Self-assembling SAS-6 Multimer Is a Core Centriole Building Block

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Centrioles are conserved microtubule-based organelles with 9-fold symmetry that are essential for cilia and mitotic spindle formation. A conserved structure at the onset of centriole assembly is a “cartwheel” with 9-fold radial symmetry and a central tubule in its core. It remains unclear how the cartwheel is formed. The conserved centriole protein, SAS-6, is a cartwheel component that functions early in centriole formation. Here, combining biochemistry and electron microscopy, we characterize SAS-6 and show that it self-assembles into stable tetramers, which serve as building blocks for the central tubule. These results suggest that SAS-6 self-assembly may be an initial step in the formation of the cartwheel that provides the 9-fold symmetry. Electron microscopy of centrosomes identified 25-nm central tubules with repeating subunits and show that SAS-6 concentrates at the core of the subunits. Recombinant and native SAS-6 self-oligomerizes into tetramers with 6-nm subunits, and these tetramers are components of the centrosome, suggesting that tetramers are the building blocks of the central tubule. This is further supported by the observation that elevated levels of SAS-6 in Drosophila cells resulted in higher-order structures resembling central tubule morphology. Finally, in the presence of embryonic extract, SAS-6 tetramers assembled into high density complexes, providing a starting point for the eventual in vitro reconstruction of centrioles.

Centrioles are conserved microtubule-based organelles with 9-fold symmetry organized around a central tubule and spoke structure termed the “cartwheel.” This conserved 9-fold symmetry is also shared with basal body and cilia (1–3). Such architectural similarity is indicative of the role of the centriole as a template for cilia formation. Cilia are microtubule-rich cellular organelles involved in signaling pathways and sensory functions in eukaryotic cells (4, 5). Centrioles also recruit a pericentriolar matrix to form centrosomes, major microtubule organizing centers required for cell proliferation and differentiation (6, 7). In ciliated cells, one of the centrioles inherited by the daughter cell migrates to the plasma membrane after cell division to serve as a template for the cell cilium. However, when a centriole duplicates, the new centriole does not use the existing centriole as a template (8, 9), raising the question of how the new centriole establishes its 9-fold symmetry.

Centriole biogenesis is a multistep process that includes an intermediate known as the procentriole (10). The procentriole has an internal cartwheel structure that is required for the stability of the centriole and the 9-fold symmetry of cilia (11). The cartwheel has a central tubule from which nine spokes emanate, each attaching to centriolar microtubules (supplemental Fig. S1A). Based on the structure of the cartwheel, it is expected that the central tubule would itself display 9-fold symmetry (12) (supplemental Fig. S1A). It has been proposed that the central tubule generates the 9-fold symmetry of the centriole (13, 14). However, the composition of the central tubule and the mechanisms underlying its biogenesis remain unknown.

SAS-6 is a coiled-coil protein that is required for centriole biogenesis (15, 16). Overexpression of SAS-6 produces multiple procentrioles (17). In Chlamydomonas and Tetrahymena, SAS-6 localizes to the center of the cartwheel (11, 18). Null mutants of SAS-6 in Chlamydomonas (11) and Drosophila (11, 19) lack the cartwheel. The SAS-6 null mutants of Chlamydomonas and Drosophila are also defective in establishing the 9-fold symmetry of centrioles. In Caenorhabditis elegans, RNAi studies show that SAS-6 is required for the formation of a centriole central tube. Direct evidence that SAS-6 is required for central tube assembly in worms comes from time resolved electron tomography of C. elegans embryos demonstrating that centriole formation begins with assembly of a central tube, a process that requires SAS-6 (21). This study also implied that the tube could itself organize 9-fold symmetry, but it was not clear if the process was driven by steric constraints or was patterned by intrinsic cues on the outer surface of the tube. It was proposed that SAS-6 brings together nine pre-centriolar units called ena-tosomes, which then form a tube-like centriole precursor (22) and that SAS-6 is the repeating subunit of the central tubule (12).

To investigate the mechanism of SAS-6 function, we employed a Drosophila model that allows for biochemical isolation...
and electron microscopy (EM) imaging of centrioles and cartwheels. In vertebrates the cartwheel is transient and is restricted to the "procentriole" stage (23). In worms the cartwheel is not apparent (21), and in protozoa it is restricted to the proximal end of basal bodies (18, 24). Drosophila centrioles are structurally similar to the procentrioles of higher organisms (7), and cartwheels are constitutive components of Drosophila centrioles. EM studies of in situ centrioles in Drosophila embryos reported that the cartwheel extends through the full length of the centriole (25).

