Trypanosome IFT mutants provide insight into the motor location for mobility of the flagella connector and flagellar membrane formation

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Summary

The flagella connector (FC) of procyclic trypanosomes is a mobile, transmembrane junction important in providing cytotactic morphogenetic information to the daughter cell. Quantitative analyses of FC positioning along the old flagellum, involving direct observations and use of the MPM2 anti-phosphoprotein monoclonal reveals a 'stop point' is reached on the old flagellum which correlates well with the initiation of basal body migration and kinetoplast segregation. This demonstrates further complexities of the FC and its movement in morphogenetic events in trypanosomes than have hitherto been described. We used intraflagellar transport RNAi mutants to ablate the formation of a new flagellum. Intriguingly the FC could still move, indicating that a motor function beyond the new flagellum is sufficient to move it. When such a FC moves, it drags a sleeve of new flagellar membrane out of the flagellar pocket. This axoneme-less flagellar membrane maintains appropriate developmental relationships to the cell body including following the correct helical path and being connected to the internal cytoskeleton by macula adherens junctions. Movement of the FC in the apparent absence of intraflagellar transport raises the possibility of a new form of motility within a eukaryotic flagellum.

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Introduction

Procyclic (insect form) cells of the African trypanosome, *Trypanosoma brucei*, contain a set of single organelles that are replicated and segregated with fidelity throughout the cell cycle (Gull, 1999). Two cellular events that are clearly pivotal to ensuring accurate morphogenesis are the formation of a new flagellum and the segregation of the single mitochondrial genome (the kinetoplast). Both of these events are critically dependent upon functions embedded within the basal body, a canonical microtubule organising centre that acts as a 'master organiser' of trypanosome cellular morphogenesis.

The basal body serves two functions in flagellum formation, acting to template the formation of the 9+2 microtubule axoneme and providing a platform for the recruitment of proteins involved in intraflagellar transport (IFT). IFT is the evolutionarily conserved mechanism used to build most eukaryotic flagella and cilia, and involves non-vesicular transport of IFT particles and their associated protein cargo along axonemal microtubules beneath the ciliary or flagellar membrane (Rosenbaum and Witman, 2002; Scholey, 2003).

Although flagellum-related functions are associated with the distal end of the basal body, kinetoplast (mitochondrial genome) segregation depends upon the tripartite attachment complex (TAC) a filamentous structure that attaches the proximal end of the basal body to the kinetoplast DNA network (Ogbadoyi et al., 2003). The TAC ensures that microtubule-dependent separation of basal bodies acts to segregate the kinetoplast DNA and results in their correct spatial positioning within the dividing cell (Robinson and Gull, 1991).

Coordination of flagellum formation and basal body segregation with other cell cycle activities, such as cleavage furrow ingression, is vital and it appears that outgrowth of the flagellum itself operates to orchestrate many of these events. As the new flagellum is built during each cell cycle, the dividing trypanosome uses a transmembrane mobile junction (the flagellar connector) to connect the tip of the growing new flagellum to the side of the old one (Briggs et al., 2004; Moreira-Leite et al., 2001). As the new flagellum extends, it remains physically attached to the cell body via a sub-pellicular flagellum attachment zone (FAZ) and it is this attachment that provides morphogenetic information used by the trypanosome during cytokinesis (Gull, 1999; Ploubidou et al., 1999; Robinson et al., 1995). Consequently we have proposed that the flagella connector, by acting to direct flagellum outgrowth, plays a pivotal role in morphogenesis, translating structural information (such as polarity, axis, alignment and helicity) embedded in the old cytoskeleton to the development of the new cytoskeleton. This phenomenon is an example of cytotaxis (Beisson and Sonnerborn, 1965; Moreira-Leite et al., 2001; Sonnerborn, 1964).

Although there are many types of cell-cell junctions in eukaryotes, none of those described in metazoans are thought to be actively mobile. They do, however, have an influence on cell and tissue shape and form. We have described the structure of the flagella connector and shown that it is formed very early, while the new flagellum is still in the flagellar pocket. We know little about how it moves or disconnects and then disassembles. Moreover, our earlier descriptions provided some intriguing early illustrations of spatio-temporal phenomena in relation to FC placement and position and basal body morphogenetic events in the trypanosome cell cycle.

