Basal body movements orchestrate membrane organelle division and cell morphogenesis in *Trypanosoma brucei*

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Summary

The defined shape and single-copy organelles of *Trypanosoma brucei* mean that it provides an excellent model in which to study how duplication and segregation of organelles is interfaced with morphogenesis of overall cell shape and form. The centriole or basal body of eukaryotic cells is often seen to be at the centre of such processes. We have used a combination of electron microscopy and electron tomography techniques to provide a detailed three-dimensional view of duplication of the basal body in trypanosomes. We show that the basal body duplication and maturation cycle exerts an influence on the intimately associated flagellar pocket membrane system that is the portal for secretion and uptake from this cell. At the start of the cell cycle, a probasal body is positioned anterior to the basal body of the existing flagellum. At the G1–S transition, the probasal body matures, elongates and invades the pre-existing flagellar pocket to form the new flagellar axoneme. The new basal body undergoes a spectacular anti-clockwise rotation around the old flagellum, while its short new axoneme is associated with the pre-existing flagellar pocket. This rotation and subsequent posterior movements results in division of the flagellar pocket and ultimately sets parameters for subsequent daughter cell morphogenesis.

Key words: Flagellar pocket, Flagellum, Centriole, Microtubule, Basal body, Electron tomography, Cytoskeleton, Trypanosome, Secretion

Introduction

An intrinsic property exhibited by eukaryotic cells is the ability to duplicate and segregate their organelles with high fidelity (Lowe and Barr, 2007). Often this process produces essentially identical progeny via a symmetrical division. At other times, the process results in two non-identical daughter cells. These duplicative phenomena are intrinsically linked to morphogenesis of cell shape, form and polarity in the daughter cells. Classic examples of asymmetrical divisions are found in the early development of some embryos, such as in *Caenorhabditis elegans* (Hyman and White, 1987), whereas the production of identical progeny is characteristic of tissue growth and is often exquisitely seen in the protists (Gull et al., 2004). In the unicellular eukaryotes, the fidelity of the processes whereby the cytoskeletal and membrane components of the cell are duplicated is so well orchestrated and linked to cell morphogenesis that the resulting cells and populations can be identified purely on morphological grounds (Aly et al., 2009; de Souza, 2009; Svard et al., 2003).

In many protests, the cytoplasmic organisation demonstrates a high order of complexity, with discrete microtubule bundles and rootlets, as well as filamentous and fibrous structures (Beisson and Jerka-Dziadosz, 1999; Gull, 1999). In addition, protists display very precise localisations of membrane organelles whose position is orchestrated, at least in part, by the highly ordered cytoskeleton (Becker and Melkonian, 1996). In many cases, we have good overall descriptions of the basic cellular architecture, but the order of events and the mechanisms by which these defined structures

are duplicated and then positioned to allow the division process to result in identical cells is rather unclear. Understanding the linkage between centriole/basal body duplication, associated cytoskeletal remodelling and plasma membrane differentiations for sensory or secretory functions is becoming important. The influence of the centriole/basal body cycle on associated membranes is a central issue in understanding ciliogenesis, primary cilia, the immunological synapse in cytotoxic T lymphocytes and asymmetrical division in stem cells (Dawe et al., 2007; Stinchcombe et al., 2006; Yamashita, 2009).

The African trypanosome *Trypanosoma brucei* is typical of most protistan cells in having a very highly ordered cell shape and form, together with a precisely structured arrangement of cytoplasmic organelles. The procyclic trypanosome cell, which lives in the insect vector mid-gut, is vermiform in shape, with a more tapered anterior end that defines the normal direction of movement. The single flagellum emerges from an invagination of the plasma membrane called the flagellar pocket and is attached in a left-handed helical pattern along the full remaining length of the cell (Gull, 1999; Robinson et al., 1995; Sherwin and Gull, 1989). This attachment is mediated by a defined flagellum-attachment zone consisting of a filament structure, a set of rootlet microtubules termed the microtubule quartet and a set of macula adherens junctions (Lacomble et al., 2009; Sherwin and Gull, 1989; Vaughan et al., 2008).

Inheritance of the left-handed helical attachment is influenced by a unique transmembrane mobile junction that forms inside the

flagellar pocket at the earliest stage of new flagellum formation. The tip of the new flagellum is connected via a transmembrane junction (the flagella connector) to the lateral aspect of the old flagellum (Briggs et al., 2004; Moreira-Leite et al., 2001). This allows inheritance of pattern and form in a cytotactic manner (Beisson, 2008; Beisson and Sonneborn, 1965; Sonneborn, 1964). Internally, there are individual copies of the Golgi complex and mitochondrion, whose mass of DNA is configured into a structured kinetoplast. This kinetoplast is always positioned close to the proximal end of the basal body and probasal body complex, a phenomenon mediated by the series of filaments in the tripartite attachment complex (TAC) that links the kinetoplast to the basal bodies (Ogbadoyi et al., 2003). This facilitates not only kinetoplast positioning, but also segregation of the replicated mitochondrial genome (Robinson and Gull, 1991).