Here, we report that in Drosophila, the central tubule of the cartwheel has repeating subunits and that SAS-6 localizes at the central tubule. Recombinant SAS-6 produced in Escherichia coli or Pichia pastoris forms tetrameric structures that are fractionated at 7.4 S. Most of the native SAS-6 exists as 7.4 S structures, which is the most common soluble form of SAS-6. Native SAS-6 is also found as part of 50 S structures and centrosomes. Elevated levels of SAS-6 in Drosophila cells resulted in higher order structures built from multiple central tubule-like structures. Affinity purification of these high density SAS-6 complexes contained tetramers. Upon disassembly, these higher order complexes resulted in tetramers. In vitro assembly of these tetramers could then be assembled into high density complexes in the presence of embryonic extract. Collectively these data suggest that the SAS-6 tetramers serve as building blocks of a higher order central tubule at the core of centriole architecture.

MATERIALS AND METHODS

Transgenic Constructs—The generation of ana1 and sas-6 fused with GFP was described previously (28). The flies were grown according to standard procedures and maintained at 25 °C.

Drosophila Embryo and S2 Cell Extract Preparation—Drosophila embryo extract was prepared as described previously (26). Briefly, Drosophila embryos of 0–12 h or tissue-cultured S2 cells were homogenized in extract buffer containing 80 mM K-Pipes, pH 6.8, 1 mM MgCl2, 1 mM Na3EGTA, 14% sucrose, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture (Sigma) plus EDTA-free complete tablets (Roche Applied Science). A similar buffer with 500 mM KCl was used. The inhibitor mixture (Sigma) plus EDTA-free complete tablets were solved in a buffer containing 80 mM K-Pipes, pH 6.8, 1 mM MgCl2, 1 mM Na3EGTA, 1 mM GTP, and 500 mM KCl. The maternal requirement (1:5000, Sigma), mouse anti-Misato (1:5000, Santa Cruz), mouse anti-V5 (1:10,000, Abcam), mouse anti-GFP (1:5000, Roche Applied Science), mouse anti-FLAG (1:1000), and mouse anti-Myc (1:200). The secondary antibodies (1:5000) used were conjugated with peroxidase (Vector labs).

For immunofluorescence, antibodies were rat anti-α-tubulin (1:200, Chemicon) and mouse anti-γ-tubulin (1:200, Sigma). All fluorescent secondary antibodies were from Jackson ImmunoResearch and used at 1:200: Cy5 goat anti-mouse and rhodamine donkey anti-rat. 4',6-Diamidino-2-phenylindole (1 μg/ml; Sigma) was used to stain DNA.

Immunofluorescence and Staining—Testis from transgenic flies expressing SAS-6-GFP and ANA1-GFP were dissected in saline solution (0.7% NaCl) and fixed 5 min in formaldehyde (3.7% in PBS). The slides were then mounted in mounting media (Biomedia) and examined using a Leica TCS SP5 confocal microscope. Images were processed using Adobe Photoshop.

Drosophila S2 cells expressing SAS-6-GFP or SAS-6-GFP-FLAG were established by co-transfecting p(UAST)-SAS-6-GFP or p(UAST)-SAS-6-GFP-FLAG with hygromycin-resistant plasmid. The cells were grown on concanavalin-coated coverslips for 1 h at 25 °C before they were fixed using formaldehyde (3.7% in PBS) for 5 min. After washing with PBS, the cells were permeabilized with PBS, 0.1% Triton X-100 for 10 min and blocked with PBS, 1% bovine serum albumin, 0.1% Triton X-100 for 45 min. Antibody staining was performed for 1 h at room temperature followed by three washes with PBS. The slides were then mounted in mounting media (Biomedia) and examined using a scanning confocal fluorescence microscope as described above.