We have now used quantitative analyses of FC positioning along the old flagellum; use of the MPM2 anti-phosphoprotein monoclonal antibody reveals that a 'stop point' is reached, which correlates well with the initiation of basal body migration and kinetoplast segregation. This demonstrates further complexities of the FC and its movement in morphogenetic events in trypanosomes than have hitherto been described. There are three notable motility operations in a eukaryotic flagellum: in addition to the tubulin polymerisation-driven extension of the new flagellum axoneme, there is the dynein ATPase-driven sliding of microtubules that leads to flagellar beating (Sale and Satir, 1977) and finally, the IFT motility that moves cargo to and from the flagellum tip (reviewed by Rosenbaum and Witman, 2002; Scholey, 2003). We have used an approach involving IFT RNAi mutants to ablate the possibility of all of these functions operating in the new flagellum and then specifically asked whether the FC can still form and if it did, whether or not the FC could actually move. Intriguingly, it can, indicating that a motor function outside the new flagellum is sufficient to move it. When the FC moves, it drags a sleeve of new flagellar membrane out of the flagellar pocket. This axoneme-less flagellar membrane maintains appropriate developmental relationships to the cell body, including following the correct helical path and being connected to the internal cytoskeleton by macula adherens junctions. Since the FC moves in the apparent absence of IFT our results raise the possibility of a new form of motility within a eukaryotic flagellum.

Results

A 'critical point' in flagellar connector migration

Previous work by Robinson et al. (Robinson et al., 1995) revealed that during the trypanosome cell cycle, a strict correlation exists between flagellum elongation and basal body separation. This study also demonstrated that when the new to old flagellum length ratio was plotted against inter-basal body distance, the data were biphasic with the graph showing an apparent inflection point. Before this inflection point the new flagellum elongated rapidly while basal body separation remained minimal, whereas after the inflection point the increase in new flagellum length slowed and there was a corresponding increase in the rate of basal body separation. It was suggested that this point in the cell cycle was a morphological event marker representing 'an intrinsic, cryptic transition point in the trypanosome cell cycle' (Robinson et al., 1995).

As this work predated recent experimental findings describing the existence of the flagellar connector, a structure that is intimately associated with new flagellum growth, we repeated this cytological analysis using electron micrographs of whole cytoskeletons representative of cells throughout the cell cycle. The following measurements were taken from this heterogeneous population: (1) distance between basal bodies; (2) length of the old flagellum; (3) length of the new flagellum and (4) distance of FC migration along the old flagellum. The collected data from these experiments are represented as a ratio of old flagellum length to account for small variations in cell length between individual cells.

Fig. 1A plots the growth of the new flagellum against the inter-basal body distance and demonstrates that the new flagellum initially extends without a significant increase in

