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Cep120 promotes microtubule formation through a unique tubulin binding C2 domain☆

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ABSTRACT

Centrioles are microtubule-based structures that play essential roles in cell division and cilia biogenesis. Cep120 is an important protein for correct centriole formation and mutations in the Cep120 gene cause severe human diseases like Joubert syndrome and complex ciliopathies. Here, we show that Cep120 contains three consecutive C2 domains that are followed by a coiled-coil dimerization domain. Surprisingly, unlike the classical C2 domains, all three Cep120 C2 domains lack calcium- and phospholipid-binding activities. However, biophysical and biochemical assays revealed that the N-terminal Cep120 C2 domain (C2A) binds to both tubulin and microtubules, and promotes microtubule formation. Structural analyses coupled with mutagenesis identified a highly conserved, positively charged residue patch on the surface of Cep120 C2A, which mediates the interaction with tubulin and microtubules. Together, our results establish Cep120 C2A as a unique microtubule-binding domain. They further provide insights into the molecular mechanism of Cep120 during centriole biogenesis.

1. Introduction

Centrioles are microtubule-based structures that organize the mitotic spindle during cell division and are used as templates for the formation of cilia and flagella (reviewed in Bornens, 2012). The centriole biogenesis process involves the spatially and temporally regulated recruitment of key protein components. At the start of the G1/S transition of the cell cycle, daughter centrioles emerge close to the mother centriole and grow during the ensuing cell cycle to reach their final length in the G2 phase (reviewed in Cunha-Ferreira et al., 2009; Firat-Karalar and Stearns, 2014; Gönczy, 2012; Jana et al., 2014; Lattao et al., 2017; Nigg and Stearns, 2011). The centriole duplication process is initiated with the assembly of a central ninefold symmetric "cartwheel" structure adjacent to the mother centriole (reviewed in Cunha-Ferreira et al., 2009; Firat-Karalar and Stearns, 2014; Gönczy, 2012; Jana et al., 2014; Lattao et al., 2017; Nigg and Stearns, 2011). Subsequently, an outer wall made up of nine symmetrically arranged microtubule triplets is formed around the cartwheel to generate the cylindrical centriole that displays a length and a diameter of approximatively 500 nm and 250 nm, respectively (Winey and O'Toole, 2014).

Centriolar microtubules display unique growth dynamics and post-translational modifications, features that are tightly controlled in cells. Remarkably, they grow ~4 orders of magnitude slower than cytoplasmic microtubules and are highly stable and non-dynamic (Kinoshita et al., 2001). As a consequence, the length of the centriole is tightly controlled as well and although a number of proteins are known to participate in the centriole elongation process, yet the molecular mechanism of centriole length control is poorly understood. An emerging common feature characterizing proteins involved in centriole length control is the presence of microtubule/tubulin-binding domains that could affect the dynamics and/or structure of centriolar microtubules and thus centriole length (Comartin et al., 2013; Lin et al., 2013; Schmidt et al., 2009; Sharma et al., 2016).

A number of centriolar proteins are implicated in controlling centriole length including CPAP, Cep120, Centrobin, SPICE1, and CP110 (Comartin et al., 2013; Gudi et al., 2011; Lin et al., 2013; Schmidt et al., 2009). Our previous work led to the identification of human CPAP as a microtubule regulator that imparts slow and processive centriolar microtubule growth (Sharma et al., 2016). However, the centriole

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elongating activity of CPAP in cells is dependent on the presence of the centriolar protein Cep120 (Comartin et al., 2013; Lin et al., 2013). Similarly to CPAP, Cep120 is required for centriole duplication and its overexpression leads to overly elongated centrioles (Comartin et al., 2013; Lin et al., 2013). Conversely and again similar to CPAP, Cep120 depletion strongly inhibits procentriole formation by inhibiting the centriole biogenesis process at an early stage (Mahjoub et al., 2010). Mutations in the Cep120 gene leads to gross centriole defects (Lin et al., 2013) and to severe human diseases including Jeune asphyxiating thoracic dystrophy (Shaheen et al., 2015) and Joubert syndrome (Roosing et al., 2016). Although it was suggested that Cep120 co-localizes with microtubules in cells via its N-terminal region (Lin et al., 2013), a molecular understanding of its microtubule-binding activity and the mechanism by which Cep120 promotes centriole elongation is currently lacking.

In this study, we show that human Cep120 contains three C2 domains in its N-terminal region, which are followed by a coiled-coil dimerization domain. None of the three C2 domains display the C2 characteristic calcium- or lipid-binding properties; however, we found that the N-terminal C2 domain (C2A) constitutes a novel tubulin/microtubule-binding domain. Using high resolution structural information, we mapped the tubulin-binding site on the surface of Cep120 C2A, which allowed us to highlight the distinguishing features of a tubulin/microtubule-binding C2 domain. We further show that the tubulin- and microtubule-binding activity of Cep120 C2A promotes microtubule assembly.

2. Materials and methods

2.1. Cloning and protein production

The human Cep120 constructs C2A (residues 1–151), C2B (residues 160–340), C2C (residues 450–610) and coiled coil (residues 707–925) were amplified from a human cDNA library and cloned into a pET-based bacterial expression vector containing either N-terminal thioredoxin-6xHis or 6xHis cleavable tag using the restriction free positive selection method (Olieric et al., 2010). The Cep120 C2A plasmid was used as a template for site directed mutagenesis (R69A, R72A, K76A, R100A) Mutations were introduced using the QuickChange mutagenesis approach (Stratagene). All clones were verified by sequencing.