Immunoelectron Microscopy of Isolated Centrosomes—Fractions from sucrose gradient-containing centrosomes were fixed in 3.7% paraformaldehyde plus 0.1% glutaraldehyde in 80 mM K-Pipes, pH 6.8, 1 mM MgCl2, 1 mM Na3EGTA at 4 °C for 10 min. The fixed centrosomes were spun onto previously glow-
discharged ACLAR coverslips (Ted Pella, Inc., Redding, CA) coated with polylysine (Sigma). Using 3% glutaraldehyde, the centrosomes were post-fixed for 10 min followed by 1% osmium tetroxide and 0.5% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.4, for 15 min at 4 °C. The specimens were stained overnight with 1% aqueous uranyl acetate at 4 °C followed by dehydration through graded cold alcohol series and brought to room temperature with absolute alcohol. The samples were embedded onto epon/araldite using standard protocol and remounted for thin serial sections. Thin sections of 70 nm were cut using ultra cut microtome, and the thin sections were collected on Formvar-coated copper grids. The specimens were post-stained with 1% uranyl acetate in 50% methanol followed by aqueous lead citrate and viewed in a Tecnai G Spirit BioTWIN transmission electron microscope (FEI, Hillsboro, OR) operated at 80 kV. For immunolabeling of centrosomes, the fixation was modified. Centrosomes on the coverslips were initially blocked with a buffer containing 2% bovine serum albumin and 0.1% cold water fish skin gelatin in Tris-buffered saline. The samples were labeled using chicken anti-SAS-6 or anti-GFP diluted to 1:20 in the same buffer and then with rabbit anti-chicken followed by protein A with 5-nm colloidal gold.

Negative Stain Electron Microscopy and EM Tomogram—The sucrose from the fractions was removed by buffer exchange using PD-10 Superdex-25 columns (GE Healthcare) with a buffer containing 80 mM K-Pipes, pH 6.8, 1 mM MgCl₂, 1 mM Na₂EGTA, and 500 mM KCl. 3.5 μl of sample was placed onto a glow-discharged copper grid for 1 min. The excess sample was blotted using filter paper, and the grids were washed with a drop of deionized water twice. The grids were then stained with two drops of freshly prepared 0.75% uranyl acetate at 4 °C and were air-dried subsequent to blotting with filter paper. The grids were observed in a Jeol 1200EX electron microscope. Protease inhibitors and 40 mM β-mercaptoethanol were added, and the cell extract was mixed well at 4 °C and then subjected to centrifugation at 30,000 × g for 30 min to sediment the membranes and aggregates. The supernatant-containing soluble form of SAS-6 fusion protein was bound with a nickel-nitrilotriacetic acid column, and the bound material was eluted with 250 mM imidazole. The purified protein was fractionated using Superdex-200 column (GE Healthcare). The standard markers were obtained from Bio-Rad.

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Tomogram reconstructions were performed by the EM/CAT package within the PRIISSM software developed at University of California, San Francisco. Final tomograms were filtered using anisotropic diffusion filter (35) or Fourier low-pass filters for anisotropic diffusion does not preserve the individual protein densities. Rotational spectra analysis was performed using the Xmipp (34) package by Fourier-Bessel decomposition (36). Tomogram segmentation and volume rendering was performed using ImageJ (33) and University of California, San Francisco chimera (37), respectively. Briefly, semiautomatic segmentation was carried out by selecting three independent regions of interest corresponding to the central tubule, spokes, and microtubules on the different tomogram slices. Voxels outside each independent regions of interest were set to 0 before pseudo-color assignment on each region of interest based on voxel intensity. Volumes from regions of interest and tomograms were combined to generate the final video stream.

E. coli Strains, Constructs, and Protein Purification—Drosophila SAS-6 cDNA was cloned into pDONR 221 (Invitrogen) and then recombined into the pet23b vector previously modified for the gateway system (Invitrogen). The cDNA was tagged with V5 and His₆ at its C terminus (a kind gift from Betten-Dias). The construct was transformed into E. coli strain BL21 (Stratagene) and grown in LB medium to absorbance 0.60 nm. The protein expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were pelleted and disrupted by lysozyme treatment followed by sonication. The lysate was centrifuged at 30,000 × g for 30 min to sediment the membranes and aggregates. The supernatant-containing soluble form of SAS-6 fusion protein was bound with a nickel-nitrilotriacetic acid column, and the bound material was eluted with 250 mM imidazole. The purified protein was fractionated using Superdex-200 column (GE Healthcare). The standard markers were obtained from Bio-Rad.

P. pastoris Strains, Constructs, and Protein Purification—P. pastoris strain GS115 (Invitrogen) was used for heterologous expression of SAS-6. YPDS and Zeocin agar plates that contained 1% (w/v) yeast extract, 2% (w/v) Tryptone, 2% (v/v) dextrose, 1 mM sorbitol, and 100 mg/ml Zeocin were used for transformation. The expression trial and 1 liter of culture were grown in BMGY (1% (w/v) yeast extract, 2% (w/v) Tryptone, 100 mM potassium phosphate, pH 6.0, 1.34% (v/v) yeast nitrogen base, 4 × 10⁻⁵ % (v/v) biotin, and 1% glycerol). TOP 10 F0 E. coli (Invitrogen) was used for the formation of the construct.