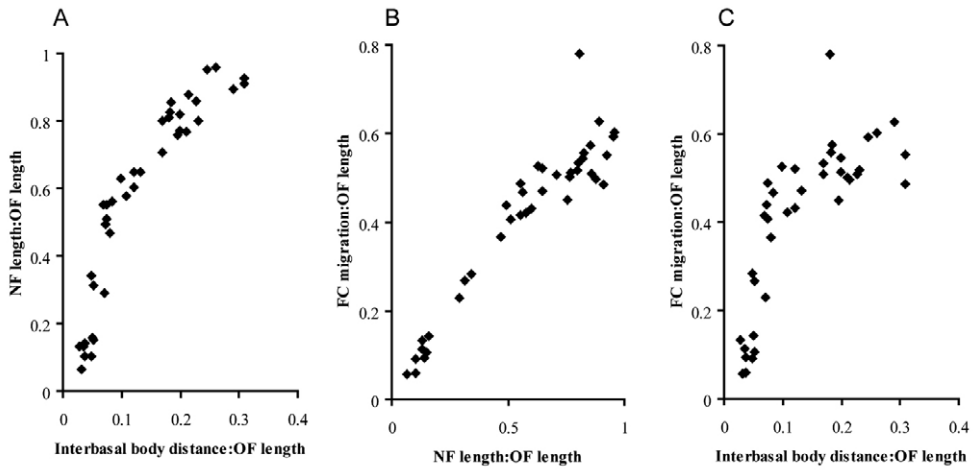

Fig. 1. Graphs showing relationships between flagellar connector movement and basal body migration in procyclic form trypanosomes. Electron micrographs of whole-mount cytoskeletons were analysed and the following cytological measurements recorded: (1) distance between basal bodies; (2) length of the old flagellum (OF); (3) length of the new flagellum (NF) and (4) distance of FC migration along the old flagellum. Measurements are expressed as a ratio to old flagellum length to account for individual variation in cell length. (A) Graph showing relationship between new flagellum length and inter-basal body distance. (B) Graph showing relationship between FC migration and new flagellum length. (C) Graph showing relationship between FC migration and inter-basal body distance.

inter-basal body distance. However, when the new flagellum has elongated to ~0.5 of the old flagellum length (inter-basal body to old flagellum length ratio equal to 0.1) an inflection point is observed. After this point there is a rapid increase in inter-basal body distance, i.e. both the basal body and kinetoplast are segregating. Although this result merely confirms the previous analysis of Robinson et al. (Robinson et al., 1995), we then asked if this inflection point was also seen when we analysed the position of FC migration along the old flagellum. Fig. 1B shows this analysis and reveals that there is indeed an inflection point in the rate of FC migration at around 0.45-0.50 (new to old flagellum length ratio). Moreover, it is clear from these data that although new flagellum length eventually extends to match the length of the old flagellum, the FC itself never actually migrates to the distal tip of the old flagellum. Rather, it extends to a maximum distance corresponding to ~0.6 of the old flagellum length.

Finally, an analysis of FC migration versus inter-basal body migration also demonstrated this inflection point at around 0.5 (FC position:old flagellum length) and 0.1 of inter-basal body distance (Fig. 1C). Before the FC reached 0.5 of old flagellum length, the basal bodies subtending the two flagella remain tightly associated. However, subsequent to the FC reaching this inflection point further extension of the new flagellum is reflected in increasing inter-basal body separation rather than further movement of the FC along the old flagellum (Fig. 1C).

Thus these analyses indicate that in the initial growth period, the basal bodies are close together and the movement of the FC precisely matches the extension kinetics of the new flagellum. Although this could be taken as evidence to support a model in which FC movement is merely a function of axoneme extension, this model clearly cannot be correct after the inflection point. After the FC has migrated to ~0.5 new flagellum:old flagellum length ratio, the inflection point (which we now term the 'critical point' in new flagellum growth) identifies an event characterised by basal body segregation (i.e. an increase in inter-basal body distance). During basal body segregation, although the length of the new flagellum continues to increase, there is little movement of the FC along the old flagellum. FC movement ceases at around 0.6 of the length of the old flagellum at a final stop point on the old flagellum length and, at least in normal cultured procyclic cells, never travels to the extreme distal tip of the old flagellum. A cartoon depicting the measurements taken and the correlation between inter-basal body distance and FC migration is shown in Fig. 3C.

Phosphoproteins and the FC

In a previous study investigating the ultrastructure of the flagellar connector we suggested that insights into FC assembly and/or disassembly might be provided by considering desmosome formation in cultured epithelial cells; a process that appears to be regulated by reversible protein phosphorylation. To investigate this, we used commercially available anti-phosphopeptide antibodies. One such monoclonal antibody, MPM2, recognises a large number of cytoskeletal and cytosolic proteins in many eukaryotic cells; most of which are specifically phosphorylated at the onset of mitosis (Westendorf et al., 1994). However, the use of this phospho-specific antibody on *T. brucei* cells gave a remarkable result; namely that MPM2 is a reliable and cell cycle independent marker for key cytoskeletal structures involved in trypanosome morphogenesis, including the FC.