Replication and segregation of these organelles and structures takes place within a cell cycle characterised by a periodic S phase for both nuclear and kinetoplast DNA (Sherwin and Gull, 1989; Woodward and Gull, 1990). We have previously described the 3D architecture of the basal body and flagellar pocket complex using a combination of standard electron microscopy (EM) and electron tomography (Lacomble et al., 2009). The order and timings of basal body maturation, probasal body genesis and flagellum formation have been documented by our laboratory (Bastin et al., 1999; Kohl et al., 1999; Sherwin and Gull, 1989; Woodward and Gull, 1990). However, it is still unclear how these processes operate spatially in relation to each other and how the cytoskeletal events are coordinated with membrane morphogenesis to allow development of a new flagellum and flagellar pocket. Also, although the timings and order of some events are clear, there are conundrums involved in explaining how these are orchestrated in the cell cycle.

These inheritance patterns require explanation in 3D, and hence we have used EM tomography to investigate the early stages of the cell cycle of procyclic and bloodstream trypanosomes. We selected cells at G1–S stages and built tomograms and 3D models to understand the morphogenesis of the flagellum and flagellar pocket. In addition, we used a large library of micrographs of random thin sections and negatively stained, whole-mount cytoskeletons throughout the cell cycle. These approaches have revealed that the probasal body, which is initially positioned on one side of the basal body of the existing flagellum, matures, elongates and forms the new flagellar axoneme. When the new flagellar axoneme has initiated extension, its basal body undergoes a dramatic circumferential, rotational journey around the old flagellum, in addition to a posteriorly directed lateral movement. The combination of these two movements forms a new flagellar pocket by division of the existing one. Specific microtubule arrays perform distinct organisational functions during this process in relation to membrane folding and positioning.

Results
Pre-mitotic repositioning of the new basal body
Negatively stained whole mounts of trypanosome cytoskeletons have been very useful for examining key events of cell morphogenesis; in these, it is possible to detect a variety of cytoskeletal features as small as a probasal body within the context of the whole cell (Sherwin and Gull, 1989). Cytoskeletons from a cell at the beginning of the cell cycle contain only one flagellum (Fig. 1A), whereas when the cell approaches mitosis, it contains two (Fig. 1B). Even at this low magnification, however, a
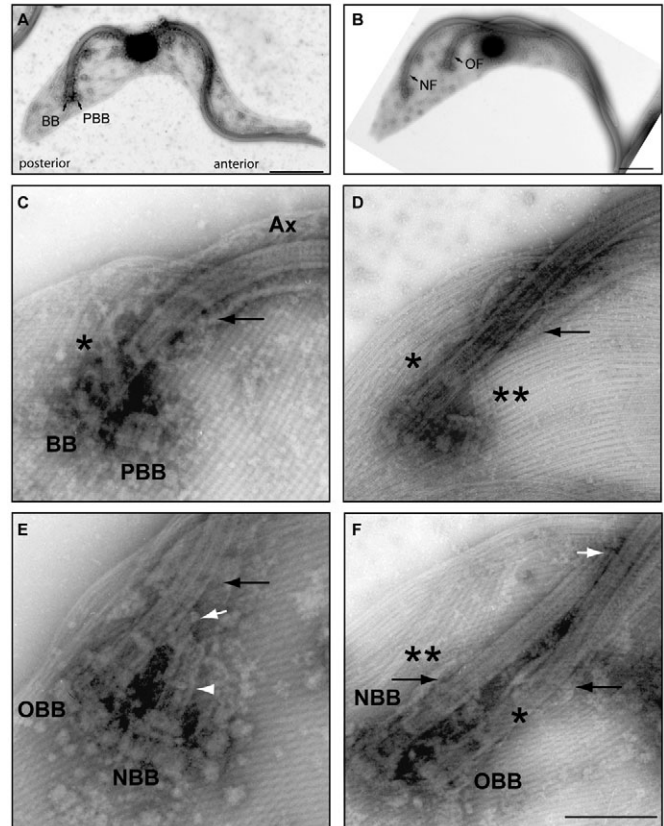

Fig. 1. Repositioning of the basal body. Negatively stained whole-mount cytoskeletons illustrate a repositioning of the new basal body. (**A**) A G1 cell with a single flagellum nucleated from the basal body (BB). The probasal body (PBB) is located anterior to the basal body. (**B**) Pre-mitotic cell with two flagella. The new flagellum is positioned posterior to the old flagellum. (**C**) A higher magnification view of a G1 cell to illustrate the positioning of the PBB relative to the BB, the axoneme (Ax) and collar (arrow). The microtubule quartet (labelled *) initiates between the BB and PBB. (**D**) The first marker of initiation of cytoskeletal morphogenesis is a new microtubule quartet (labelled **). (**E**) The probasal body has now matured to the point that it can form a new flagellum. There is an extension of the transition zone (white arrowhead), and a very short axoneme distal to the transition zone, yet the connection of the new flagellum to the old via the flagella connector (white arrow) has already been established The new flagellum basal body (NBB) is positioned anterior to the old flagellum basal body (OBB). (**F**) The new flagellum basal body (NBB) is now positioned posterior to the old flagellum basal body (OBB), even though the new flagellum is still short (white arrow). Scale bars: 2 μm (A,B), 500 nm (C–F).

conundrum is apparent; the new flagellum extends from the probasal body formed in the previous cell cycle, and this new flagellum (NF) is always located at the posterior end of the cell (Fig. 1B). One might therefore imagine that the probasal bodies would be formed on the posterior side of their respective basal bodies and thus be in the correct position for subsequent elongation as the new flagellum in the next cell cycle. However, examination of Fig. 1A,B shows this not to be the case. The probasal bodies (PBB) are formed on the anterior side of their respective basal bodies. Therefore, a movement of the probasal body must occur during flagellum and cell morphogenesis. To gain insight into the timing of this transition, we analysed a collection of over 500 micrographs of appropriate cell cycle stages and dissected the

order of appearance of cytoskeletal structures throughout cell morphogenesis.