SAS-6 coding sequence from (AT29216) was inserted into pPICZB between sites EcoRI and NotI to generate SAS-6-Myc-His₆. One microgram of SacI-linearized plasmid was transformed into P. pastoris GS115 cells by electroporation (Bio-Rad micropulsor) in a 0.2-cm cuvette. One milliliter of 1 mM sorbitol was added, and the electroporated cells were incubated for 1 h at 30 °C. Then 1 ml of YPD was added to the cell solution and incubated with shaking for 1 h. The resulting solution was spread onto YPDS + Zeocin plates and incubated at 30 °C for 3 days. The yeast colonies were picked aseptically and grown with 1 ml of YPD (yeast extract/peptone/dextrose) tubes containing 1 mg/liter selective marker Zeocin. Each colony was PCR screened for the presence of the insert and then screened for protein expression. For the expression trial the medium BMMY was used, and the culture was induced with 0.5% methanol every 24 h. The protein expression was monitored every 8 h until the optimum level was reached at 72 h. For preparative cultures the pre-grown nursery cultures from minimal media were transferred to 6-liter flasks for the protein expression. The cultures were microscopically checked for any contamination.

The yeast cells were spun down at 5000 rpm, washed with distilled H₂O, and resuspended with lysis buffer that contained 6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 100 mM NaCl, 2 mM EGTA, 100 mM Na₂VO₄, and 0.1% TritonX1100 and rapidly frozen using liquid nitrogen. The frozen cells were mechanically disrupted using a domestic mixer under frozen conditions or ball mill (Retsch). The disrupted cells were verified under light microscope. Protease inhibitors and 40 mM β-mercaptoethanol were added, and the cell extract was mixed well at 4 °C and then subjected to centrifugation at 30,000 × g. The clear supernatant was used for affinity purification using nickel-nitrilotriacetic acid resins. The binding buffer contained 50 mM imidazole to prevent nonspecific binding, and the final elution was carried out using 250–500 mM imidazole. When required the protein sample was concentrated using Millipore spin columns.

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RESULTS

SAS-6 Is a Component of the Centriole Central Tubule—To gain structural insights into the centriole central tubule, we biochemically isolated centrosomes from Drosophila embryo extract and processed them for thin-section EM as previously described (26). Our thin-section EM of chemically fixed isolated Drosophila centrosomes showed that the central tubule is 25–30 nm in outer diameter (n = 15) (supplemental Fig. S1B) as demonstrated previously (25). This is similar in size to the central tubules of most of the model organisms (supplemental Table 1) with the exception of the hexapod Acerentomon microrhinus, where the diameter of the central tubule varied according to the centriole diameter (27). Analysis of cryo-EM tomograms of centrosomes suggested that the central tubule is an extended structure along the axis of the centriole (supplemental Movies 1 and 2). This supported that the central tubule is a polymer of repeating subunits as was previously proposed (supplemental Fig. S1) (17).

We then examined whether SAS-6 is a component of the central tubule in Drosophila. Work in human cells has shown that SAS-6 localizes to the proximal end of centrioles (17). SAS-6-GFP localization in the elongated centrioles of Drosophila spermatocytes showed that SAS-6 is restricted to the proximal end (Fig. 1A). It was previously reported that SAS-6 is present throughout the full length of spermatocyte centrioles when SAS-6 gene products were expressed using a strong promoter (19).

We then performed ultrastructural immunolocalization of SAS-6 using isolated Drosophila centrosomes. The centrosomes were isolated from extracts of transgenic Drosophila embryos expressing SAS-6-GFP. For immunostaining, we used GFP and SAS-6 antibodies separately. Additionally, to distinguish the pericentriolar material (PCM) portion of the centrosome from the centriole core, we immuno-stained the centrosomes for Asl, a PCM protein (28) (Fig. 1D). Analysis of immunogold labeling of cross-sectioned centrioles showed that in Drosophila, SAS-6 is specifically localized to the central tubule at the center of the centriole (Fig. 1, Bi and Ci). In longitudinally sectioned centrioles, preferential SAS-6 labeling was found at the center part of the centriole (Fig. 1, B, ii–iv, and C, ii–iv). Similar results were previously reported in Chlamydomonas (11) and Tetrahymena (18, 20). These studies indicate that SAS-6 has an evolutionarily conserved function as a component of the cartwheel and central tubule.