Immunolocalisation studies using MPM2 in association with a panel of monoclonal antibodies recognising trypanosome cytoskeletal structures reveals that MPM2 recognises the flagellum axoneme, the flagellum attachment zone (FAZ), the distal tip of both new and old flagellum, the FC and the basal and probasal body (Fig. 2A-C). The trypanosome shown in Fig. 2A is co-labelled with MPM2 and the anti paraflagellar rod antibody ROD1. This image of a postmitotic 2K2N cell with two flagella clearly demonstrates that the MPM2 staining associated with the flagellum (green) is present on the axoneme and not the extra-axonemal PFR, as there is no observable co-localisation with ROD1 (red). The other line of MPM2 labelling observable in this image is located on the cell body and co-labelling experiments with the antibody L6B3 (data not shown) indicates that this labelling corresponds to the FAZ: the structure responsible for physically attaching the flagellum to the trypanosome cell body. Immunogold-labelling electron microscopy also reveals that MPM2 specifically labels the FAZ

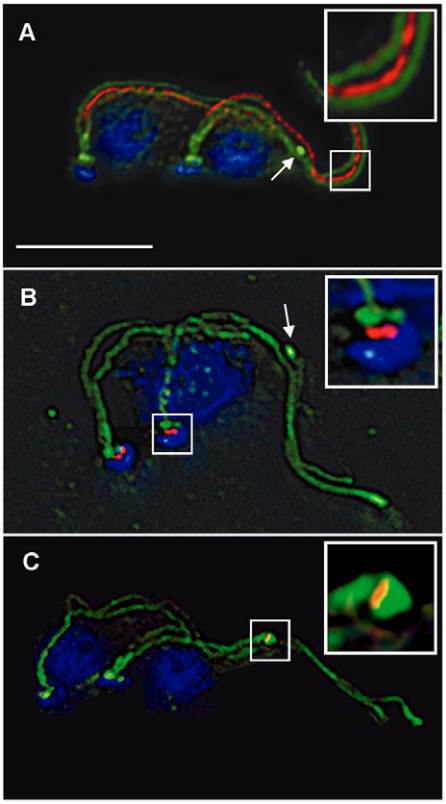

Fig. 2. Visualisation of MPM2-positive phosphoproteins in procyclic trypanosomes. Immunofluorescence images of *T. brucei* cytoskeletons co-labelled with the anti-phospho-epitope antibody MPM2 (green) and (A) ROD1, a specific marker for the extra-axonemal PFR structure; (B) BBA4, a specific marker for the proximal pole of both basal and probasal body structures; and (C) AB1, a specific marker for the central component of the flagella connector (red). Nuclear and kinetoplast DNA are labelled with DAPI (blue). The sections identified by the white boxes are enlarged in the top right hand corner of each panel to allow closer inspection of the co-labelling pattern observed. Bar, 10 μm.

filament rather than microtubule components of the FAZ (data not shown).

In Fig. 2B, a dividing cell is co-labelled with MPM2 (green) and BBA4 (red). The monoclonal antibody BBA4 specifically labels the proximal end of trypanosome basal and probasal bodies. Consequently, the co-labelling pattern observed in Fig. 2B suggests that MPM2-positive phosphoproteins are localised towards the distal end of both the basal and probasal body, although it is evident that a more intense labelling is associated with the mature basal body subtending the flagellum. Specific MPM2 labelling of the axoneme and FAZ are again observed in this image.

Examination of trypanosomes possessing two flagella revealed an intense and invariant focus of MPM2 labelling associated with the distal tip of the new flagellum (Fig. 2A-C). Although the tip of the old flagellum is also labelled, closer inspection of the MPM2 staining pattern on the new flagellum shows that MPM2 provides another marker for the FC. Co-labelling with AB1, the previously characterised monoclonal antibody that labels the interstitial component of the FC between the new and old flagellum, demonstrates that AB1 labelling is precisely positioned between the two foci of MPM2 labelling. Our interpretation of this labelling pattern is that MPM2 detects the filamentous components of the FC within the new and old flagella. It is evident from Fig. 2C that MPM2 labelling is particularly marked on the lateral aspect of the old flagellum, in a region immediately adjacent to the AB1 localisation. This region is associated with extensive electron dense filamentous structures connecting the FC to specific outer doublets of the old flagellum axoneme. The quantitative cytological measurements shown in Fig. 1 and discussed above were also recorded from these MPM2-stained cells. The results obtained are in accord with these previous data (supplementary material Fig. S1).

