Structure Report

Localization and orientation of the γ-Tubulin Small Complex components using protein tags as labels for single particle EM

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γ-Tubulin Small Complex (γ-TuSC) is the universally-conserved complex in eukaryotes that contains the microtubule (MT) nucleating protein: γ-tubulin. γ-TuSC is a heterotetramer with two copies of γ-tubulin and one copy each of Spc98p and Spc97p. Previously, the structure of γ-TuSC was determined by single particle electron microscopy (EM) at 25 Å resolution. γ-TuSC is Y-shaped with a single flexible arm that could be the key to regulating MT nucleation. EM gold labeling revealed the locations of γ-tubulin at the top of the Y. In vivo Fluorescence Resonance Energy Transfer (FRET) suggested the relative orientations of Spc98p and Spc97p but did not distinguish which large subunit formed the flexible arm. Here, using fluorescent proteins as covalently attached tags, we used class averages and 3-D random conical tilt reconstructions to confirm the in vivo FRET results, clearly demonstrating that the Spc98p/97p C-termini interact directly with γ-tubulin. Most significantly we have determined that the flexible arm belongs to Spc98p and our data also suggests that the N-termini of Spc98p and Spc97p are crossed. More generally, our results confirm that despite their small size, covalently-attached fluorescent proteins perform well as subunit labels in single particle EM.

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Microtubule Organizing Centers (MTOCs) organize the spatial and temporal patterns of microtubules by controlling their nucleation. Although the morphology of MTOCs varies greatly among different phyla, all eukaryotes utilize γ-tubulin (Tub4p in yeast) to nucleate microtubules (Wiese and Zheng, 2006). γ-Tubulin is found in the 300 kDa γ-Tubulin Small Complex (γ-TuSC), a highly conserved heterotetramer with two copies of γ-tubulin and one each of Spc98p and Spc97p. While both purified γ-tubulin and γ-TuRC are potent MT nucleators, nucleating activity for γ-TuSC is barely detectable even though it is the only γ-tubulin complex within the highly functional Saccharomyces cerevisiae Spindle Pole Body (SPB) (Gunawardane et al., 2000; Vinh et al., 2002).

Recently, we determined the structure of the S. cerevisiae γ-TuSC at moderate resolution using single particle electron microscopy (Kollman et al., 2008). The structure is Y-shaped and γ-tubulin is located in lobes at the tips of the two arms (Fig. 1A). One of the arms adopts different conformations due to a limited rotation about its base. Significantly, in all of the observed conformations, the γ-tubulins are held in nucleation incompatible orientations. We have suggested that this configuration may provide a way of regulating γ-TuSC activity, and that further movement of the flexibly attached arm is required to bring the two γ-tubulins together in a nucleation compatible orientation (Kollman et al., 2008).

The location of γ-tubulin within γ-TuSC was determined by directly labeling His-tagged γ-tubulin with Ni-NTA nanogold. All attempts to label Spc98p and Spc97p in the same way using either 6×His or 12×His tags were unsuccessful due to very low labeling yields. Although the relative orientations of Spc98p and Spc97p were suggested by in vivo Fluorescence Resonance Energy Transfer (FRET) of dual-labeled complexes (Fig. 1B), these experiments were unable to distinguish Spc98p and Spc97p in the EM structure. Further, because the FRET experiments examined γ-TuSCs assembled at the SPB, there was the potential for confusing intra- and intercomplex FRET signals. Thus, we sought a more direct way to determine the locations and orientations of the γ-TuSC components.

Previous reports have shown the benefits of using small proteins as covalent labels for localization in EM. In several cases two-dimensional averages were used to localize tagged components (Alcid and Jurica, 2008; Bertin et al., 2008; Li et al., 2008;...
TuSC:Spc98pCFP, we were unable to locate the tag in the raw micrographs and in the 2-D class averages, possibly because the tag localizes on top or behind the complex.

Overall, our data confirm the locations of the N- and C-termini of Spc98p and Spc97p inferred from our previous in vivo FRET results (Kollman et al., 2008). In addition to confirming the Spc98p/97p subunit polarity, new structural features have been revealed. Importantly, we have shown that Spc97p forms the rigid arm of γ-TuSC, while Spc98p contains the flexible arm. Unexpectedly, the N- and C-terminal tags on Spc97p are on opposite sides of γ-TuSC. The tag at the N-terminus of Spc97p is more localized and favors a set of positions diagonally opposite to its C-terminus. We suggest that this indicates that the Spc97p and Spc98p are crossed, resulting in a crossed N-termini. Additionally, the N-terminal region of Spc98p appears to be inherently flexible, a finding consistent with sequence alignments of Spc97p and Spc98p which show Spc98p to have an additional ~150 amino acids at its N-terminus. In our previous study (Kollman et al., 2008) we suggested that movement of the flexibly attached arm regulates γ-TuSC activity. Our 2-D averages and 3-D reconstructions clearly identify Spc98p as the subunit containing the flexible arm. The identification of the flexible subunit will help focus future studies involving mutagenesis and/or post-translational modifications on Spc98p to help understand the mechanism of regulation of MT nucleation by γ-TuSC.

Our data also confirms the termini localization indicated by our previous in vivo FRET study and further suggests that the N-termini are crossed. Together this allows us to update our model of γ-TuSC from the one seen in Fig. 1B to the one in Fig. 4. Finally, our 2D averages and 3-D reconstructions suggest that covalently-attached fluorescent protein tags are ideally suited to EM localization even at moderate resolutions.

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