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The C-terminal α-helix of SPAS-1, a *Caenorhabditis elegans* spastin homologue, is crucial for microtubule severing

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ABSTRACT

Spastin belongs to the meiotic subfamily, together with Vps4/SKD1, fidgetin and katanin, of AAA (ATPases associated with diverse cellular activities) proteins, and functions in microtubule severing. Interestingly, all members of this subgroup specifically contain an additional α-helix at the very C-terminal end. To understand the function of the C-terminal α-helix, we characterised its deletion mutants of SPAS-1, a *Caenorhabditis elegans* spastin homologue, in vitro and in vivo. We found that the C-terminal α-helix plays essential roles in ATP binding, ATP hydrolysing and microtubule severing activities. It is likely that the C-terminal α-helix is required for cellular functions of members of meiotic subgroup of AAA proteins, since the C-terminal α-helix of Vps4 is also important for assembly, ATPase activity and in vivo function mediated by ESCRT-III complexes.

1. Introduction

Spastin (SPAS-1 in *Caenorhabditis elegans*) is a member of the AAA (ATPases associated with various cellular activities) protein family (Hazan et al., 1999; Matsushita-Ishiodori et al., 2007). It has been demonstrated that spastin has an ATP-dependent microtubule severing activity (Roll-Mecak and McNally, 2010). Spastin is a causative agent for human disease, hereditary spastic paraplegia (HSP) (Hazan et al., 1999; Svenson et al., 2001) and defects in microtubule severing activity of mutant spastin are considered to be a cause of axonal degeneration in HSP McDermott et al., 2003; Sherwood et al., 2004; Trotta et al., 2004; Evans et al., 2005; Salinas et al., 2005; Wood et al., 2006; Yu et al., 2008a). AAA proteins generally assemble into a hexamer, whose formation is critical for their function. It has been demonstrated that human spastin forms a hexamer in an ATP-dependent (White et al., 2007) or -independent (Pantakani et al., 2008) manner and that *Drosophila* spastin and *C. elegans* SPAS-1 form a hexamer in an ATP-dependent manner (Roll-Mecak and Vale, 2008). In contrast, we recently found that SPAS-1 forms a hexamer in an ATP-independent manner, when highly concentrated SPAS-1 was analysed in vitro (Matsushita-Ishiodori et al., 2009). In addition, we demonstrated that SPAS-1 interacts with microtubules through MTBD (microtubule binding domain) of SPAS-1 and thus is enriched on microtubules, leading to form a hexamer in an ATP-independent manner (Matsushita-Ishiodori et al., 2009). It is noteworthy, however, that there is no direct evidence so far that a hexamer is the form bound to microtubules. It could be a hexamer or a superstructure of several hexamers.

Together with Vps4/SKD1 (VPS-4 in *C. elegans*), fidgetin (FIGL-1), and katanin (MEI-1), spastin (SPAS-1) belongs to the meiotic subfamily of AAA proteins (Fröhlich, 2001; Lupas and Martin, 2002). Spastin, katanin and fidgetin have been demonstrated to function in microtubule severing (McNally and Vale, 1993; Evans et al., 2005; Roll-Mecak and Vale, 2005; Zhang et al., 2007; Roll-Mecak and McNally, 2010). In contrast, Vps4 forms a dodecameric double-ring structure (Scott et al., 2005; Gonciarz et al., 2008; Yu et al., 2008b; Landsberg et al., 2009) and functions in endocytic trafficking, virus budding and cytokinesis mediated with ESCRT-III complexes (Hurley and Hanson, 2010). Interestingly, all members of this subgroup specifically contain an additional α-helix at the very C-terminal end (Scott et al., 2005). It has been revealed that the C-terminal α-helix is important for ATPase activity and microtubule severing of *Drosophila* spastin (Roll-Mecak and Vale, 2008) and for Vps4 assembly and ATPase activity in vitro and function in vivo (Vajjhala et al., 2008).

Here, we examined the importance of the C-terminal α-helix of the microtubule severing enzyme SPAS-1 of *C. elegans*. We demonstrate that the C-terminal α-helix plays an essential role in ATP binding, ATP hydrolysing and microtubule severing activities of SPAS-1.

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2. Materials and methods

2.1. Cell culture, transfection and immunocytochemistry

Full-length cDNA fragments for *spas-1* was cloned into pcDNA3 (Matsushita-Ishiodori et al., 2007). Site-directed mutagenesis in *spas-1* was carried out by using QuikChange II XL Site-directed mutagenesis Kit (Stratagene). Introduction of desired mutation was confirmed by DNA sequencing. HEK293 cells were cultured in DMEM supplemented with 10% FBS and were transfected by using FuGENE6 (Roche) according to the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde and treated with a Cy3-conjugated anti-FLAG antibody (Sigma) and an FITC-conjugated anti-a-tubulin antibody (DM1A; Sigma) for immunofluorescence microscopy.