The order of events is illustrated more precisely in Fig. 1C–F. High-magnification views of negatively stained whole-mount cytoskeletons at the posterior end of the cell reveal cytoskeletal features such as the basal body (BB), probasal body (PBB), the flagellar collar (arrow) and the microtubule quartet (*). During cell division, each of these structures must be replicated once. In a G1 cell (Fig. 1C), the basal body (BB) form the single flagellar axoneme (Ax) with a probasal body (PBB) on its anterior side. The microtubule rootlet, which is termed the microtubule quartet (Fig. 1C, *), is initiated between the basal body and probasal body and crosses the flagellum before tracking helically to join the main subpellicular microtubules. The collar is apparent as a near-circular structure with a finger-like projection (Fig. 1C, black arrow). The first indication of initiation of cytoskeletal morphogenesis is likely to occur at the G1–S border and is the elongation of a new microtubule quartet (Fig. 1D, **) anterior to the old one (Fig. 1D, *) from the appropriate region of the old probasal body. Subsequently, the probasal body matures and elongates to form a transition zone and axoneme (Fig. 1E, white arrowhead). In addition, after the axoneme has elongated around 240nm from the basal plate, its tip connects to the lateral aspect of the old flagellum (Fig. 1E, white arrow) via the flagella connector (Briggs et al., 2004; Davidge et al., 2006; Moreira-Leite et al., 2001). Fig. 1F illustrates cells subsequent to this cell cycle stage. New probasal bodies are formed, a new collar is detectable (black arrows), and the axoneme is extended to ~2 µm. However, the new and old flagella basal bodies have not yet moved apart, as per the situation in Fig. 1B. Nevertheless, at this stage, the conundrum becomes apparent. The new flagellum with associated structures now lies on the other side of the old flagellum (contrast Fig. 1F with 1E).

Flagellar repositioning and flagellar pocket duplication

Negatively stained cytoskeletons are highly informative, but provide only two-dimensional views of cells that have lost their membranes. We therefore turned to thin section analyses to gain different views of the flagella in the context of the flagellar pocket. Fig. 2A presents a thin section of a stage that is likely to be similar to those in Fig. 1A,C. The probasal body is associated with the old flagellum on its anterior side. Fig. 2B illustrates a cell where the new flagellum (NF) has formed and elongated. It has invaded the bulge side of the pre-existing pocket (Lacomble et al., 2009) and again occupies the anterior position relative to the old flagellum. This cell displays the characteristics of S phase in that filamentous structures are associated with both antipodal sites of the kinetoplast (Liu et al., 2005). Fig. 2C illustrates a cell whose new flagellum has switched to a posterior position. In addition, a membrane profile (hereafter termed the ridge), protrudes into the volume of the single flagellar pocket (arrow). These images demonstrate that the new flagellar pocket is not formed de novo, but rather by division of the existing pocket.

The ridge appears to represent the earliest recognisable stage of flagellar pocket division. By this stage, kinetoplast elongation has progressed further and exhibits a more elongated form; presumably it is in division. Fig. 2D shows the membrane and cytoplasmic invagination of the ridge; the pocket then develops into a major area of cytoplasm (arrow). Subject to the plausible assumption that the cytoskeleton affects membrane shape, rather than vice versa, further separation of the basal bodies then presumably segregates the flagellar pocket into two distinct entities, one surrounding the
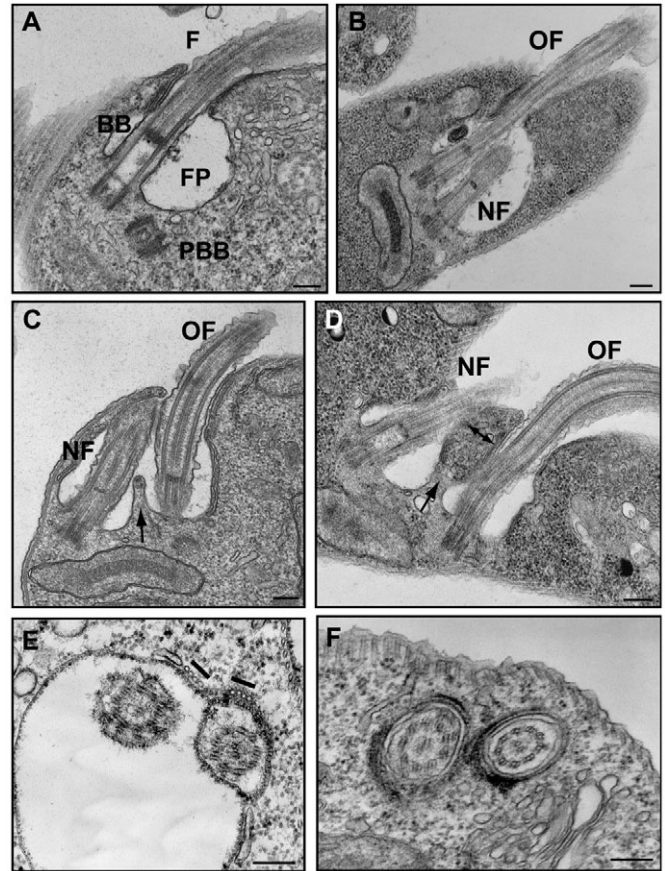