Recombinant SAS-6 Produced in E. coli and P. pastoris Self-oligomerizes to Form Tetramers—To check if SAS-6 can form subunits similar to those found in the centriole central tubule, we produced and characterized recombinant SAS-6 in E. coli and in P. pastoris, expression systems that do not have centrioles. In E. coli, the SAS-6-V5-His fusion protein was expressed as a ~55-kDa protein found mostly in inclusion bodies (supplemental Fig. S2Aii). However, affinity purification from large volume cultures allowed us to obtain a detectable amount of soluble protein (supplemental Fig. S2Aii). First, we analyzed its oligomerization potential using sucrose gradient sedimentation and size exclusion chromatography. In a 5–40% linear gradient, we found an intense signal centered at 7.4 S (Fig. 2A). In size exclusion chromatography, the majority of SAS-6 was eluted in a manner consistent with a complex with a molecular weight slightly higher than 158 kDa, approximately the size of a SAS-6 tetramer and also at the boundary of the exclusion volume (supplemental Fig. S2B). These data are consistent with SAS-6 possessing homophilic binding properties forming multimers.

We further tested if recombinant SAS-6 produced from E. coli could interact with native SAS-6. We mixed SAS-6-V5-His with fly embryonic extract containing SAS-6-GFP and employed nickel affinity resin to recover the SAS-6-V5-His along with its binding partners. Elution of the resin followed by Western analysis showed that recombinant SAS-6-V5-His specifically binds SAS-6-GFP but not Misato, a non-relevant fly mitochondrial protein (Fig. 2B).

We then examined the purified 7.4 S structures using negative-stain EM after removing the sucrose by buffer exchange. Importantly, the recombinant SAS-6 generated objects with a diameter of ~12 nm with four ~6-nm stain excluding regions arranged in 4-fold symmetry (Fig. 2C). The four-stain-excluding regions had identical densities, suggesting that these structures are tetramers made of SAS-6 monomers. Analysis of the purity of the samples by silver staining detected SAS-6 as the major component (Fig. 2D).

We conducted additional experiments in P. pastoris, an eukaryotic expression system that may have better protein expression and folding properties. Using Myc antibody, we detected the affinity-purified SAS-6-Myc-His fusion protein as a monomer at ~55 kDa in reducing SDS-PAGE (supplemental Fig. S2C). However, in the absence of the reducing agent β-mercaptoethanol, we detected oligomerized SAS-6 as a smear at higher molecular weight as well as a distinct signal that corresponds to a size of ~150 kDa (Fig. 2E). In size exclusion chromatography, we detected a SAS-6 peak between 670 and 158 kDa, including a peak at the boundary of the exclusion volume (supplemental Fig. S2D). SAS-6 expressed from P. pastoris also bound in a homophilic manner with endogenous SAS-6 from embryonic extract (Fig. 2F).

Like the recombinant SAS-6 produced from E. coli, the density sedimentation of affinity-purified recombinant SAS-6 from P. pastoris identified a 7.4 S peak. However, we also observed SAS-6-positive fractions around 50 S and higher (Fig. 2G). Negative-stain EM analysis of pooled high density fractions identified SAS-6 tetramers (Fig. 2, Hi and Hii) similar to those formed by recombinant SAS-6 produced in E. coli and also agglomerates of tetramers (Fig. 2Hiiti), some of which exhibited a curved assembly. One question that remains is how free tetramers can be present in the high density fractions. One possibility is that the higher order structures disassociated into stable tetrameric building blocks during the sucrose removal and buffer exchange process that preceded negative staining of fractions. In an attempt to increase the number of particles, we concentrated the SAS-6-containing samples and found that SAS-6 was lost during the course of concentration (supplemental Fig. S2F), possibly due to its tendency to aggregate at high concentrations.

Native SAS-6 Exists as Tetrameric Structures—To test if the SAS-6 tetramers are functionally relevant, we performed a bio-
chemical characterization of native SAS-6. A major limitation of using biochemical methods to study centrioles is that most cells have only two centrioles. To get enough starting material, we harvested large amounts of Drosophila embryos expressing SAS-6-GFP from Drosophila population cages. We fractionated Drosophila embryonic extracts by velocity sedimentation. In a 5–40% linear gradient with 100 mM KCl, a wide distribution of SAS-6-positive fractions was detected. Importantly, under this condition most of SAS-6 was distributed starting around 7.4 S, where tetramers are fractionated (Fig. 3A).
SAS-6 Tetramer Is a Core Centriole Building Block

