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Research Paper

Identification and localization of SAS-6 in the microsporidium *Nosema bombycis*

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ABSTRACT

The centriole in eukaryotes functions as the cell's microtubule-organizing center (MTOC) to nucleate spindle assembly. The evolutionarily conserved protein SAS-6 constitutes the center of the cartwheel assembly that scaffolds centrioles early in their biogenesis. Microsporidia possess the spindle plaque instead of centriole as their MTOC to nucleate spindle assembly. However, little is known about the components of spindle plaques in microsporidia. In our present study, we identified a SAS-6 protein in the microsporidium *Nosema bombycis* and named it as NSAS-6. The NSAS-6 gene contains a complete ORF of 1104 bp in length that encodes a 367-amino acid polypeptide. NSAS-6 consists of a conserved N-terminal domain and a coiled-coil domain. The high identity of SAS-6 homologous sequences from microsporidia indicates that SAS-6 is a conserved protein in microsporidia. Immunolocalization in sporoplasms, intracellular stages and mature spores showed that NSAS-6 probably localizes to the nucleus of *N. bombycis* and exists throughout the life cycle of *N. bombycis*. These results suggest that NSAS-6 is required in cell morphogenesis and division in *N. bombycis*. The function and structure of NSAS-6 should be the focus for further studies, which is essential to elucidate the role of SAS-6 in spindle plaque assembly.

1. Introduction

Centrioles are microtubule-based cylindrical structures that exhibit nine-fold symmetry and facilitate the organization of centrosomes, flagella, and cilia (Bettencourt-Dias and Glover, 2007). Previous work using *Caenorhabditis elegans*, *Drosophila*, and humans as model systems has identified several key centriole components, including SPD-2/CEP192, ZYG-1/PLK4, SAS-5/Ana2/STIL, SAS-6, and SAS-4/CPAP, and delineated a conserved ZYG-1/PLK4-mediated regulatory pathway governing centriole assembly (Gonczy, 2012). Similar to centrioles as the microtubule-organizing center (MTOC) of cells in humans, the spindle pole body (SPB) in yeast is a gigadalton macromolecular structure composed of six electron-dense layers arranged in a stack spanning the nuclear envelope (Bullitt et al., 1997; O'Toole et al., 1999). The major components of the SPB have been identified and localized, and their arrangement has been defined by tomography and cryoelectron microscopy. To date, 27 proteins have been identified, among which 18 were localized to subregions of the spindle pole (Rout and Kilmartin, 1990; Spang et al., 1996; Sundberg et al., 1996; Wigge et al., 1998). SPB duplication includes the formation of a satellite, expansion into a cytoplasmic duplication plaque, half-bridge growth and subsequent insertion into the nuclear envelope (Adams and Kilmartin, 2000).

Microsporidia are a group of obligate intracellular eukaryotes that infect almost all of vertebrates and invertebrates (Becnel and Andreadis, 1999; Canning et al., 1986; Weber et al., 1994). Similar to centrioles and basal bodies in most eukaryotes, spindle plaques are microtubule-based structures that function as MTOCs to nucleate spindle assembly in microsporidia. In microsporidian cells, the fine structure of spindle plaque closely resembles the SPB of yeasts (Desportes and Théodoridès, 1979; Vávra, 1976), and its components are also found both internally and externally to the nuclear envelope (Alfa and Hyams, 1990). Externally the spindle plaque in microsporidia is a lenticular electron-dense area of stratified material situated in a depression of the nuclear envelope, whereas the internal part of the spindle plaque, where the spindle microtubules converge, is an aggregation of electron-dense material on the internal nuclear membrane (Vávra and Larsson, 1976).

The highly conserved protein SAS-6 constitutes the center of the cartwheel assembly that scaffolds centrioles early in their biogenesis (Breugel et al., 2011). SAS-6 has been widely studied in different organisms including *C. elegans*, *Homo sapiens*, *Danio rerio*, *Trypanosoma*

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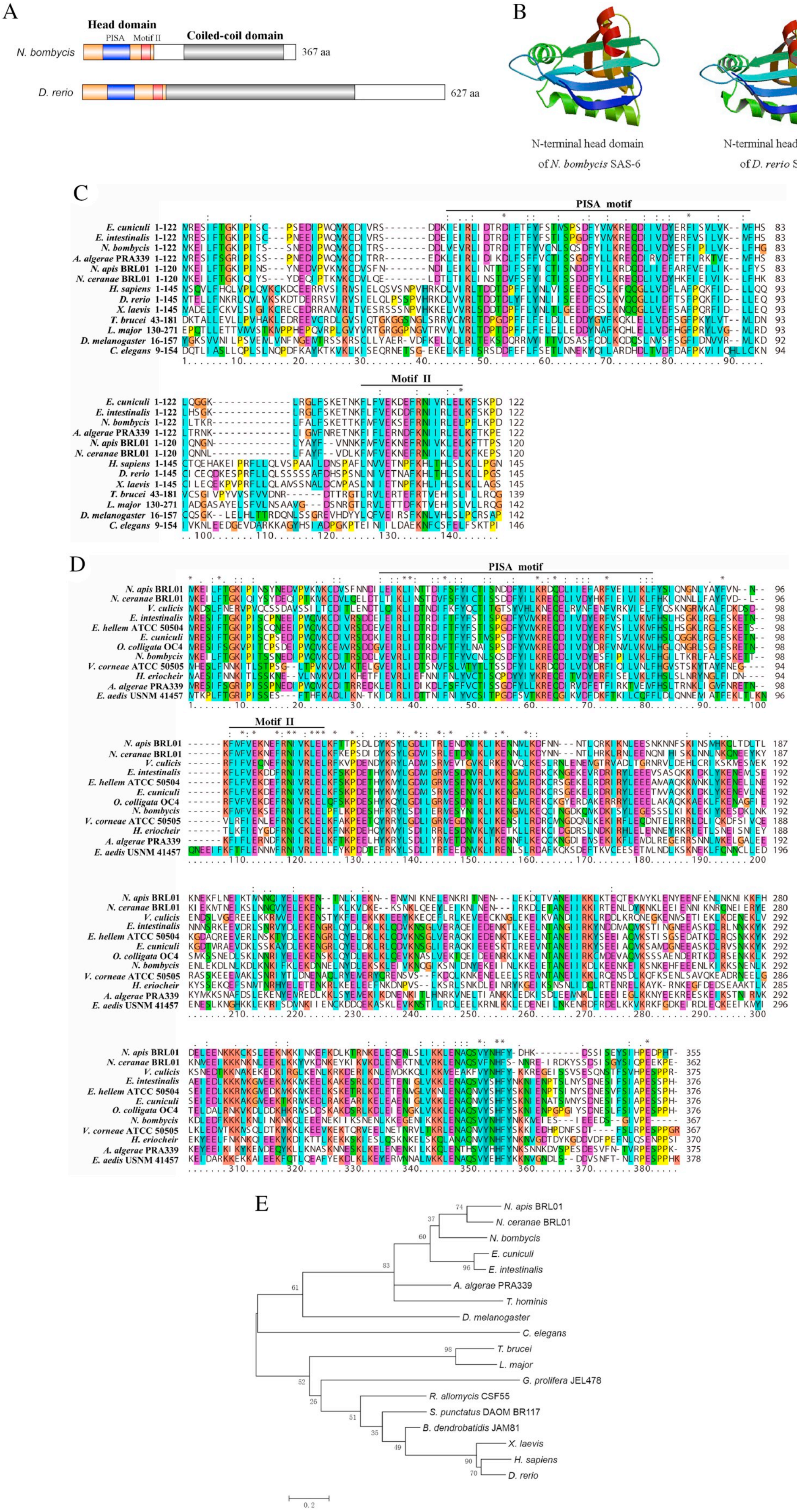