A remnant of the FC is retained on the old flagellum following cytokinesis

We have previously shown that there is considerable variability in the timing of FC detachment, which can occur at various points, in mid to late (and even post) cell cleavage (Briggs et al., 2004). However, data presented in Fig. 1 demonstrate that maximal point of FC migration along the old flagellum (the stop point) is remarkably consistent.

Given the intriguing MPM2 localisation pattern we observed at the FC (on the new flagellum tip but particularly the lateral aspect of the old flagellum), we then asked whether MPM2 labelling could still be observed in cells where the FC had already detached; thus providing temporal insights into FC disassembly during the cell cycle. Examination of 2K2N cells in which the FC has already detached revealed a focus of MPM2 labelling retained on the old flagellum for the remainder of the cell cycle; we have termed this the FC remnant. Indeed, we believe that loss of this specific signal may only occur after cytokinesis. Although MPM2 labelling on trypanosomes is largely invariant through the cell cycle, a more detailed examination of 1K1N cells (i.e. post cytokinesis trypanosomes) revealed heterogeneity within this population. In ~13% of 1K1N cells, a bright MPM-positive dot is still observed half-way along the old flagellum immediately anterior to the nucleus and precisely at the maximal position reached by the FC during the previous cell division cycle (Fig. 3A). We suggest that this represents the remnant of the FC structure left on the old flagellum post cytokinesis – a view buttressed by a quantitative analysis invariantly positioning this MPM2 positive structure at the FC migration stop point (see Fig. 3B). In Fig. 3B this discrete focus of MPM2 labelling on 1K1N cells is superimposed upon a graph plotting a ratio of FC migration along the old flagellum against length of the new flagellum in biflagellated cells. The MPM2 focus precisely correlates with the stop point that is located approximately 0.5-0.6 along the old flagellum length. As we never observe this MPM2 labelling of the stop point on cells that have initiated formation of a new flagellum, we take this to indicate that the MPM2-positive phosphoproteins recognised at this location must be removed (or dephosphorylated) before the G1-S transition (Woodward and Gull, 1990). Furthermore MPM2 labelling of the FC remnant, either on 2K2N cells in which the new flagellum has already disconnected or on 1K1N cells post cytokinesis, never extends beyond the stop point. This suggests that the FC is incapable of independent movement after the new flagellum has detached and raises several interesting questions regarding the precise nature and location of the motor driving FC migration.

Where is the FC motor?

Our observations raise the issue of the location of the motor that moves the connector out from the flagellar pocket and along the old axoneme. We have reasoned previously (Briggs et al., 2004) that the FC might be passively driven by the extension of the new flagellum. A motor for the FC might, however, be in the old flagellum or a combination of both. We know the FC is built in the flagellar pocket very soon after pro-basal body extension, in readiness for nucleation of the new flagellar axoneme. We generated trypanosome cell lines in which we could inducibly ablate the expression of key proteins involved in intraflagellar transport (IFT) such as the IFT particle protein *Tb*CHE2 (Fujiwara et al., 1999) and hence inhibit new axoneme extension. Using the *Tb*CHE2 RNAi mutant we specifically asked whether the FC could still be assembled, and moreover subsequently migrate along the old flagellum in cells unable to construct a new flagellum. Knockdown of *Tb*CHE2 mRNA had a dramatic effect on the extension of the new flagellum but no significant effect on the maintenance or integrity of the old flagellum (these results are mirrored by studies ablating other IFT components) (Kohl et al., 2003). Electron microscopic observations on RNAi cells showed that although the axoneme was not formed in the new flagellum, a FC was formed in the flagellar pocket (Fig. 4A).