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To test if SAS-6 tetramers are part of the centrosome, we disrupted centrosomes at high salt conditions and analyzed using velocity sedimentation. Treating centrosomes with 0.5 M KCl did not affect SAS-6 distribution and was fractionated in the high density fractions at the bottom of the sucrose gradient (Fig. 3D). However, when centrosomes are treated with 1.5 M KCl, SAS-6 was found around 7.4 S where tetramers are fractionated, and only a small amount of SAS-6 was still detected in the centrosome fraction (Fig. 3Dii). Therefore, the observed SAS-6 finding suggests that the most stable soluble form of SAS-6 in cells is tetramers, and therefore, the tetramers could serve as intermediate building blocks in assembling the higher order central tubule structures.
SAS-6 Tetramer Is a Core Centriole Building Block

at 7.4 S is detached from the centrioles, suggesting that SAS-6 in the centriole central tubule is composed of tetramers. Elevated Levels of SAS-6 in Drosophila Cells Induce Microtubule Organizing Centers That Contain Central Tubule-like Structures—A previous report showed that overexpression of SAS-6 in Drosophila embryos induces large structures that function as microtubule organizing centers (19). These structures appeared to be hollow tubes of 2.2 times larger in diameter than endogenous centrioles. This led us to speculate that SAS-6 overexpression in Drosophila cells would lead to ectopic SAS-6 structures stable enough to be fractionated and analyzed by EM. We generated Drosophila S2 cells stably overexpressing SAS-6-GFP. These cells showed at least a 25-fold increase in SAS-6 as compared with untransfected control cells (supplemental Fig. S4A). However, γ-tubulin was observed in similar amounts in the control cells and in SAS-6-GFP cells, suggesting that elevation of SAS-6 levels does not lead to an increase in other centrosomal proteins.

Immunofluorescence analysis revealed that SAS-6-GFP predominantly localized at the mitotic spindle poles or centrosomes during mitosis with little distribution in the cytoplasm (supplemental Fig. S4Bi). During interphase, most cytoplasmic SAS-6-GFP concentrated into multiple GFP foci emanating microtubule asters (supplemental Fig. S4Bi), suggesting that elevated levels of SAS-6 induce the assembly of ectopic structures. Indeed, thin-section EM analysis on an interphase cell confirmed that these structures are distinct and are absent from untransfected control cells (supplemental Fig. S4, C and D).

Fractionation of SAS-6-GFP cell extract in the presence of 100 or 500 mM KCl indicated that the ectopic structures were denser than 50 S (Fig. 4A). Although they are capable of recruiting γ-tubulin (Fig. 4, Ai), these structures were distinct from normal centrosomes that are fractionated at the high density at the end of the gradient (Fig. 4A). In the presence of 500 mM KCl, γ-tubulin was stripped from these structures without affecting their density (Fig. 4, Ai), indicating that γ-tubulin is not required to stabilize them.

As in embryonic extracts, upon negative stain EM analysis we detected tetrameric objects in the SAS-6-positive 50 S fractions (Fig. 4B). Importantly, the fractions denser than 50 S contained large and elongated higher order structures (Fig. 5, Ci) harboring identical rings (n = 13) of ~25 nm in diameter (Fig. 5, C, ii and iii). The presence of these higher order ring structures in fractionated Drosophila cell extract and their absence in yeast suggests that an unknown centriolar factor is required for the organization of SAS-6 tetramers into stable higher order central tubule-like structures.

7.4 S SAS-6 Tetramers Are Intermediates in the Disassembly of Higher Order Structures—To evaluate directly whether or not the 7.4 S tetrameric structures are structural intermediates during the assembly and disassembly of higher-order structures, we took a biochemical approach. We established Drosophila cells stably expressing SAS-6-GFP-FLAG. Cell extracts were subjected to velocity sedimentation in a linear sucrose gradient of 15–60%, allowing for isolation of low density (around 7.4 S) and high density (50 S and higher) structures, as described in Fig. 4A. The low and high density pools were then separately subjected to affinity purification using affinity resins coated with FLAG antibody. We then performed velocity sedimentation of purified SAS-6 complexes obtained from both low and high density fractions (Fig. 5A). As expected, the SAS-6 complex purified from the low density pool was fractionated at 7.4 S, indicating that the low density SAS-6 complex is stable (Fig. 5Ai).

Importantly, after velocity sedimentation the purified high density SAS-6 complexes also fractionated at 7.4 S even though they were expected to fractionate above 50 S (Fig. 5Ai). The observation that 7.4 S structures result when high density (50 S and above) SAS-6 complexes are subjected to velocity sedimentation indicates that stable 7.4 S structures are constitutive structural components of less stable higher order structures.