Fig. 1. SAS-6 is a conserved protein containing PISA and motif II. (A) Schematic representation of the SAS-6 protein of *Nosema bombycis* and *Danio rerio.* Head domain is indicated in orange, PISA domain in blue, Motif II in red and the coiled-coil domain in grey. (B) The three-dimensional structure of the N-terminal domain of NSAS-6 and *D. rerio* SAS-6 shown in a ribbon diagram and rainbow-colored from N (blue) to C terminus (red). (C) Multiple sequence alignment of the N-terminal domain from SAS-6 homologs from *N. bombycis* (APF31939)*, Encephalitozoon cuniculi* (AGE96042.1)*, Encephalitozoon intestinalis* (XP_003072335.1), *Anncaliia algerae* PRA339 (KCZ80640.1), *N. apis* BRL01 (EQB59849.1), *N. ceranae* BRL01 (XP_002995353.1), *Homo sapiens* (Q6UVJ0), *Xenopus laevis* (Q6NRG6), *D. rerio* (Q7ZVT3), *Caenorhabditis elegans* (O62479), *Trypanosoma brucei* (EAN77009.1), *Leishmania major* (CBZ13059.1), and *Drosophila melanogaster* (Q9VAC8). Identical residues (*), residues conserved in size and hydrophocity/hydrophilicity (:), and residues conserved in size or hydrophocity/hydrophilicity (.) are indicated. Orange: glycine (G); gold: proline (P); blue: small and hydrophobic amino acids (A,V,L,I,M,F,W); green: hydroxyl and amine amino acids (S,T,N,Q); Magenta: negatively charged amino acids (D,E); red: positively charged amino acids (R,K); dark blue: histidine (H) and tyrosine (Y). (D) Multiple sequence alignment of SAS-6 homologs from microsporidia *N. bombycis* (APF31939), *E. cuniculi* (AGE96042.1)*, E. intestinalis* (XP_003072335.1), *A. algerae* PRA339 (KCZ80640.1), *N. apis* BRL01 (EQB59849.1), *N. ceranae* BRL01 (XP_002995353.1), *Vittaforma corneae* ATCC 50505 (XP_007603773.1), *Ordospora colligata* OC4 (XP_014564501.1), *Hepatospora eriocheir* (ORD99325.1), *Encephalitozoon hellem* ATCC 50504 (XP_003886714.1), *Vavraia culicis* (XP_008073631.1) and *Edhazardia aedis* USNM 41457 (EJW05015.1). (E) Maximum likelihood phylogenetic tree based on the homologous sequences of N-terminal region of SAS-6 in *N. apis* BRL01 (EQB59849.1), *N. ceranae* BRL01 (XP_002995353.1), *N. bombycis* (APF31939), *E. cuniculi* (AGE96042.1)*, E. intestinalis* (XP_003072335.1), *A. algerae* PRA339 (KCZ80640.1), *Trachipleistophora hominis* (ELQ75431.1), *D. melanogaster* (Q9VAC8), *C. elegans* (O62479), *T. brucei* (EAN77009.1), *L. major* (CBZ13059.1), *Gonapodya prolifera* JEL478 (KXS22160.1), *Rozella allomycis* CSF55 (EPZ35149.1), *Spizellomyces punctatus* DAOM BR117 (KND01540.1), *Batrachochytrium dendrobatidis* JAM81 (EGF78297.1), *H. sapiens* (Q6UVJ0), *X. laevis* (Q6NRG6), *D. rerio* (Q7ZVT3). The protein sequences were aligned using ClustalW. Gaps and ambiguously aligned sites were excluded for subsequent phylogenetic analyses. The maximum likelihood (ML) tree was constructed using MEGA 6.0 software and bootstrap analyses were performed employing JTT model-based distance matrices generated from 1000 resamplings of the alignments. Labels on branches indicate bootstrap values in percentages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

