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Cryo-electron tomography and 3-D analysis of the intact flagellum in *Trypanosoma brucei*

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ABSTRACT

Trypanosoma brucei is a uni-cellular protist that causes African sleeping sickness. These parasites have a flagellum that is attached to the cell body and is indispensible for its motility. The flagellum consists of a canonical 9 + 2 axoneme and a paraflagellar rod (PFR), an intricate tripartite, fibrous structure that is connected to the axoneme. In this paper we describe results from cryo-electron tomography of unperturbed flagella. This method revealed novel structures that are likely involved in attaching the flagellum to the cell. We also show the first cryo-electron tomographic images of a basal body in situ, revealing electron dense structures inside its triplet microtubules. Sub-tomogram averaging of the PFR revealed that its distal region is organized as an orthorhombic crystal.

1. Introduction

Trypanosoma brucei (*T. brucei*) is a single cell parasite that causes the African sleeping sickness (human African trypanosomiasis), endemic in sub-Saharan Africa. *T. brucei* is transmitted by the tsetse fly (*Glossina* spp.). Throughout 2009, an estimated 30.000 humans were infected (WHO, 2010). Currently, available treatments have severe side effects, and resistance against these drugs is increasing (Pyana et al., 2011; Wilkinson and Kelly, 2009). Detailed structural and cell biological studies of these parasites might reveal important novel drug targets. Furthermore, *T. brucei* belongs to the kinetoplastids, a group of protozoa that is evolutionary distant from humans, which renders it an excellent organism to study cellular evolution in eukaryotes.

T. brucei cells maintain their characteristic slender shape due to the sub-pellicular microtubule array, an arrangement of mostly parallel microtubules located directly under the cell membrane (Gull, 2003; Robinson et al., 1995). The cells are motile due to a single flagellum that grows from a basal body situated below a secretory organelle called the flagellar pocket, which is located near the cell's posterior (Fig. 1A) (Lacomble et al., 2009; Webster, 1989). The flagellum is attached to the cell body over its entire length, except for a short segment that protrudes beyond the cell's anterior end. This attachment is important because silencing of the *fla1* gene by RNAi, which detaches the flagellum, decreases cellular viability and causes failure in cytokinesis (LaCount et al., 2002; Nozaki et al., 1996). Attachment is accomplished by a "flagellum attachment zone" (FAZ), a specialization of the cell's cortex positioned on the inside of the plasma membrane just opposite the flagellum. This zone includes a gap in the sub-pellicular microtubule array that contains the so-called "microtubule quartet" (MTQ), a sub-set of the subpellicular MTs that are nucleated at the base of the flagellar pocket, and are anti-parallel to the other MTs (Fig. 1B) (Sherwin and Gull, 1989; Webster, 1989). This gap also contains the "FAZ filament", an electron dense fiber that runs parallel to the MTQ, and the macula adherens, junctional complexes between cell body and the flagellum (Vickerman, 1969). Nonetheless, the ways in which these cytoplasmic specializations might bind the flagellum to the cell surface have remained obscure. The images obtained in this study reveal novel structures that may be important for flagellar attachment.

The flagellum in *T. brucei* comprises two major structures, the conserved 9 + 2 axoneme (9 doublet microtubules and two central pair microtubules) and the more kinetoplastid specific paraflagellar rod (PFR; (Vaughan, 2010) Fig. 1C). The PFR is a paracrystalline fiber that is arranged parallel to the axoneme along the extracellular part of the flagellum (de Souza and Souto-Padron, 1980; Portman and Gull, 2010; Vickerman, 1962). The PFR constitutes a platform for metabolic enzymes and signaling factors (Oberholzer et al., 2007; Pullen et al., 2004; Ridgley et al., 2000). It is essential for motility (Bastin et al., 1998; Santrich et al., 1997) and thus, cell viability in the bloodstream form that causes the human infection (Broadhead et al., 2006; Griffiths et al., 2007).

Previous structural studies on the PFR have revealed three structurally distinct regions: the proximal, intermediate and distal

