The SAS-5 N-terminal domain is a tetramer, with implications for centriole assembly in C. elegans

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Introduction

Centrioles are the fundamental units for both centrosome formation and cilium biogenesis. Caenorhabditis elegans has become an important model organism in studying centrosome duplication and centriole assembly because of its experimental tractability and the availability of powerful genetic and genomic tools. Five proteins have been implicated in centriole biogenesis following work in C. elegans, namely ZYG-1, SPD-2, SAS-4, SAS-5, and SAS-6. Their structural and functional orthologs have also been identified in other species. All five of these proteins localize to centrioles and are required for centriole duplication. Depletion of any of them blocks centriole assembly.

Two research groups independently discovered the recruitment hierarchy of the five centriolar proteins during centriole duplication. Furthermore, it was reported previously that SAS-5 binds specifically to a very narrow region of the SAS-6 central coiled coil through its C-terminal domain (CTD, residues 391–404). Here, we further demonstrate by both static light scattering and small angle X-ray scattering that the SAS-5 N-terminal domain (NTD, residues 1–260) forms a tetramer. Specifically, we found that the tetramer is formed by SAS-5 residues 82–260, whereas residues 1–81 are intrinsically disordered. Taking these results together, we propose a working model for SAS-5-mediated assembly of the multi-layered central tube structure.

Results and Discussion

The N-terminal domain of SAS-5 is composed mostly of helical structures. We recently reported that SAS-5 binds specifically to the central region of the SAS-6 coiled coil via its CTD.

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We performed further bioinformatic analysis of SAS-5 to find out its overall domain arrangement and structure compositions. GlobPlot V2.3,24 which was employed to predict intrinsic disorder and globularity of SAS-5, suggested that the N-terminal region of SAS-5 (residues 1–260) forms a folded structure whereas the C-terminal one third of the protein (residues 261–404) is mostly disordered (Fig. 1A). Secondary structure predictions by PSIPRED V3.35 suggested that the SAS-5 NTD is composed mostly of helical structures, with residues 1–81 less ordered than the rest in this domain (Fig. 1B). Primary sequence alignment of SAS-5 from three Caenorhabditis species also demonstrated that the N-terminal 81 residues are less conserved than the central and the C-terminal regions (Fig. 1C). To investigate the structure of SAS-5, we cloned the SAS-5 NTD into an expression vector that adds a cleavable maltose binding protein (MBP) tag plus a 10 × His tag to the N terminus of the protein (Fig. 1D). The SAS-5 NTD was eluted at 57 ml on the Superdex-200 16/60 column (GE Healthcare) after removal of the tag and was relatively pure (Fig. 1E and F). We then subjected the purified protein to circular dichroism (CD) experiments to measure the secondary structures and to measure the melting temperature \((Tm)\) of the SAS-5 NTD. Consistent with the secondary structural predictions, we found that the protein is predominantly \(\alpha\)-helical, with a far-UV CD spectrum of the protein displaying two characteristic negative peaks at 209 nm and 223 nm (Fig. 1G). \(Tm\) was measured to be \(-50°C\) (Fig. 1H), indicating that the SAS-5 NTD indeed forms a folded domain.

The SAS-5 N-terminal domain forms a tetramer. Since the elution volume of a protein in gel filtration chromatography is determined not only by the molecular weight (M.W.) but also by the overall shape of the molecule, it is not advisable to evaluate the oligomeric state of a protein using gel filtration chromatography. We performed static light scattering (SLS) experiments to assess the oligomeric state of SAS-5. The result suggested that the SAS-5 NTD has an M.W. of 120–160 kDa (Fig. 1I), and could be either a tetramer (\(31 \times 4 = 124\) kDa) or a pentamer (\(31 \times 5 = 155\) kDa). To further verify the oligomeric state of the SAS-5 NTD, we recloned residues 1–260 of SAS-5 into KiM2a, a pMAL-derived vector that adds an MBP tag to the N terminus of the target protein (Fig. 2A). To minimize the flexibility of the linker, we used only two alanines (AA) to connect the tag and SAS-5. The fusion protein was overexpressed in Escherichia coli strain BL21(DE3) and pure protein eluted from an amylose column was applied onto a Superdex S-400 16/60 column (GE Healthcare). The MBP-AA-SAS5-NTD was eluted at 70.4 ml with a symmetric elution peak and the protein seemed of high purity on an SDS-PAGE gel (Fig. 2B). SLS experiments showed that it has an M.W. of 290 ± 10 kDa, suggesting that the protein forms a tetramer (Fig. 2C).