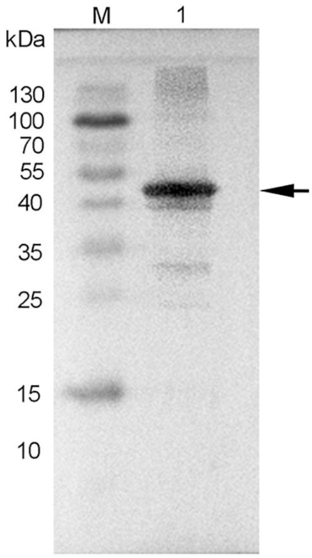

Fig. 2. Western blot of recombinant NSAS-6. Lane M: protein marker; Lane 1: NSAS-6 protein detected using the mouse anti-His tag antibody. Arrow indicates the expressed NSAS-6 protein.

brucei, *Leishmania major*, and *Drosophila melanogaster*, but has not been found in *Saccharomyces cerevisiae* (Breugel et al., 2011, 2014; Hu et al., 2015; Leidel et al., 2005; Rodrigues-Martins et al., 2007). SAS-6 is a member of an evolutionarily conserved protein family that has a conserved N-terminal domain and a coiled-coil domain (Breugel et al., 2011). The structures of SAS-6 from L. *major* and *D. rerio* have been demonstrated (Breugel et al., 2011, 2014). BLAST analysis has showed that homologous SAS-6 sequences are present in *N. bombycis* and other microsporidia. However, the SAS-6 in microsporidia has not been studied extensively. The present study aimed to assess the characteristics and function of SAS-6 in the *N. bombycis*, which may pave the way for further studies in other microsporidia.

2. Materials and methods

2.1. *N. bombycis* spores and host cell cultivation

BmN cells were used to culture *N. bombycis* spores. The cells were cultivated at 27 °C in TC-100 insect medium (AppliChem, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Gibco, Grand Island, Australia). Spores of *N. bombycis* were obtained from the Pathology Department of the Sericultural Research Institute of Chinese Academy of Agricultural Sciences (Zhenjiang, China).

2.2. Bioinformatic analysis

We searched the MicrosporidiaDB (http://microsporidiadb.org/) using BLAST and the SAS-6 sequence of *D. rerio* as query. A homologous sequence in *N. bombycis* was detected and designated as *NSAS-6* (GenBank Accession Number KX769814). The isoelectric point (PI) and molecular weight of NSAS-6 were predicted using ExPaSy proteomic tools (http://web.expasy.org/compute_pi/). The signal peptide and the transmembrane domain were predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Subcellular localization was predicted using Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/). Protein domains prediction was conducted using SMART (http://smart.embl-heidelberg.de/). The N-terminal region of NSAS-6 was used to find homologous sequences in NCBI (https://www.ncbi.nlm.nih.gov/). The homologous sequences were compared using ClustalX. Phylogenetic tree based on the homologous sequences of N-terminal region of NSAS-6 in selected organisms was constructed using MEGA 6.0. The three-dimensional structure of N-terminal region of NSAS-6 was predicted using SWISS-MODEL (https://swissmodel.expasy.org/). To analyze the relation between SAS-6 and other elements in spindle plaque duplication, we also searched for proteins such as STIL or Polo-like Kinase (PLK4) in MicrosporidiaDB, which commonly interact with SAS-6 in most organisms.

2.3. Gene cloning, recombinant protein expression and purification

In this study, we cloned the *NSAS-6* gene using specific primers: the forward primer 5′-CGGAATTCATGAAAGAAATCCTTTTTAC-3′ containing an *Eco*RI restriction site, and the reverse primer 5′-CCAAGCTTACTCAGGTACTATCCCACTA-3′ containing a *Hind* III restriction site. Genomic DNA was extracted from *N. bombycis* as previously described (Fredricks et al., 2005) and used as template. PrimeSTAR® HS (Premix, Takara, China) and a thermal cycler (MG96+, LongGene Scientific, Hangzhou, China) were used for PCR amplification. The PCR conditions were as follows: 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s; and 72 °C for 10 min. The PCR products were separated on a 1% agarose gel, purified with the MiniBEST DNA fragment purification kit (TaKaRa, Dalian, China) and cloned into the pMD19-T vector (TaKaRa). Then, the vector was transformed into competent Top10 *E. coli* strain cells. Positive clones were confirmed by blue-white selection and the isolated plasmids were sequenced by Sangon BioTech (Shanghai, China). Then, the recombinant plasmid pMD19-T-*NSAS-6* and the pET28a expression vector were digested with *Eco*RI and *Hind*III, ligated, and transformed into Top10 cells. Positive recombinant plasmids were confirmed by enzyme digestion and then transformed into *E. coli* BL21 (DE3) cells. These bacterial cells were induced for 4 h at 37 °C with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After induction, bacterial cells were lysed in precooled PBS buffer (pH = 7.4) using supersonic waves, harvested by centrifugation at 12000 rpm for 15 min, and re-suspended in 8 M urea followed by centrifugation at 12000 rpm for 15 min to extract inclusion bodies proteins. The protein samples were boiled for 10 min in SDS extraction buffer. SDS-PAGE and Western blot were performed to confirm the expression of the recombinant protein using a histidine tag-specific antibody (Sigma-Aldrich, St. Louis, MO, USA). The expressed recombinant proteins were purified with the His-Bind purification kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The purified proteins were verified by mass spectrometry (Sangon BioTech).

2.4. Polyclonal antibody production and immunoblotting

Polyclonal antibody preparation and immunoblotting protocols to detect the NSAS-6 protein in *N. bombycis* were performed as previously described (Li et al., 2009; Wu et al., 2008, 2009; Yang et al., 2014). Briefly, rabbits (New Zealand) were subcutaneously injected every week with the NSAS-6 recombinant protein mixed with Freund's adjuvant (Sigma). Negative control rabbits were injected with PBS. After four times of injection, the rabbits were bled to collect serum.