Fig. 2. Flagellar pocket duplication. Representative, thin-section, transmission electron micrographs illustrating the early stages of flagellar pocket duplication. (**A**) A single flagellum (F) extends from a basal body (BB) within the flagellar pocket (FP). A probasal body (PBB) is located in an anterior position relative to the flagellum. (**B**) The probasal body has matured and has initiated a new flagellum (NF), which has invaded the existing flagellar pocket. The new flagellum is still located anterior to the old flagellum (OF). (**C**) The new flagellum (NF) is now posterior to the old flagellum (OF). A membrane profile, termed the ridge (arrow), protrudes into the existing flagellar pocket between the two flagella. (**D**) The ridge has now developed further, and there is more separation of the basal bodies. (**E**) Transverse section of a flagellar pocket containing two flagella and two closely associated microtubule quartets (black bars). (**F**) Transverse section of the exit point from the flagellar pocket illustrating two flagella each surrounded by an electron-dense collar structure. Scale bars: 200 nm.

new flagellum and the other surrounding the old. Fig. 2E shows a transverse section of a flagellar pocket containing two flagella. At this stage in the cell cycle, two microtubule quartets (black lines) run close to each other on the cytoplasmic face of the flagellar pocket membrane. Fig. 2F represents a transverse section at a later stage, where each flagellum is distinctly surrounded by a collar structure defining the exit point from the cell.

Taken together, the evidence from negatively stained cytoskeleton and thin sections demonstrates that some form of basal body and new flagellum repositioning must occur. Furthermore, our analyses suggest that this occurs in a coordinated manner at a major transition stage in the cell cycle. The events appear to coordinate morphogenesis and segregation of a complex set of cytoskeletal and membrane components. To address the
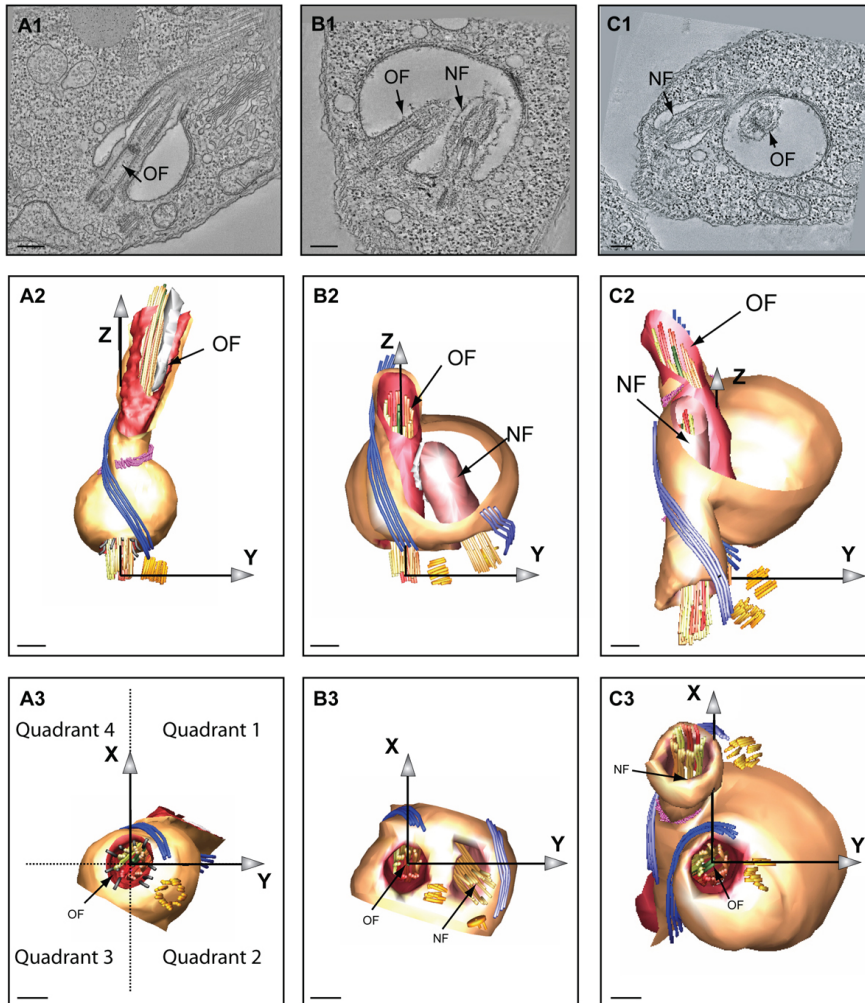
Fig. 3. Rotation of the new basal body around the old flagellum. Three representative tomograms (**A–C**) reveal different stages of basal body and flagellar pocket morphogenesis and demonstrate the rotation. A1,B1,C1 show slices through the flagellar pockets from the original tomograms. Tomogram models (A2–C3) contain Cartesian axes previously described (Lacomble et al., 2009). (A2,A3) Two views of the model of a tomogram illustrating a cell in which the probasal body is located on the bulge side of the flagellar pocket in quadrant 2. The origin of the microtubule quartet lies between the two basal bodies. (B2–B3) In this cell, the probasal body has matured and has subtended a new flagellum (NF) that has invaded the existing flagellar pocket and connected to the old flagellum (OF). The new flagellum is still positioned essentially as in A2 and A3: quadrant 2. (C2,C3) A later stage in the cell cycle just before flagellar pocket division. The new flagellum is now in a more posterior location and lies in quadrant 4. Please see text for explanation of the rotation. See supplementary material Table S1 for colour key. Scale bars: 200 nm.