To assess the overall conformation of the SAS-5 tetramer, we performed further small angle X-ray scattering (SAXS) experiments using the MBP-AA-SAS5-NTD purified after Superdex S-400 16/60 chromatography. The ab initio model of the MBP-AA-SAS5-NTD were the N termini of the four SAS-5 molecules, whereas the “body” represents the tetramer formed by residues 82–260 of SAS-5 (Fig. 2D). At the tip of each “arm” was a spherical structure which is the globular MBP tag (Fig. 2D).

What structural roles could SAS-5 play in C. elegans centriole assembly? We recently reported that the association of SAS-5 and SAS-6 is mediated by a robust interaction \((K_c = 3 \, \mu M)\) between the SAS-5 CTD and a central segment in the SAS-6 coiled coil (Fig. 3A).23 It was also shown recently that the Trichonympha basal body, which is derived from the mother centriole, contains a stack of rings formed by SAS-6.21 Putting these observations together in the context of centriole assembly, we wish to propose a hypothetical working model for how SAS-5 may connect neighboring rings in the stacking assembly of the central tube (Fig. 3B). In this model, each SAS-5 anchors its CTD onto the SAS-6 coiled coil, and the SAS-5 NTDs form a tetramer that bridges the four SAS-6 homodimers bound to the SAS-5 CTDs. Two of the four SAS-6 dimers belong to one ring (layer N) and the other two to the neighboring ring (layer N+1). In this way, the multi-layered cartwheel assembly mediated by SAS-5 would strengthen interactions both within the same ring and across neighboring rings formed by SAS-6 (Fig. 3B).

Notably, the N-terminal flexible part of SAS-5 (residues 1–81), which is 10–15 nm as demonstrated by the SAXS experiments (Fig. 2G), would be freely movable in our assembly model. This would allow the SAS-5 N terminus to reach the head group of SAS-6. An important test of this model is whether the SAS-5 N-terminal part directly interacts with the SAS-6 head groups or it has other uncharacterized functions in centriole duplication. This will be investigated in future work.

Materials and Methods

Cloning, protein expression and purification. Sequences encoding SAS-5 residues 1–260, 1–81 and 82–260 were all amplified by PCR from C. elegans cDNA and cloned into pET29a (Novagen), MalPET and/or KIM2a. MalPET and KIM2a are both pMAL-derived vectors with the former adding both MBP and 10 × His tags (cleavable by TEV protease) and the latter adding only MBP tag plus a two-Ala linker to the N terminus of SAS-5. All recombinant proteins were expressed in E. coli BL21 (DE3) cells. Cells were grown at 37°C. At an OD\(_{600}\) of 0.6–0.8, cultures were...
cold shocked on ice for 10–15 min and then shifted to 18°C. Protein induction was done overnight with 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Aldrich). Cells were harvested and resuspended in cold lysis buffer containing 20 mM TRIS-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole and 5% (v/v) glycerol.

Figure 1. For figure legend, see page 4.
Cells were broken using an EmulsiFlex-C3 homogenizer (Avestin) and cell lysate was cleared by centrifugation at 30,000 × g for 30 min. The supernatant was filtered (0.4-μm) and loaded onto a gravity amylase column (for MBP-AA-SAS-5 aa1-260/aa82-260) or a Ni-HiTrap column from GE Healthcare (for MBP-10 × His-SAS-5 aa1-260 and SAS-5 aa1-81_6 × His) pre-equilibrated in the same lysis buffer. The amylase column was washed with 10 × column volume (cv) of buffer containing 20 mM HEPES-NaOH (pH 7.2), 300 mM NaCl, 15 mM β-mercaptoethanol and 25% (v/v) glycerol; bound protein was eluted using the same buffer containing 50 mM maltose. Eluted protein was concentrated using centrifugal filters (Amicon, MWCO 10-kDa) and loaded onto a Sephacryl S-400 16/60 column (GE Healthcare) pre-equilibrated with 20 mM TRIS-HCl (pH 8.0), 100 mM NaCl and 5% (v/v) glycerol. Fractions containing target protein were pooled for SLS and SAXS experiments (Fig. 2B). For MBP-10 × His-SAS-5 and SAS-5 aa1-81_6 × His, bound proteins on the Ni-HiTrap column were eluted using the lysis buffer containing a linear gradient concentration of imidazole (20–500 mM, 20 × cv). The N-terminal MBP-10 × His tag in MBP-10 × His-SAS-5 was removed by incubating purified protein with 1% (w/v) of TEV protease while dialyzing against 2–4 L of the same lysis buffer overnight at 4°C. The dialyzed protein sample was passed through a Ni-HiTrap column to remove both released MBP-10 × His tag and uncut fusion protein. Flow-through was concentrated and further purified using a Superdex-200 16/60 column (GE Healthcare) pre-equilibrated with 20 mM TRIS-HCl (pH 8.0), 100 mM NaCl and 5% (v/v) glycerol. Fractions containing the SAS-5 NTD (elution volume ~57 ml) were pooled and concentrated to 5–10 mg/ml for later experiments (Fig. 2F).