For immunoblotting, proteins from mature spores of *N. bombycis* were extracted as previously described (Cai et al., 2011; Yang et al., 2015). Approximately 1 mL of purified spores (10^9 spores mL^{-1}) were placed in a 2-mL tube with 0.8 g of glass beads (425–600 μm; acid-washed; Sigma), then crushed with Mini-BeadBeater at 4800 rpm for 1 min and repeated six times. The suspension of crushed spores was incubated in an extraction buffer (0.125 mol/ L Tris-HCl, 5% SDS, 50% glycerol, 5% β-mercaptoethanol) at 4 °C for 6 h followed by centrifugation at 8000 rpm for 10 min. The supernatant was collected for use in subsequent experiments. The proteins of the sporoplasms were extracted by germination method. First, the mature spores were induced in 0.1 mol/L KOH for 1 h at 27 °C, then centrifuged at 8000 rpm for 10 min and washed with PBS. The sporoplasms and spore shells were separated via Percoll gradient centrifugation (Taupin et al., 2006; Yang et al., 2015). The sporoplasms

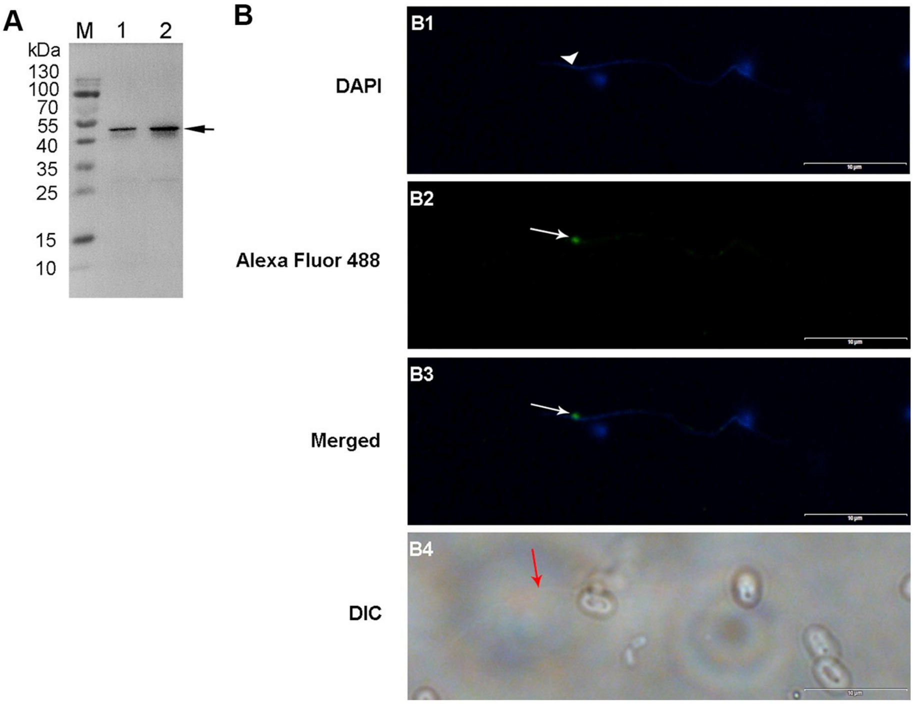

Fig. 3. (A) Immunoblot analysis of total proteins extracted from mature spores and sporoplasms of *N. bombycis* using an anti-NSAS-6 polyclonal antibody. Lane M: Marker; Lane 1: proteins of sporoplasms; Lane 2: proteins of mature spores. A 48-kDa band was detected respectively in the proteins of mature spores and sporoplasms by using an anti-NSAS-6 antibody. NSAS-6 is indicated by an arrow. (B) Immunofluorescence assay of NSAS-6 in the polar tube. Spores were induced in 0.1 mol/L KOH at 27 °C for 30 min and then incubated in PBS at 27 °C for 10 min. Samples were collected and then fixed for IFA. Samples were visualized through a fluorescence microscope after incubation with a primary antibody against NSAS-6 and DAPI. The anti-NSAS-6 serum was diluted to a ratio of 1:200. The secondary antibody was Alexa Fluor 488 (BBI Life Science)-conjugated goat anti-rabbit IgG (Sangon BioTech) at a 1:250 dilution. (B1) The sporoplasm was stained blue in the polar tube. The arrowhead indicates sporoplasm. (B2) NSAS-6 was stained green. Arrow indicates NSAS-6. (B3) Merged figure. The arrow indicates NSAS-6. (B4) The corresponding DIC image. The red arrow indicates the polar tube. Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were stained with DAPI and examined with an Olympus TH4-200 fluorescence microscope. The sporoplasm proteins were extracted using the extraction buffer mentioned above. Proteins were separated on 12% SDS-PAGE and then transferred onto PVDF membranes (Millipore, Germany) to perform Western blot analysis. Then, the membrane was blocked for 1 h at room temperature in TBST with 5% (*w*/*v*) BSA and incubated for 1 h in anti-NSAS6 serum (1:1000). After washing three times in TBST, the membrane was incubated with goat anti-rabbit IgG (1:1000, Sangon BioTech) conjugated with HRP. Finally, after washing three times in TBST, the protein bands were visualized using a chemiluminescence HRP substrate (Takara).

2.5. Immunolocalization of NSAS-6 protein in N. bombycis

BmN cells inoculated with *N. bombycis* were grown on coverslips for 24 h. The proliferative cells of *N. bombycis* were collected by disruption of the infected BmN cells followed by filtration through glass wool and fixation on glass slides (Taupin et al., 2006; Tsaousis et al., 2008). Spores were induced in 0.1 mol/L KOH at 27 °C for 30 min and then incubated in PBS at 27 °C for 10 min, 20 min or 30 min respectively. The sporoplasms were collected by centrifugation. Proliferative cells, sporoplasms, and mature spores of *N. bombycis* were placed onto poly-L-lysine-coated coverslips respectively and then fixed in 50:50 acetone:methanol (*v*/*v*) at −20 °C for 2 h before processing for IFA. After blocking with 5% BSA and 10% goat serum, the cells were incubated at room temperature in anti-NSAS6 serum (1:200 dilution), followed by incubation with goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (BBI Life Science, USA). Coverslips were mounted with DAPI-containing anti-fade mounting reagent (Vectashield, USA), and observed under an Olympus TH4-200 fluorescence microscope.