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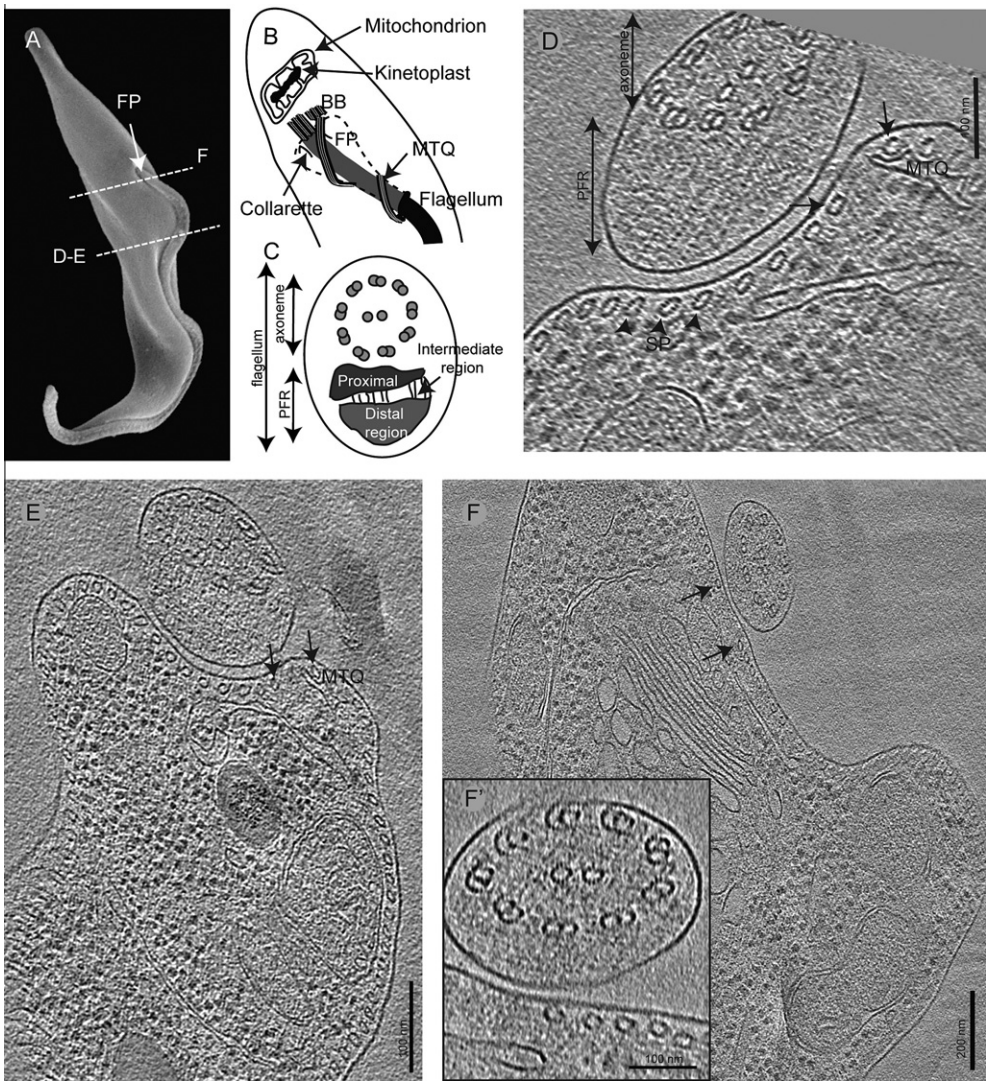

Fig.1. Vitreous sections of the flagellum in *Trypanosoma brucei*. (A) A scanning electron micrograph shows *T. brucei*'s elongated cell shape and attached flagellum that grows out of the flagella pocket (FP) (with permission from Lacomble et al. (2009)). (B) A cross-sectional cartoon of the cell's posterior end, with the FP around which the microtubule quartet (MTQ) wraps. At the proximal end of the flagellum, the basal bodies (BB) with the associated kinetoplast-containing mitochondrion. At the base of the flagellum inside the FP is the filamentous collarette structure. (C) The flagellum consists of the axoneme and the paraflagellar rod (PFR). (D–F) Slices from cryo-tomograms of frozen hydrated sections, showing the axoneme, PFR and sub-pellicular MTs (arrowheads; SP). Note the distance between flagellar and cellular membranes. (F) The PFR in this slice is thinner than in others, suggesting that this section was cut where the flagellum emerged from the flagellar pocket, the site of the PFR proximal end. This interpretation is supported both by the large gap in the sub-pellicular microtubules (arrows) underlying the membrane and the presence of the Golgi apparatus, which are also found in this region. (F′) zoomed in image of the flagellum of panel F. Thickness of slice in Z: (C) 50 nm (D) 1 nm and (E) 30 nm.

domains (Farina et al., 1986). They also showed that the PFR has links to axonemal doublet microtubules numbered 4–7 and to the flagellar attachment zone (de Souza and Souto-Padron, 1980; Koyfman et al., 2011; Sherwin and Gull, 1989). The PFR distal region consists of periodically arranged crossing filaments (de Souza and Souto-Padron, 1980; Rocha et al., 2010; Sant'Anna et al., 2005), that change their angles relative to one another, depending on the extent of flagellar bending in that region (Koyfman et al., 2011; Rocha et al., 2010).

Here we present the first structural 3D analysis of the intact *T. brucei* flagellum by cryo-electron tomography (cryo-ET). We have investigated both vitreous sections and whole, plunge-frozen cells, each of which provides structural preservation that is the best currently possible (Al-Amoudi et al., 2004; Hoenger and Bouchet-Marquis, 2011; Leis et al., 2009). Studying cells in this near-to-native state has provided insight at the macromolecular level into the flagellum attachment and the structure of the PFR. The 3D volumes obtained from cryo-ET were further analyzed by averaging sub-tomogram volumes from repetitive and structurally identical areas. The resulting increase in signal-to-noise ratio has helped to identify repeating structures in the distal region of the PFR.

2. Material and methods

2.1. Cell culture and sample preparation

T. brucei brucei, procyclic form (Lister 429), were maintained in logarithmic growth, using SDM79 medium with 20% FCS at 28 °C, as in (Höög et al., 2010). To prepare cells for whole cell cryo-ET, 4 μl of cells at a density of $\sim 3 \times 10^6$ cells/ml were pipetted onto a glow-discharged holy carbon grid (4×4 μm holes; Quantifoil, Jena, Germany) and mixed with 1 μl of concentrated 10 nm colloidal gold particles (Ted Pella, Redding, CA, USA), blotted and plunged into liquid ethane.

For the preparation of vitreous sections, cells were concentrated by centrifugation for 2 min at 600*g*, and then resuspended in 20%