2.2. Purification of proteins

DNA fragments encoding wild-type and mutant SPAS-1 were cloned into pET15b (Matsushita-Ishiodori et al., 2009). Plasmids were introduced into *Escherichia coli* BL21(DE3). Transformants were grown at 30 °C, and expression of wild-type and mutant SPAS-1 was induced by the addition of IPTG (0.5 mM), followed by growth for 3 h. SPAS-1 proteins were purified as described previously (Matsushita-Ishiodori et al., 2009). Proteins purified were suspended in Storage buffer (25 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.01% NP-40, and 10% glycerol), and stored at −80 °C. For fluorescence spectroscopy, NP-40 was omitted from purification and storage solutions.

2.3. Malachite green ATPase assay

ATPase activity was measured at 30 °C using the malachite green colorimetric assay as described previously (Akiyama et al., 1996; Matsushita-Ishiodori et al., 2009). Sodium phosphate was used as a standard.

Microtubules were prepared from porcine brain a/b-tubulin dimers as described previously (Matsushita-Ishiodori et al., 2009).

2.4. Fluorescence spectroscopy

Fluorescence spectra of tryptophan were measured in the reaction mixture either with or without 3 mM ATP. The excitation wavelength was set at 295 nm and tryptophan emission was mon-

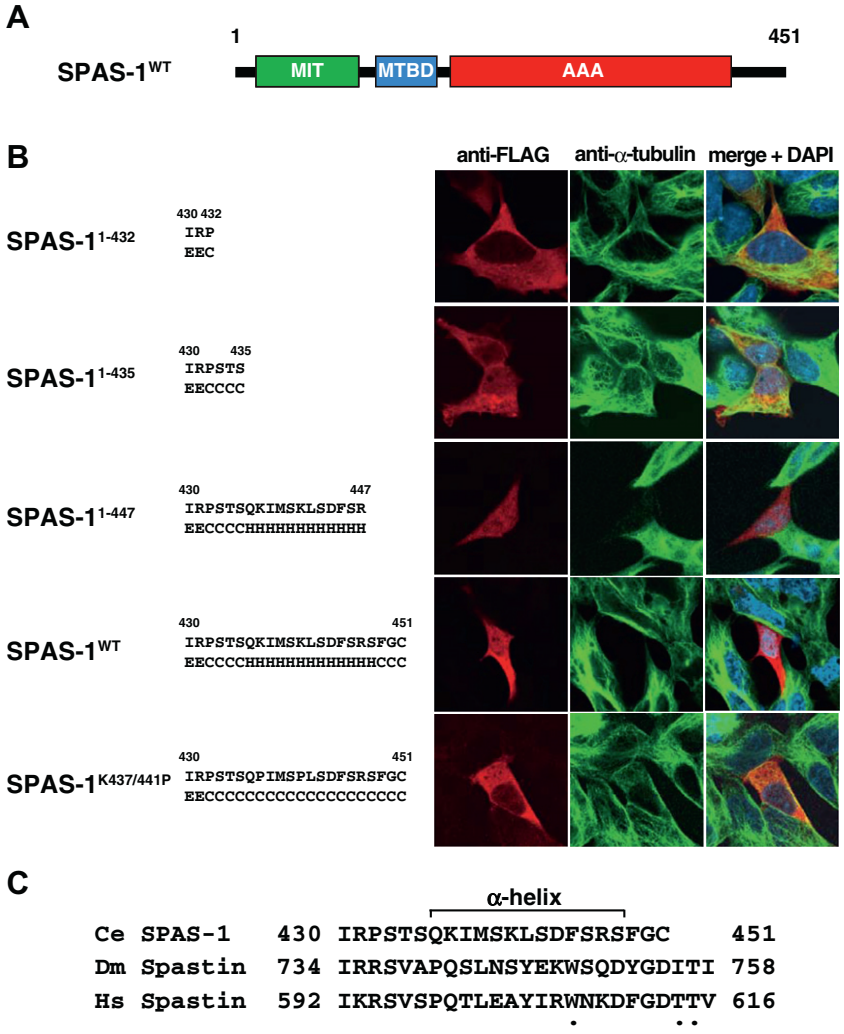

Fig.1. Requirement of the C-terminal α-helix of SPAS-1 for microtubule severing. (A) Schematic diagram of wild-type SPAS-1 (SAPS-1^{WT}) is shown. MIT, microtubule interacting and trafficking domain; MTBD, microtubule binding domain; AAA, AAA ATPase domain. (B) FLAG-tagged SPAS-1 mutants (SPAS-1$^{1–432}$, SPAS-1$^{1–435}$, SPAS-1$^{1–447}$, SAPS-1^{WT}, and SAPS-1$^{K437/441P}$) were overexpressed in HEK293 cells. Transfected cells were immunostained with anti-FLAG (left) and anti-α-tubulin (middle) antibodies. Merged plus DAPI images are also shown (right). C-terminal amino acid sequences and predicted secondary structure for each SPAS-1 mutant are presented. C, coil; E, β-sheet; H, α-helix. (C) C-terminal amino acid sequences of spastin homologues of *C. elegans, D. melanogaster* and *H. sapiens*. Amino acid residues, whose mutations have been identified from HSP patients, are shown with dots.