3. Results

3.1. Cloning and characterization of NSAS-6 gene

In this study, we have identified the *NSAS-6* gene of *N. bombycis*. DNA sequence analysis showed that the *NSAS-6* gene contains a complete ORF of 1104 bp in length that encodes a polypeptide of 367 amino acids. NSAS-6 has a theoretical molecular mass of 43.74 kD and a predicted pI of 5.72, no signal peptide or transmembrane domain. The predicted locus of NSAS-6 of *N. bombycis* is localized to the nucleus. Bioinformatics analysis showed that NSAS-6 has *a conserved* N-terminal domain and a coiled-coil domain (Fig. 1 A). The structure of SAS-6 in *D. rerio* had been determined in 2011, which suggests that SAS-6 is an important part of the centrioles (Breugel et al., 2011). Similar to that of *D. rerio*, the N-terminal domain of NSAS-6 contains two highly conserved regions (Fig. 1A and C): namely, the PISA-motif and a second motif (motif II). We found that the PISA-motif of NSAS-6 shares 42% amino acid identity with that of *D. rerio*, and that the three-dimensional structure of N-terminal domain of NSAS-6 is similar to that of *D. rerio* (DOI:https://doi.org/10.2210/pdb2y3v/pdb) (Fig. 1B). Multiple sequence alignment showed *that* NSAS-6 shares more than 40% amino acid identity with most of the selected microsporidia SAS-6*, and the homology with* SAS-6 of *Encephalitozoon cuniculi, Encephalitozoon intestinalis*, and *Nosema apis* BRL01 *was 52%, 52%, and 47%,* respectively (Fig. 1D). Phylogenetic analysis based on the N-terminal region of SAS-6 showed that all the microsporidia clustered together in the same clade which was far away from fungi basal chytrid such as *Batrachochytrium dendrobatidis*, *Spizellomyces punctatus*, *Gonapodya prolifera*, *Rozella allomycis*, and that *N. bombycis* is closely related to *N. apis* BRL01 *and Nosema ceranae* BRL01, suggesting that the SAS-6 of microsporidia share the same evolutionary origin (Fig. 1E). After searching for PLK and STIL in the database, which usually interact with SAS-6 and play an important role in centriole assembly, we found that PLK4 is present in the *N. bombycis* (GenBank Accession Number EOB11702.1) but STIL is not. Bioinformatics analysis showed that PLK4 of *N. bombycis* has 564 amino acids *and a conserved* S_TKc domain but no polo-box domain. Similarity analysis showed that PLK4 of *N. bombycis* shares 33% amino acid identity with that of human.

3.2. Recombinant protein expression, purification and immunoblot analysis of NSAS-6

To further analyze NSAS-6, the recombinant expression plasmid pET-28a-*NSAS-6* was constructed. The recombinant NSAS-6 protein was expressed as inclusion bodies in *E. coli* BL21 (DE3) and purified by using a His-Bind purification kit (GE Healthcare). Western blot analysis showed a unique positive band with an expected size of 48 kD (Fig. 2). LC-ESI-MS/