nature and mechanics of this re-orientation we turned to analyses in three dimensions using EM tomography.

The new basal body rotates around the old flagellum, influencing the morphogenesis of the flagellar pocket

To understand basal body movement during flagellar morphogenesis in three dimensions, we acquired several tomograms of cells around the G1–S boundary when the new flagellum is forming. We used the three-dimensional Cartesian axes based on the singularity of the proximal end of the old basal body previously described (Lacomble et al., 2009) to facilitate comparison of tomograms from the various cell cycle stages. The origin of this coordinate system is the centre of the basal body at its most proximal end. The z-axis runs through the basal body centre and up the proximal portion of the axoneme; the x-axis is contained by the plane of the central pair microtubules at the point at which they are nucleated; the y-axis points toward the probasal body of the old flagellum. These axes facilitate description of the orientation of each basal apparatus and, therefore comparisons between tomograms of different cell cycle stages.

Fig. 3 illustrates our findings by using three tomograms that represent different stages of basal body and flagellar pocket morphogenesis. Fig. 3A1,B1,C1 show slices through the flagellar pocket areas from the original tomograms, giving a general

impression of the original data and the relative flagellar positions. Two views of each model are then shown. The first view looks down the x-axis and allows easy observation of the sequence of events leading to new flagellar pocket formation (Fig. 3A2,B2,C2). The second view looks along the z-axis from the base of the flagellum (Fig. 3A3,B3,C3). All images are shown with the positive y direction pointing roughly towards the anterior of the cell. In Fig. 3A1–A3, the probasal body is on the anterior bulge side of the flagellar pocket. Fig. 3A2,A3 illustrate the origin of the microtubule quartet between the basal body and probasal body and its helical path around the flagellar pocket and traversing the collar.

Fig. 3B1–B3 show a cell at approximately the cell cycle stage illustrated earlier by Fig. 1E and Fig. 2B. The probasal body has matured and elongated, and the new flagellum has invaded the flagellar pocket. The flagella connector is formed, and the new microtubule quartet is elongated. The two new probasal bodies have also formed. Fig. B3 shows that, although the new basal body has now matured and extended, the original probasal body seen in Fig. 3A3 is still in essentially the same position relative to the axes. It lies in quadrant 2 of this view as illustrated in Fig. 3A3.

Fig. 3C1–C3 describe an even later stage in the cell cycle, immediately before division of the flagellar pocket. The tomogram slice shown in Fig. 3C1 illustrates that the new flagellum has

now repositioned to a more posterior site in the cell, as described in Fig. 1F and Fig. 2C,D. Fig. 3C2 shows that although there is a single flagellar pocket, it is now distorted by the process of division. There is a large bulge luminal area, but a discrete nascent new flagellar pocket is forming around the new flagellum. The repositioning of this new flagellum can be seen in Fig. 3C2 and, perhaps even more dramatically in the view of the model in Fig. 3C3. This figure shows that the new flagellum basal body now lies in quadrant 4 (see quadrants illustrated in Fig. 3A3) (Lacomble et al., 2009). The nascent new flagellar pocket and associated cytoskeletal structures were also positioned toward the negative z-axis, reflecting a more posterior location in the cell.

These tomograms strongly imply both a rotational and to some extent, a lateral movement of the new flagellar basal body and axoneme. The old flagellum appears to be the axis of rotation, thus determining division of the flagellar pocket. How are these movements orchestrated? The directionality of rotation can be determined by the position of the flagella connector at the various times during the rotation. In a late-stage dividing cell, the flagella connector is positioned opposite microtubule doublets 7, 8 and 9 of the old flagellum (Briggs et al., 2004). The position of the flagella connector in the earliest of our images is opposite microtubule doublets 5, 6 and 7 of the old flagellum. However, in a cell similar to that in Fig. 3C2, where rotation around the old flagellum is underway, the flagella connector faces microtubule doublet 2, 3 and 4 of the old flagellum. This result defines the direction of rotation as anticlockwise, looking from the basal body towards its flagellum, and emphasises the fact that rotation occurs.