We next used negative-stain EM to analyze the structures that fractionated at 7.4 S after velocity sedimentation of affinity-purified low density complexes. We detected three populations of objects with distinct morphology. Analysis of the objects (Fig. 5, B, i and ii) revealed the presence of tetrameric structures (Fig. 5Bi), tetrameric structures with appendages (Fig. 5Biv), and curved structures with recognizable subunits (Fig. 5Bv). These structures are similar to objects observed in native (Figs. 3C and 4B) and recombinant (Fig. 2, C and H) SAS-6 preparations.

Of significance, negative-stain EM analysis of the 7.4 S fractions obtained after velocity sedimentation of affinity-purified high density SAS-6 complexes also identified tetrameric objects (Fig. 5C, i and ii) and curved structures (Fig. 5Ci), present in roughly the same ratio as before (Fig. 5D). These data strengthen the argument that observed 7.4 S structures are indeed structural intermediates that are found after the disassembly of higher order structures.

To check the specificity of all these 7.4 S structures, we used silver-stain PAGE to analyze purity. The fractions used for EM analysis that sediment as low density complexes identified the presence of SAS-6 and a few other proteins (Fig. 5E). It is possible that these other proteins are SAS-6 interacting partners. Similar results were also obtained when we analyzed the fractions used for EM analysis that sediment as high density SAS-6 complexes (Fig. 5F).

7.4 S SAS-6 Tetramers Are Building Blocks of a Higher Order Complex—Having speculated that SAS-6 tetramers are components of high density native 50 S structures, we further repeatedly tested if the tetramers could indeed contribute to the assembly of higher order structures. To this end we mixed the purified 7.4 S structures of recombinant SAS-6 from E. coli (Fig. 2A) and SAS-6-GFP-FLAG from Drosophila cells (Fig. 5A) with low density embryonic extract, and the complex was subjected to velocity sedimentation. We found that combining SAS-6 from the two different sources resulted in the formation of complexes that are significantly denser than the individual
components alone. Although the recombinant endogenous SAS-6 complex resulted in 11.4 S structures (Fig. 6A), the SAS-6-GFP-FLAG with embryonic extract resulted in structures that are fractionated around 30 S (Fig. 6B). This is more similar to the native 50 S SAS-6 core. One possible explanation is that the SAS-6 protein is properly folded when expressed in Drosophila cells plus the presence of additional factors that are required to enhance the structural assembly. We, however, did not perform EM analysis of these complexes as they contained low amounts of specific complexes in cellular extract and because of the instability of higher order structures for EM processing. Collectively, the capacity of tetramers to form high density complexes suggests that the SAS-6 tetramers could serve as building blocks in the assembly of higher order structures that require endogenous factors present in embryonic extract for central tubule assembly.

DISCUSSION

Centriole biogenesis includes an early intermediate known as the procentriole (10). The cartwheel, a conserved centriolar scaffold, appears at the onset of procentriole assembly (31). The absence of a cartwheel leads to the formation of centrioles and cilia with disrupted symmetry (11, 19). To date, SAS-6 is one of the clearly conserved centriole proteins, which is a component of the cartwheel (11, 18, 20). However, the precise function of SAS-6 in general and in cartwheel formation remained unclear. The current study provides structural insights into the formation of a SAS-6...
structural unit with a 4-fold symmetry that could serve as a building block for the cartwheel central tubule. It remains unclear whether there is an upstream signal that defines the site of procentriole assembly and triggers SAS-6 tetramers to get assembled. ZYG-1 and PLK4 kinases are likely components of the upstream signaling cascade that induces centriole nucleation (21, 32). We have recently shown that the protein “Asterless” (Asl) is a PCM protein critical for centriole formation and functions early in the centriole duplication process (28). It would be interesting to further delineate the place of SAS-6, ZYG-1/PLK4, and Asl in the assembly of the central tubule and subsequent procentriole formation.

Importantly, in Chlamydomonas (11), Drosophila (19), and Tetrahymena (20), outer centriole components do appear in the absence of SAS-6, and in both Chlamydomonas and Drosophila the 9-fold symmetry is disrupted. The fact that outer centriole components, such as the microtubules, can assemble independently of the cartwheel suggests that the primary role of the cartwheel is to generate symmetry. It should be noted, however, that structural studies in C. elegans did not find a cartwheel with a central tubule. Instead, a central tube measuring 60 nm is connected directly to the microtubules (21). In this system SAS-6 physically interacts with SAS-5 (16), a protein that is not conserved in other organisms that possess centrioles. Both SAS-6 and SAS-5 are required for the formation of the