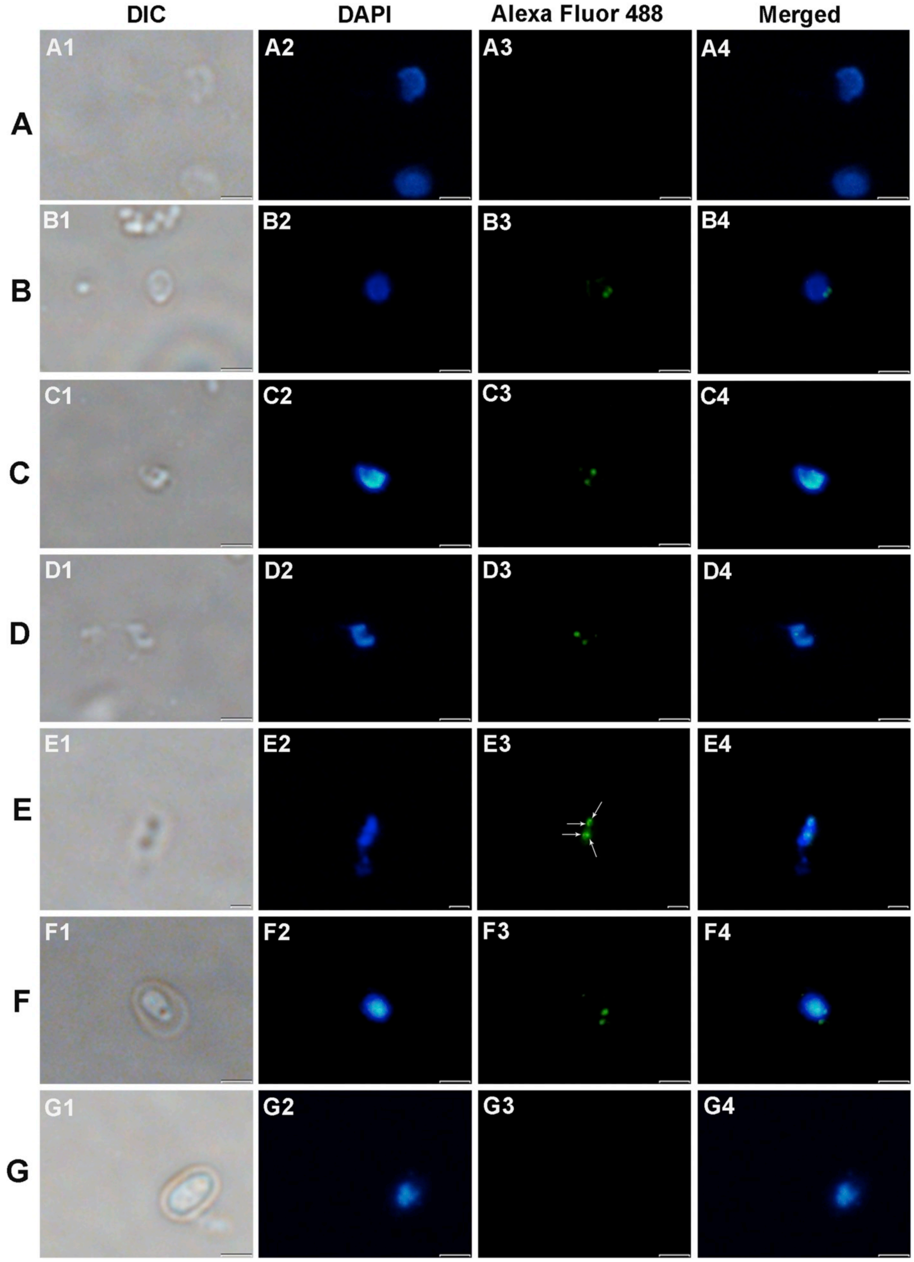

Fig. 4. Immunofluorescence assay of NSAS-6 protein. Spores were induced in 0.1 mol/L KOH at 27 °C for 30 min and then incubated in PBS at 27 °C for 10 min, 20 min or 30 min respectively. (A)(B) The induced spores were incubated in PBS at 27 °C for 10 min. (C) The induced spores were incubated in PBS at 27 °C for 20 min. (D) The induced spores were incubated in PBS at 27 °C for 30 min. (E) BmN cells inoculated with *Nosema bombycis* were grown on coverslips for 24 h and the proliferative cells of *N. bombycis* were collected by disruption of the infected BmN cells followed by filtration through glass wool. (F)(G) Mature spores. Sporoplasms, mature spores, sporonts, or meronts were visualized through a fluorescence microscope after incubation with primary antibodies against NSAS-6 (IFA) and DAPI. The anti-NSAS-6 serum was diluted to a ratio of 1:200. The secondary antibody was Alexa Fluor 488 (BBI Life Science)-conjugated goat anti-rabbit IgG (Sangon BioTech) at a 1:250 dilution. (A)(G) Only blue fluorescence is visible in negative control group treated with prebleed serum. (A1)(G1) The corresponding DIC image. (B)(C)(D)(E)(F) Blue and green fluorescence signals were observed in samples treated with NSAS-6 antiserum. (B1)(C1)(D1)(E1)(F1) The corresponding DIC image. (E3)(E4) Four green fluorescence dots appeared on the nucleus of the parasite which was undergoing division. Arrows indicate NSAS-6. Scale bar, 2 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MS analysis showed that the sequence of the recombinant NSAS-6 protein is consistent with that of NSAS-6. Additionally, SDS-PAGE and Western blot showed that the recombinant NSAS-6 protein formed inclusion bodies. Sporoplasms were acquired by germinating spores and Percoll gradient centrifugation. Fig. 3A shows the results of Western blot, indicating that NSAS-6 is an intracellular protein in *N. bombycis*.

3.3. Immunolocalization of NSAS-6

Subcellular localization prediction and Western blot analysis showed that NSAS-6 is an intracellular protein. To confirm the cellular localization of NSAS-6, IFA was performed on mature spores, sporoplasms and proliferative cells of *N. bombycis*. Spores were induced in 0.1 mol/L KOH and then incubated with PBS for 10 min. We found that the sporoplasm was released through the polar tube (Fig. 3 B1) when the polar tube was ejected from the mature spores (Fig. 3 B4). The sporoplasm nuclei were stained blue with DAPI and the NSAS-6 was positively stained in the sporoplasm with Alexa Fluor 488 (Figs. 3 B2 and B3), which suggests that the NSAS-6 enters the host cell along with the sporoplasm. The mature spores, sporoplasms, sporonts, or meronts incubated with the NSAS-6 polyclonal antibody displayed bright green fluorescence signals (Fig. 4B, E, and F), whereas no green fluorescence signals were observed in the negative control (Fig. 4A and G). Prolongation of incubation time of the induced spores in PBS from 10 min to 20–30 min resulted in a change in the shape of nucleus, namely, from being dispersed to diplocaryon (Figs. 4 B2, C2, and D2). Furthermore, four green fluorescence dots were observed on the nucleus of the parasite, which was undergoing nuclear division, suggesting that NSAS-6 was replicating (Figs. 4 E3 and E4). These results indicate that NSAS-6 is localized to the nucleus and maybe participates in karyokinesis of *N. bombycis*.

4. Discussion

SAS-6 is an evolutionarily conserved protein, which has only been reported in eukaryotes that have basal bodies or centrioles. This is the first report that proves the existence of SAS-6 in *N. bombycis*, which has spindle plaques. Sequence analysis revealed that NSAS-6 has a series of characteristics that are common to the rest of SAS-6 family. Similar to that of *D. rerio*, NSAS-6 has a N-terminal domain and a coiled-coil domain. In addition, the three-dimensional structure of the N-terminal domain of NSAS-6 is similar to that of *D. rerio*. Multiple sequence alignment of SAS-6 homologs from microsporidia shows that these parasites have high amino acid identity, and SAS-6 also has the conserved PISA-motif and motif II, suggesting that SAS-6 is a conserved protein in microsporidia. By searching for this protein with SAS-6 of fungi as queries in database, among basal fungi chytrid and zygomycete, we have found that proteins annotated as SAS-6 are only present in chytrid fungi such as *B. dendrobatidis*, *S. punctatus*, *G. prolifera*, *R. allomycis*, *Rhizoclosmatium globosum*, *Batrachochytrium salamandrivorans, Neocallimastix californiae, Anaeromyces robustus, Piromyces finnis, Piromyces* sp. E2, and not in zygomycete fungi. Phylogenetic analysis based on the N-terminal region of SAS-6 shows that *N. bombycis* fall into one group with other microsporidia *N. apis* BRL01*, N. ceranae* BRL01*, E. cuniculi*, *E. intestinalis*, *A. algerae* and *Trachipleistophora hominis*, and that the basal chytrid fungi clustered together but were far away from microsporidia. These results infer that this gene is ancestrally present in the Fungi/microsporidia but lost in many fungal groups, and that the microsporidia have a common ancient evolutionary origin.