A clockwise rotation for this morphogenetic movement would be unlikely, because the new microtubule quartet is initially anterior to the new flagellum, and would therefore have to cross through the old microtubule quartet to become posterior to it. Hence, in mechanistic terms the anticlockwise rotation facilitates the gathering of membrane to form a new flagellar pocket and the efficient segregation of cytoskeletal elements associated with it. As mentioned previously, there is also a lateral movement towards a more posterior position in association with this anticlockwise rotational movement. Examination of the tomograms, particularly Fig. 3C3 revealed the importance of this anticlockwise rotation of the new flagellum basal body around the old flagellum from quadrant 2 through 1 to 4. Its lateral movement towards the viewer in Fig. 3C3 means that the old microtubule quartet acts as a cytoskeletal rib over which the membrane of the new flagellar pocket folds and is essentially divided. This rib-like function of the old microtubule quartet is again emphasised by examination of more-complete views of the cell shown in Fig. 4. Fig. 4A shows one view of the model at this stage of the cell cycle. Fig. 4B is a view whereby the viewer is looking at Fig. 4A from the back side. This tomogram reveals that the new quartet of microtubules have moved with the new basal body and flagellum around the old to also take up this posterior position. This morphogenetic phenomenon drives the formation of the new pocket by division of the old flagellar pocket membrane. During this rotary movement, a new collar is formed, serving to delimit the new flagellar pocket. Our tomography also shows that the new collar forms on the nascent pocket (Fig. 4) and any subsequent lateral segregation of the basal bodies would simply facilitate separation of the new pocket from the old along the old microtubule rib. Here, one can also see the pocket at this division
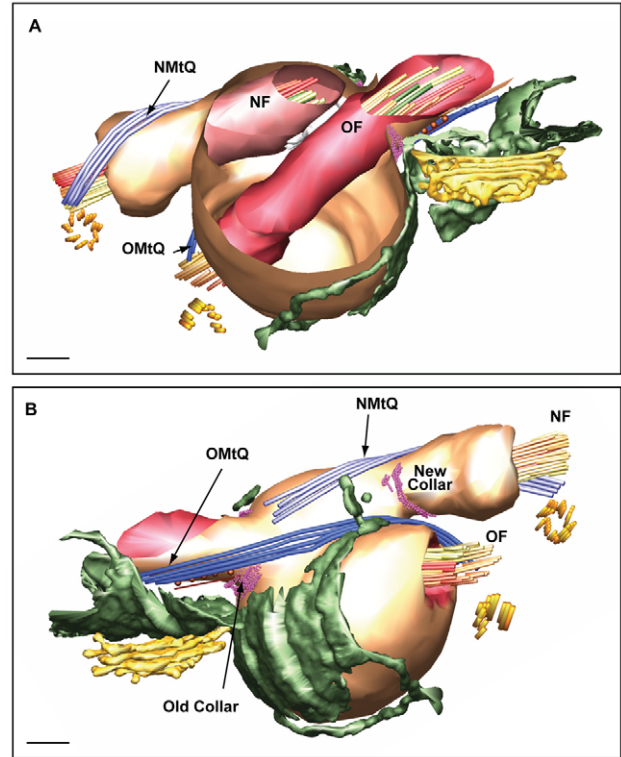

Fig. 4. New flagellar pocket morphogenesis. Two views of a model of a tomogram illustrating the formation of a flagellar pocket. (**A**) A nascent pocket has formed around the new flagellum (NF), along with a new microtubule quartet (NMtQ). (**B**) Position of the old and new microtubule quartets and old and new collars. See supplementary material Table S1 for colour key. Scale bars: 200 nm.

stage, which emphasises the organisational positioning of the old and new microtubule quartets and the association of the Golgi complex adjacent to the neck region (Lacomble et al., 2009).

The events above describe the process in the well-studied procyclic form of *T. brucei*. The bloodstream form of this parasite exhibits many similarities and some differences in cellular organisation. We asked whether the basic mechanics described above are also typical of this life cycle stage. Whole-mount cytoskeletons of bloodstream form trypanosomes are very similar to those of the procyclic form. The probasal body initially lies anterior to the old flagellum early in the cell cycle (Fig. 5A) and shifts to the posterior side of the old flagellum (Fig. 5B). Tomographic analysis confirmed a similar rotation (Fig. 5C,D), with comparable implications for flagellar pocket morphogenesis.