Human CPAP cc (residues 898–1053) was amplified from form a HsCPAP clone (Kitagawa et al., 2011) and inserted into a pET-based bacterial expression vector containing a N-terminal 6xHis cleavable tag using the restriction free positive selection method.

Protein production was performed in the *E. coli* expression strain Bl21(DE3) in LB broth by inducing with 0.5 mM IPTG at an OD_{600} of 0.4 to 0.6 over-night at 20 °C. Cells were harvested by centrifugation at 4 °C, 3500*g* for 15 min and lysed by sonication in a buffer containing 50 mM HEPES, pH 8.0, 500 mM NaCl, 10 mM Imidazole, 10% Glycerol, 2 mM β-mercaptoethanol, and proteases inhibitors (Roche). The crude extracts were cleared by centrifugation at 20,000*g* for 20 min and the supernatants were filtered through a 0.4 μm filter before purification.

Protein purification was performed by immobilized metal-affinity chromatography (IMAC) on HisTrap HP Ni^{2+} Sepharose columns (GE Healthcare) at 4 °C according to the manufacturer's instructions. The thioredoxin-6xHis or 6xHis tags were cleaved overnight by 3C protease during dialysis against lysis buffer (without proteases inhibitors). Cleaved samples were reapplied onto an IMAC column to separate the cleaved products from the respective tags and potentially uncleaved protein. Processed proteins were concentrated and further purified on a HiLoad Superdex 75 16/60 size exclusion chromatography column (GE Healthcare) equilibrated in 20 mM Tris HCl, pH 7.5, 150 mM NaCl, 2 mM DTT. Protein fractions were analyzed by Coomasie stained SDS-PAGE. Fractions containing the target protein were pooled and concentrated by ultrafiltration. Protein concentrations were estimated by UV at 280 nm and the pure proteins were aliquoted, flash frozen in liquid nitrogen and stored at −80 °C.

Noteworthy, C2B required the use of *E. coli* BL21-CodonPlus (DE3)-RIPL competent cells (Agilent) to be expressed in reasonable amounts. Besides, the coiled-coil proteins were kept in high salt conditions (500 mM NaCl) during all purification steps.

2.2. Bioinformatics analysis

The analysis of Cep120 C2A amino acid conservation across evolution was performed using ConSurf (Ashkenazy et al., 2016). The Cep120 C2A structure (PDB ID 4ICW, chain A) was used as input. We performed a search using HMMER against the UNIREF90 database with an E-value cut-off of 0.0001, minimal% ID of 35%, maximal% ID of 95% and 2 iterations. Out of 150 sequence hits found, 138 were aligned using MAFFT (Katoh et al., 2017). The resulting multiple sequence alignment was subsequently used for ConSurf calculations using the Bayesian-based estimate of the evolutionary conservation of amino acids (Mayrose et al., 2004).

2.3. Circular dichroism (CD) spectroscopy

CD spectroscopy was performed at 20 °C using a Chirascan spectropolarimeter (Applied Photophysics) with a 1 mm path-length cell. Proteins were prepared in PBS buffer (20 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) at 0.15 mg/ml. Spectra were recorded from 260 to 195 nm at an interval of 1 nm and were repeated three times. For each spectrum, the three scans were averaged and subtracted by the averaged spectrum of the PBS buffer.

Thermal stability was assessed using a 1 °C/min temperature ramp between 10 °C and 90 °C and measured by circular dichroism at 222 nm.

2.4. Size exclusion chromatography followed by multi-angle light scattering (SEC-MALS)

SEC-MALS experiments were performed in high salt conditions (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 2 mM DTT) using a S-200 30/300 analytical size exclusion chromatography column connected in-line to a miniDAWN TREOS light scattering and Optilab T-rEX refractive index detector (Wyatt Technology). Measurements were carried out at 20 °C and the Cep120_CC sample was used at 4 mg/ml (injected volume: 30 μL). Data analysis was performed using the software package provided by the instrument.

2.5. Crystallization and structure solution

Cep120 C2A_K76A and C2C domain protein crystals were obtained by the sitting drop vapor diffusion method in a condition containing 20% PEG 3350 and 0.2 M NaSCN for C2A_K76A, and in the Morpheus A10 condition for Cep120 C2C (0.03 M magnesium chloride hexahydrate; 0.03 M calcium chloride dehydrate, 0.1 M Tris(Base)/BICINE pH 8.5, 20% v/v Ethylene glycol and 10% w/v PEG 8000). For producing heavy atom derivatives of C2C crystals, 1 μL of 100 mM Samarium (III) acetate hydrate was added to a 1 μL drop containing C2C crystals for 10 min. Crystals were subsequently flash frozen in liquid nitrogen. X-ray diffraction experiments were performed at the X06DA beamline of the Swiss Light Source, Switzerland.

A 360° native dataset at 100 K was collected at 1 Å wavelength for Cep120 C2A_K76 crystals. Data were processed using XDS and scaled using XSCALE (Kabsch, 2010). The structure was solved by the molecular replacement method using wild type the Cep120 C2A structure as a model (PDB ID 4ICW) using Phaser in PHENIX (Adams et al., 2011). The Cep120 C2C structure was solved by single wavelength anomalous dispersion (SAD) method. A 360° dataset was collected at 1.85 Å wavelength at 100 K. Data were processed using XDS and scaled using XSCALE. Substructure determination and initial phasing were performed using AutoSol in PHENIX. For both the structures, further model