In this study, NSAS-6 is localized to the nucleus of sporoplasms, intracellular stages, and mature spores during the life cycle of *N. bombycis*, and the structure that SAS-6 localizes to arises by duplication of the old ones in the replicating cell of *N. bombycis*, which coincides with the finding that the new spindle plaques arise by division of the old ones in microsporidia (Larsson, 1986). Although NSAS-6 is present in *N. bombycis*, current knowledge about its role in spindle plaque assembly is little. SAS-6 has been identified in various eukaryotes and is required for centriole assembly, probasal body biogenesis, and flagellum assembly (Breugel et al., 2011, 2014; Gonczy, 2012; Hu et al., 2015; Leidel et al., 2005; Rodrigues-Martins et al., 2007). Moreover, there is a conserved PLK4-mediated regulatory pathway governing centriole assembly, and PLK4, SAS-6, and STIL are core components of the centriole assembly pathway in human (Gonczy, 2012). In human cells, PLK4 is needed for the continued presence of centriolar SAS-6 and STIL. SAS-6 is recruited but not maintained at procentrioles in PLK4-deficient human cells (Strnad et al., 2007). By searching the database, we also found that PLK4 is present in the microsporidium *N. bombycis*. However, the PLK4 of *N. bombycis* is very special because it lacks the polo-box domain (PBD). Polo-like kinases (PLKs) usually have a highly conserved protein kinase domain in the amino-terminal region and a non-catalytic conserved Polo-box domain towards the C-terminus (Barr et al., 2004; Vaid et al., 2016). The PBD of PLK mediates interaction of PLK with some of its substrates to localize it to different subcellular structures to regulate different stages of cell cycle (Lowry et al., 2004). Structural studies have revealed that the PBD might function as a docking site for various proteins phosphorylated at serine or threonine residues (Elia et al., 2003). In *N. bombycis*, the relationship between PLK4 and NSAS-6 remains unclear. SAS-6 is essential for the presence of STIL at centrioles, whereas STIL is required for centriolar SAS-6 in human cells (Tang et al., 2011). STIL is a centrosomal protein that is essential for zebrafish embryonic development (Vulprecht et al., 2012). Although STIL was not found in the microsporidia, NSAS-6 has been identified as an important element in the development of *N. bombycis*. Thus, *N. bombycis* may have a protein that has a similar function to that of STIL in human or zebrafish. The interaction between NSAS-6 and related proteins such as PLK4 in *N. bombycis* requires further study, which is essential to elucidate the role of NSAS-6 in spindle plaque assembly.

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References

Adams, I.R., Kilmartin, J.V., 2000. Spindle pole body duplication: a model for centrosome duplication? Trends Cell Biol. 10, 329–335.

Alfa, C.E., Hyams, J.S., 1990. Many routes lead to pole. Nature 348, 484.

Barr, F.A., Sillje, H.H., Nigg, E.A., 2004. Polo-like kinases and the orchestration of cell division. Nat. Rev. Mol. Cell Biol. 5, 429–440.

Becnel, J.J., Andreadis, T.G., 1999. Microsporidia in insects. In: Wittner, M. (Ed.), The Microsporidia and Microsporidiosis. ASM Press, Washington, DC, pp. 447–501.

Bettencourt-Dias, M., Glover, D.M., 2007. Centrosome biogenesis and function: centrosomics brings new understanding. Nat. Rev. Mol. Cell Biol. 8, 451–463.

Breugel, M.V., Hirono, M., Andreeva, A., Yanagisawa, H., Yamaguchi, S., Nakazawa, Y., Morgner, N., Petrovich, M., Robinson, C.V., Johnson, C.M., Veprintsev, D., Zuber, B., 2011. Structures of SAS-6 suggest its organization in centrioles. Science 331, 1196–1198.

Breugel, M.V., Wilcken, R., McLaughlin, S.H., Rutherford, T.J., Johnson, C.M., 2014. Structure of the SAS-6 cartwheel hub from Leishmania major. elife 3, e01812.

Bullitt, E., Rout, M.P., Kilmartin, J.V., Akey, C.W., 1997. The yeast spindle pole body is assembled around a central crystal of Spc42p. Cell 89, 1077–1086.

Cai, S., He, X., He, X., Qiu, H., Li, G., He, Y., 2011. A protocol for fast and efficient preparation of genomic DNA and total proteins of *Nosema bombycis*. Science of Sericulture 37, 1019–1024.

Canning, E.U., Lom, J., Dykova, I., 1986. The microsporidia of vertebrates. In: Academic Press. United Kingdom, London.

Desportes, I., Théodoridès, J., 1979. Etude ultrastructurale d'Amphiamblys laubieri n. sp. (microsporidie, metchnikovellidae) parasite dune gregarine (*Lecudina* sp.) d'une echiurien abyssal. Protistologica 15, 435–457.

Elia, A.E.H., Cantley, L.C., Yaffe, M.B., 2003. Proteomic screen finds pSer/pThr binding domain localizing Plk1 to mitotic substrates. Science 299, 1228–1231.

Fredricks, D., Smith, C., Meier, A., 2005. Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J. Clin. Microbiol. 43, 5122–5128.

Gonczy, P., 2012. Towards a molecular architecture of centriole assembly. Nat. Rev. Mol. Cell Biol. 13, 425–435.

Hu, H., Liu, Y., Zhou, Q., Siegel, S., Li, Z., 2015. The centriole cartwheel protein SAS-6 in *Trypanosoma brucei* is required for probasal body biogenesis and flagellum assembly. Eukaryot. Cell 14, 898–907.

Larsson, R., 1986. Ultrastructure, function and classification of microsporidia. In: Corliss, J.O., Patterson, D.J. (Eds.), Progress in Protistology. Biopress, Ltd., Bristol, United Kingdom, pp. 325–390.