Discussion

Centrioles and basal bodies have been implicated in the establishment of shape, polarity and form in diverse eukaryotic cells. For example, in *Caenorhabditis elegans*, the first division of the embryo is asymmetrical, producing a larger anterior daughter cell. Nuclear and centrosomal rotation, and positioning of the centrosome and spindle lead to the asymmetry of cleavage. In addition, the centrosome appears to have an influence on the establishment of anterior–posterior polarity in the early embryo (Hyman and White, 1987). Issues of axis and polarity are crucial for the development of single-celled protists. In these organisms,
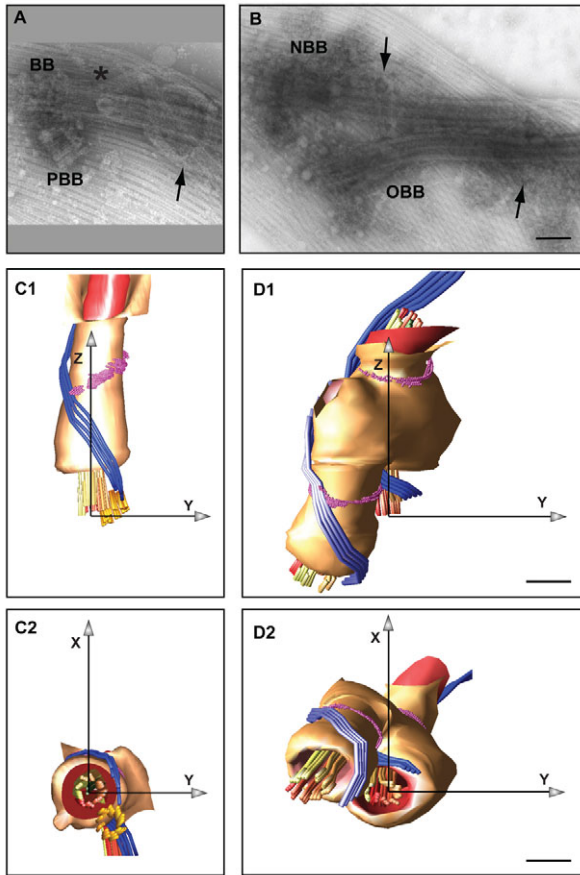
Fig. 5. Flagellar pocket morphogenesis in the bloodstream form. The basic mechanics described for the procyclic form are also found in the bloodstream form. (**A**) G1 cell with a single flagellum nucleated from the single basal body (BB). The probasal body (PBB) is located anterior to the basal body. (**B**) Premitotic cell with two flagella. The new flagellum and basal body (NBB) are posterior to the old flagellum basal body (OBB). (**C1,C2**) Alternative views of a model of a tomogram with a flagellar pocket containing a single flagellum, a probasal body positioned in quadrant 2 and the origin of the microtubule quartet between the two basal bodies. (**D1,D2**) Alternative views of a model of a tomogram where the new flagellum basal body has rotated around the old flagellum basal body and a new nascent flagellar pocket has formed. Scale bars: 200 nm (A,B), 250 nm (C1–D2).

the centriole–basal-body complex is strategically positioned with an associated, highly defined set of microtubule rootlets and/or sets of striated filament appendages. These microtubules and microfilament appendages have defined positions, polarities and orientations that assist in defining overall shape and form (Brown et al., 1976; Hibberd, 1975; Holmes and Dutcher, 1989). Therefore, it is impossible to separate our understanding of the duplication and segregation of centrosomes and basal bodies from the wider implications for cell morphogenesis. In many cells, light microscopy has shown that morphogenesis involves particular centrosomal or basal body movements, and electron microscopy has allowed the definition of the arrangements of component filaments and rootlets (Brugerolle, 1992; Nohynkova et al., 2006; Wright et al., 1985). In only a few cases do we know much about the detailed coordination of centrosomal/basal body duplication in terms of the subsequent inheritance of cell pattern and polarity. The trypanosome offers a useful cell in which to study such events.

Here, we have used an integrated approach combining over 1000 conventional electron micrographs of cells and cytoskeletons with electron tomography to provide a 3D analysis of the morphogenesis of the basal body complex and flagellar pocket in *T. brucei*. A major conclusion is that the new basal body complex undergoes a rotational movement around the basal body of the old flagellum. This motion is essential for correct placement of organelles as new structures are laid down in anticipation of cell division. It has always appeared counterintuitive that the probasal body is positioned at the anterior side of the basal body, and yet the new flagellum is always formed at the posterior side. Given that migration of the new flagellum into the posterior end of the dividing cell is crucial for segregation of the mitochondrial genome in the kinetoplast (Robinson and Gull, 1991), then genesis and positioning of the probasal body at the posterior side of the basal body would seem be the more reasonable option. However, our tomography clearly indicates that the genesis of the new flagellum is initiated in an anterior position, necessitating the morphogenetic movement of the new flagellum around the old. The structures seen also imply that this motion facilitates flagellar pocket division. The new flagellar pocket is not formed de novo, but is derived from the old one during new flagellum formation and basal body rotation.

The structures and forces responsible for this spectacular rotation remain unknown. Since the basal bodies are physically linked to the kinetoplast, the motor could lie in the kinetoplast; however, we and others have previously shown this to be unlikely, because basal bodies can segregate in the absence of kinetoplast segregation (Ploubidou et al., 1999; Robinson and Gull, 1991; Zhao et al., 2008). However, there might be implications for kinetoplast organisation from this morphogenetic rotation of the new basal body. There are distinct patterns of DNA replication in the kinetoplast of *T. brucei* and other kinetoplastid parasites (Ferguson et al., 1994; Liu and Englund, 2007; Perez-Morga and Englund, 1993). It seems likely that the tripartite attachment complex filaments linking the basal body and kinetoplast are already engaged with the probasal body when it matures and elongates to form the transition zone and axoneme (Ochsenreiter et al., 2008; Ogbadoyi et al., 2003; Zhao et al., 2008). If so, then the rotational movement around the old basal body would also exert an influence on the duplicating kinetoplast, which would subsequently segregate, remaining connected by the nabelschnur structure (Gluenz et al., 2007). Therefore, the morphogenetic movements described here for the new basal body complex might provide a mechanism for this process.