**FIGURE 5.** 7.4 S SAS-6 structures are stable components of higher order structures. A, velocity sedimentation of FLAG tag affinity-purified low density (i) and high density (ii) SAS-6 complexes is shown. Both complexes fractionate around 7.4 S, indicating that the 7.4 S structures are stable structural intermediates that remain intact after the disassembly of higher order structures. B, negative-stain EM of low density SAS-6 complexes fractionated at 7.4 S (boxed fraction in Ai) identifies tetrameric objects (iii), tetrameric objects with appendages (iv), and subunit-rich curved structures (v). B, i and ii, show the particles in a wide field. C, negative-stain EM of high density SAS-6 complexes fractionated at 7.4 S (boxed fraction in Aii) also identifies tetrameric objects (ii) and curved structures (iii). C, i shows the particles in a wide field. D, shown is distribution of particles in a 0.45-μm field (n = 20) after sedimentation of low (7.4 S) and high density (50 S) SAS-6 complexes. E, shown are a silver stain (i) and Western blot of the boxed fraction from Ai that was used for EM analysis. The blot was probed with anti-FLAG (ii) and anti-SAS-6 (iii). FLAG, fraction containing affinity-purified SAS-6 complex; Control, affinity-purified cell extracts obtained from cells stably expressing SAS-6-GFP. Note that anti-FLAG (ii) recognizes SAS-GFP-FLAG, whereas anti-SAS-6 (iii) recognizes both the fusion protein and the endogenous SAS-6. F, shown are a silver stain (i) and Western blot of the boxed fraction from Aii that was used for negative-stain EM analysis. The blot was probed with anti-FLAG (ii) and anti-SAS-6 (iii).
order structures (Fig. 5A). Importantly, the ability of SAS-6 tetramers to form higher order structures in vitro when combined with embryonic extract suggest that the SAS-6 tetramers serve as stable intermediates in assembling higher order structures. This indicates that in addition to SAS-6 tetramers, central tubule assembly also requires other unknown factors present in vivo.

The finding that the abundance of SAS-6 present in the cell exist as tetramers (Fig. 3A and supplemental Fig. S3) that tetramers result from disassembly of SAS-6 higher-order structures (Fig. 5A), and that tetramers assemble into higher order structures (Fig. 6) suggest that SAS-6 tetramers and additional factors are required to assemble into a higher order complex. Therefore, future biochemical and in vitro reconstitution studies should be able to uncover the factors that are essential for the assembly and stability of the central tubule.

Previously, it was proposed that nonameric rings of SAS-6 form the central tubule with one subunit thick (17). Future structural studies are required to test this model. Our studies show that SAS-6 forms stable tetramers, and therefore, the central tubule may be made of tetramers. This can be tested in the future by generating mutations in SAS-6 that prevent the formation of tetramers and lead to defects in central tubule. These studies will require the identification of amino acids that are found at the interphase between SAS-6 monomers.

Theoretically, tetramers can be organized into the central tubule at least in three ways. (i) A ring of tetramers where each tetramer is placed perpendicular to the axis of the central tubule is one possibility. In this case, the central tubule wall in the cross-section would be of two subunits thick. Precise measuring of the width of the central tubule wall after fine structural studies would test this model. (ii) A ring of tetramers that faces the central tubule axis resulting in a one-subunit thick central tubule wall is another possibility. This organization cannot be fit into a 9-fold symmetry as circumference of 9 tetramers would be larger than that of the central tubule; given that each tetramer has a 12-nm in diameter, any closed disk would have a perimeter of at least 108 nm (12 nm × 9). From the experimental data, we have observed that the central tubule has a perimeter that can be estimated to be between 79 nm (π × 25 nm) and 94 nm (π × 30 nm) (considering the diameter of the central tubule is 25–30 nm), which is less than the minimum perimeter.
required for a stack of disks (108 nm). (iii) A helic of tetramers is a third possibility. If a 9-fold symmetrical central tubule one subunit thick is assumed, then the central tubule would be organized into 4.5 tetramers per turn so that one turn corresponds to a nine-subunit repeat. Future detailed structural studies are required to elucidate the internal organization of SAS-6 in the central tubule.

It was also proposed that SAS-6 organizes the procentriole by orienting and holding a preassembled structure that corresponds to 1/9 of the centriole, which are named as enatosomes (19, 22). It is possible that SAS-6 tetramers and their assembly to higher order structures are the basic structural units mediating the organization of enatosomes.

It has been decades since EM was first used to reveal the centriole structure, and multiple RNAi and genetic studies have indicated a role for SAS-6 early in centriole formation (11, 15–17, 19, 21), but technical challenges have prevented analysis of the central tubule and its internal organization. The results from the current study suggest that SAS-6 self-assembles into tetramers that serve as building blocks during assembly of a cartwheel central tubule.

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