Leidel, S., Delattre, M., Cerutti, L., Baumer, K., Gonczy, P., 2005. SAS-6 defines a protein family required for centrosome duplication in C. elegans and in human cells. Nat. Cell Biol. 7, 115–125.

Li, Y., Wu, Z., Pan, G., He, W., Zhang, R., Hu, J., Zhou, Z., 2009. Identification of a novel spore wall protein (SWP26) from microsporidia *Nosema bombycis*. Int. J. Parasitol. 39, 391–398.

Lowry, D.M., Mohammad, D.H., Elia, A.E., Yaffe, M.B., 2004. The polo box domain: a molecular integrator of mitotic kinase cascade and polo-like kinase function. Cell Cycle 3, 128–131.

O'Toole, E.T., Winey, M., Mcintosh, J.R., 1999. High-voltage electron tomography of spindle pole bodies and early mitotic spindles in the yeast *Saccharomyces cerevisiae*. Mol. Biol. Cell 10, 2017–2031.

Rodrigues-Martins, A., Bettencourt-Dias, M., Riparbelli, M., Ferreira, C., Ferreira, I., Callaini, G., Glover, D.M., 2007. DSAS-6 organizes a tube-like centriole precursor, and its absence suggests modularity in centriole assembly. Curr. Biol. 17, 1465–1472.

Rout, M.P., Kilmartin, J.V., 1990. Components of the yeast spindle and spindle pole body. J. Cell Biol. 111, 1913–1927.

Spang, A., Geissler, S., Grein, K., Schiebel, E., 1996. γ -Tubulin like Tub4p of *Saccharomyces cerevisiae* is associated with the spindle pole body substructures that organize microtubules and is required for mitotic spindle function. J. Cell Biol. 134, 429–441.

Strnad, P., Leidel, S., Vinogradova, T., Euteneuer, U., Khodjakov, A., Gonczy, P., 2007. Regulated HsSAS-6 levels ensure formation of a single procentriole per centriole during the centrosome duplication cycle. Dev. Cell 13, 203–213.

Sundberg, H.A., Goetsch, L., Byers, B., Davis, T.N., 1996. The spacer protein Spc110p targets calmodulin to the central plaque of the yeast spindle pole body. J. Cell Biol. 133, 111–124.

Tang, C.J., Lin, S.Y., Hsu, W.B., Lin, Y.N., Wu, C.T., Lin, Y.C., Chang, C.W., Wu, K.S., Tang, T.K., 2011. The human microcephaly protein STIL interacts with CPAP and is required for procentriole formation. EMBO J. 30, 4790–4804.

Taupin, V., Méténier, G., Vivarès, C.P., Prensier, G., 2006. An improved procedure for Percoll gradient separation of sporogonial stages in *Encephalitozoon cuniculi* (Microsporidia). Parasitol. Res. 99, 708–714.

Tsaousis, A.D., Kunji, E.R., Goldberg, A.V., Lucocq, J.M., Hirt, R.P., Embley, T.M., 2008. A novel route for ATP acquisition by the remnant mitochondria of *Encephalitozoon cuniculi*. Nature 453, 553–556.

Vaid, R., Sharma, N., Chauhan, S., Deshta, A., Dev, K., Sourirajan, A., 2016. Functions of Polo-like Kinases: a Journey from yeast to Humans. Protein Pept. Lett. 23, 185–197.

Vávra, J., 1976. Structure of the microsporidia. In: Bulla, L.A., Cheng, T.C. (Eds.), Comparative Pathobiology. Plenum Press, New York, pp. 87–110.

Vávra, J., Larsson, J.I.R., 1976. Structure of the microsporidia. In: Wittner, M. (Ed.), The Microsporidia and Microsporidiosis. ASM Press, Washington, DC, pp. 10–20.

Vulprecht, J., David, A., Tibelius, A., Castiel, A., Konotop, G., Liu, F., Bestvater, F., Raab, M.S., Zentgraf, H., Izraeli, S., Kramer, A., 2012. STIL is required for centriole duplication in human cells. J. Cell Sci. 125, 1353–1362.

Weber, R., Bryan, R.T., Schwartz, D.A., Owen, R.L., 1994. Human microsporidial infections. Clin. Microbiol. Rev. 7, 426–461.

Wigge, P.A., Jensen, O.N., Holmes, S., Soues, S., Mann, M., Kilmartin, J.V., 1998. Analysis of the *Saccharomyces* spindle pole by matrix-assisted laser desorption/ionization (MALDI) mass spec trometry. J. Cell Biol. 141, 967–977.

Wu, Z., Li, Y., Pan, G., Tan, X., Hu, J., Zhou, Z., Xiang, Z., 2008. Proteomic analysis of spore wall proteins and identification of two spore wall proteins from *Nosema bombycis* (Microsporidia). Proteomics 8, 2447–2461.

Wu, Z., Li, Y., Pan, G., Zhou, Z., Xiang, Z., 2009. SWP25, a novel protein associated with the *Nosema bombycis* endospore. J. Eukaryot. Microbiol. 56, 113–118.

Yang, D., Dang, X., Peng, P., Long, M., Ma, C., Qin, J.J., Wu, H., Liu, T., Zhou, X., Pan, G., Zhou, Z., 2014. NBHSWP11, a microsporidia *Nosema bombycis* protein, localizing in the spore wall and membranes, reduces spore adherence to host cell BmE. J. Parasitol. 100, 623–632.

Yang, D., Pan, G., Dang, X., Shi, Y., Li, C., Peng, P., Luo, B., Bian, M., Song, Y., Ma, C., Chen, J., Ma, Z., Geng, L., Li, Z., Tian, R., Wei, C., Zhou, Z., 2015. Interaction and assembly of two novel proteins in the spore wall of the microsporidian species *Nosema bombycis* and their roles in adherence to and infection of host cells. Infect. Immun. 83, 1715–1731.