B, the mutation of Q89R was predicted to locate on the putative microtubule-binding surface in the CH domains. Replacement of a polar amino acid to a basic amino acid would affect electrostatic interaction with microtubules and might alter microtubule dynamics. To test this possibility, we examined the mutant protein for its effect on a microtubule assembly assay *in vitro*. Tubulin was purified from bovine brain and a polymerization assay was performed at 37 °C in the presence of the wild type or mutant Mal3 proteins purified from bacteria. The mutant protein

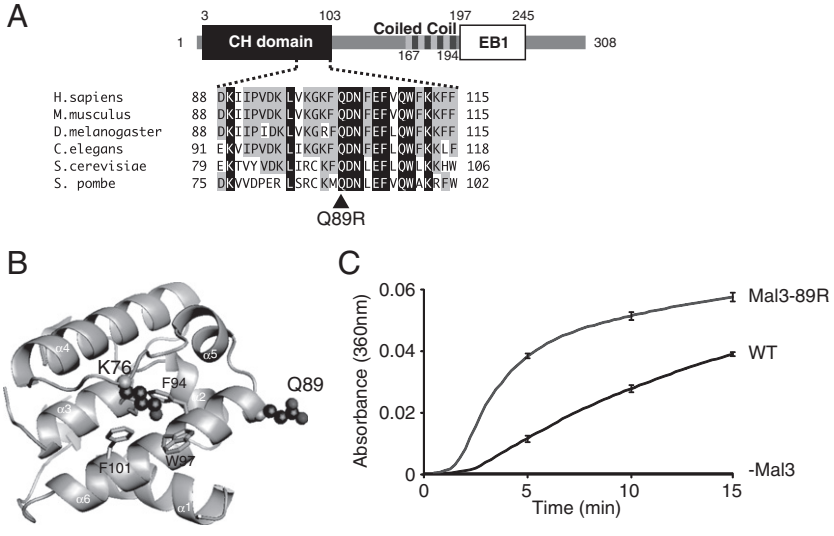

Fig. 1 – Phenotype of the *mal3-89R* mutant. (A) Mutation site of *mal3-89R*. The mutation site is shown with a domain structure of Mal3 and the amino acid sequences of other members of the EB1 family. Amino acids conserved among all the members are shaded in black and in more than three in gray. (B) Location of the *mal3-89R* mutation. Q89R and conserved hydrophobic residues are labeled and shown as ball-and-stick. The model was built using MODELLER [52] and represented by PyMOL Molecular Graphic System (http://www.pymol.org). (C) Effect on *in vitro* microtubule assembly assay. Wild type Mal3 and Mal3-89R proteins were examined for their ability to promote the microtubule-assembly.