It is unlikely that the force driving this movement is simply axonemal elongation. In intraflagellar transport mutants, where the growth of a new axoneme is prevented, basal body segregation still occurs to some extent (Absalon et al., 2008; Davidge et al., 2006). Indeed, after segregation, a flagellar sleeve representing an 'empty' new flagellum lies on the correct posterior side of the old flagellum (Davidge et al., 2006). The flagella connector is another possibility, although the lack of a connector structure in the bloodstream form argues against this (Moreira-Leite et al., 2001). Another contender for the site of the force would be the microtubule quartet (rootlet microtubules). Our analyses now place initiation of the microtubule quartet as the very first indication of new flagellum and pocket morphogenesis in *T. brucei*. We show here that early nucleation of the new microtubule quartet precedes probasal body extension or new probasal body formation. We suggest that part of the importance of this schedule is to establish the first cytoskeletal contact with the flagellar pocket membrane on behalf of the nascent

basal body complex, facilitating orientation and docking of the existing probasal body when it matures and elongates to form the transition zone and axoneme. If the organising centre for the quartet is also specifically aligned via the probasal body then the microtubules will extend in a left-handed helical pattern reminiscent of the old quartet, and so connect with a specific zone of the flagellar pocket. The basal body and microtubule quartet remain in intimate connection as the rotational movement occurs. Thus the rotational movement occurs before the new flagellum has emerged from the flagellar pocket and before the microtubule quartet has elongated fully to join the sub-pellicular array. This raises the possibility that the force is in some way sited at one or both of the microtubule quartets.

Two events of importance follow; first, the division of the flagellar pocket and second, the further separation of the basal bodies and kinetoplasts after the rotational movement has placed the new basal body on the posterior side of the old. In terms of formation of the new flagellar pocket, a nascent flagellar pocket is formed within the existing flagellar pocket by the initial rotational and posterior movement of the new basal body. We suggest that the old microtubule quartet acts here as a cytoskeletal rib, over which the flagellar pocket membrane is drawn, allowing the new flagellar pocket to be structurally defined as a consequence of the rotary movement. The direction and polarity of this set of rootlet microtubules is therefore crucial to morphogenesis. The importance of microtubule rootlet involvement in cell membrane morphogenesis is seen in other organisms. In *Chlamydomonas reinhardtii*, a microtubule rootlet has been shown to be essential in positioning the cleavage furrow during cytokinesis (Ehler and Dutcher, 1998; Ehler et al., 1995). We also know that in trypanosomes, the flagellum attachment zone is of key importance in determining the plane of cleavage (Kohl et al., 2003; Robinson et al., 1995). The identification of molecular components of the microtubule quartet will be crucial if we are to understand the detailed role of this cytoskeletal structure (Vaughan et al., 2008). In addition to forming the basal area of the flagellar pocket by movements and structures associated with the new basal body, a new flagellar neck and collar region must be formed around the new flagellum before it emerges from the cell body (Bonhivers et al., 2008; Lacomble et al., 2009). Our tomography shows that the new collar forms on the nascent pocket. This cytoskeletal structure is a key factor in defining the membrane of the new flagellar pocket and facilitating its separation from both the old pocket and the membrane of the nascent flagellum.

The second issue is the more lateral movement apart of the basal bodies that also segregate the mitochondrial genome (Robinson and Gull, 1991). During this movement, the new flagellar axoneme is elongating and is attached to the old flagellum via the flagella connector (Briggs et al., 2004; Davidge et al., 2006; Moreira-Leite et al., 2001). The connector forms at one position, and during the morphogenetic rotational transit, the flagella connector exhibits both rotational and lateral movement along the old flagellum membrane. These movements mean that by the time the new flagellum exits the flagellar pocket it is located in the characteristic position along the posterior side of the old flagellum. The morphogenetic transit places the new flagellum tip and connector in a position to engage with the appropriate side of the old flagellum before it exits the pocket.

Again, the description of the motions begs the question as to why the new flagellum needs to be on the posterior side of the old flagellum. The answer appears to be that being on that side will facilitate the extensive lateral movement of the new basal body towards the extending posterior end of the cell, so allowing wide segregation of the kinetoplast genomes. If the new basal body remained on the anterior side then a posteriorly directed movement would result in a collision of the new basal-body–kinetoplast complex with the old complex. The force for this more lateral movement apart of the basal bodies and kinetoplasts might well involve various combined actions of the flagella connector, the FAZ and interactions with sub-pellicular microtubules.

In summary, rotational movement is crucial for new flagellar pocket formation and thus for cellular morphogenesis. Repositioning on the posterior side of the old flagellum is a key factor in facilitating the subsequent lateral movement of the new basal body complex towards the posterior end of the vermiform-shaped trypanosome. These events have been clarified thanks to an extensive combination of electron microscopy and electron tomography. This approach is likely to be valuable for elucidating other cytoskeletal-remodelling phenomena in protistan and metazoan cells deserving of explanation beyond the resolution of the light microscope.

Materials and Methods

Cell culture, sample preparation for electron microscopy and cellular electron tomography methods are exactly as carried out in our previous electron tomography paper (Lacomble et al., 2009).

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/17/2884/DC1

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