Static light scattering (SLS). The SLS studies were performed on an Agilent 1260 Infinity LC System (Agilent Technologies). The liquid chromatography equipment consists of an HPLC system (Agilent Technologies) connected in series with a triple-angle laser light scattering detector (miniDAWN TREOS), a UV detector at 280 nm (Agilent technologies) and a refractive index detector (RI-101, Shodex). Fifty to 100 μl of protein samples (~1–5 mg/ml) were eluted from a Superdex-200 10/300 GL column (GE Healthcare) at a flow rate of 0.5 ml per min. Data analysis was performed using the Astra software (Wyatt technology).

Circular dichroism (CD). CD measurements were performed on a Chirascan™-plus unit (AppliedPhotophysics) with a 0.5-mm pathlength cuvette. Protein samples of 0.2 mg/ml were used in both wavelength scan and melting temperature measurement (λ = 209 nm). Data points were exported and curves were generated using Windows Excel-2013 (Microsoft).

Small angle X-ray scattering (SAXS). SAXS data for the MBP-tagged SAS-5 NTD and the SAS-5 aa1-81_6 × His were collected following standard procedures on the BM29 BioSAXS beamline of the ESRF at Grenoble, France using a robotic sample changer and a Pilatus 1M detector. The scattering patterns were measured at the solute concentrations of 1, 4 and 8 mg/ml using a sample detector distance of 2.841 mm and a wavelength of λ = 1.55 Å. To monitor for radiation damage, ten one-second exposures were collected for each protein sample and no radiation effect was observed. The data were normalized to the intensity of the transmitted beam, and the scattering of the buffer was subtracted. Data collection, processing and analysis are performed automatically using the beamline software BsxCuBE with help of the beamline scientist Dr. Adam Round.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Author’s Contributions
ES, RQ and GD designed the experiments and performed data analyses. ES, RQ and JL performed experiments. GD supervised the project and wrote the manuscript.
Figure 2. For figure legend, see page 6.
Figure 2. The SAS-5 NTD forms a tetramer with residues 1–81 being intrinsically disordered. (A) Expression construct of the SAS-5 NTD fused to MBP with a two-Ala linker. (B) Elution profile of the MBP-tagged SAS-5 NTD on the Sephacryl S-400 16/60 gel filtration column (GE Healthcare); fractions were checked on a 12% (v/v) SDS-PAGE gel. (C) An SLS profile of purified MBP-tagged SAS-5 NTD suggested a tetrameric form of the protein (73 × 4 = 292 kDa). (D) Two different views of the ab initio low resolution shape reconstructed by GASBOR. A hypothetical model of the complex (right) showed that the SAS-5 NTD forms a tetramer with the N-terminal stretch of residues being flexible as predicted in Figure 1A and B. The blobs at the tips of the four arms are the MBP moiety. (E) An SLS profile of purified SAS-5 residues 1–81 suggested it forms a monomer in solution. (F) A far-UV CD spectrum of purified SAS-5 residues 1–81 suggested the protein contains mostly random coils. (G) Green beads show the ab initio low resolution shape of SAS-5 residues 1–81 reconstructed by GASBOR. For comparison, a 70-residue α-helix, which is ~10 nm long, is shown on the right. (M) An SLS profile of the MBP-tagged SAS-5 residues 82–260 suggested this part of the protein forms a tetramer (63 × 4 = 252 kDa).

Figure 3. A hypothetical model of how the tetrameric SAS-5 NTD might strengthen the assembly of the central hub in centriole assembly. (A) Schematic drawing of the SAS-5/SAS-6 complex reported previously.15 (B) A working model showing how the tetrameric SAS-5 complex could connect four SAS-6 dimers in two neighboring rings during central hub assembly.

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