In normal trypanosomes, a G1 cell possesses a basal body subtending the flagellum and a pro-basal body alongside. In the G1-S transition, the pro-basal body extends a transition zone, ‘docks’ with the flagellar pocket membrane and then the new flagellum axoneme extends into the flagellar pocket, ultimately forming a new flagellar pocket. In the *Tb*CHE2 induced RNAi mutant, all of these events occur except extension of the axoneme. However, thin-section electron microscopy revealed an intriguing phenotype, the new flagellar axoneme was absent but a new flagellar membrane had extended out of the flagellar pocket (Fig. 4B,C) pulled by the apparent movement of the FC up the length of the old flagellum. For the description below we term this structure the new flagellum sleeve. The sleeve is clearly positioned in the correct place and extended out of the

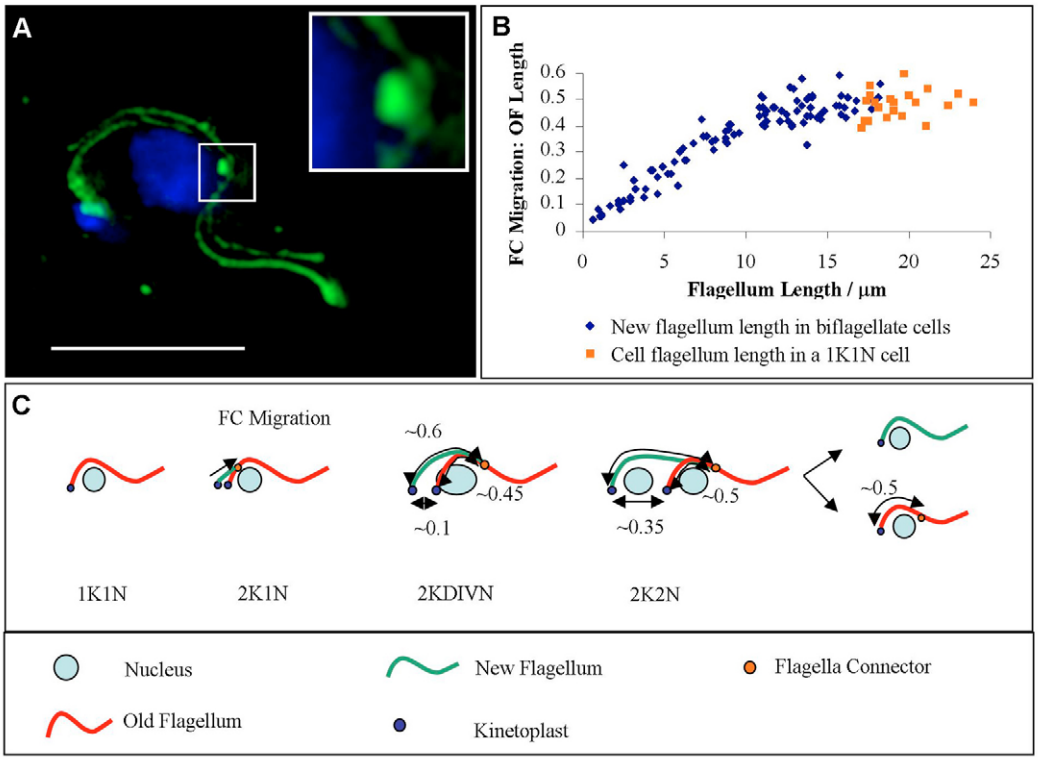

Fig. 3. An MPM2-positive structure is retained on the old flagellum post cytokinesis enabling distinction between procylic cells post division. (A) Immunofluorescence image of a representative 1K1N procyclic trypanosome labelled with the anti-phospho-epitope antibody MPM2 (green), showing a focus of MPM2 labelling (FC remnant) at the defined stop point of FC migration. Nuclear and kinetoplast DNA are labelled with DAPI (blue). (B) Quantitative analysis of FC migration as revealed by MPM2 labelling of procyclic cells. The graph plots FC migration (as a ratio of old flagellum length) against new flagellum length in biflagellate cells (blue diamonds). The position of the FC remnant on the old flagellum for a population of 1K1N cells (red squares) is overlaid to demonstrate that the observed focus of MPM2 on 1K1N cells precisely correlates with the defined FC stop point. The reduced overlap between the datasets is a reflection of under representation of very late division cells in the biflagellate population. (C) Cartoon summarising the correlation between the temporal and spatial positioning of the new and old flagellum, the FC and inter basal body separation as deduced from the cell measurement data shown in Fig. 1 and supplementary material Fig. S1. This cartoon also depicts the asymmetric MPM2-staining pattern predicted for 1K1N cells following cytokinesis. DIVN, dividing nucleus; K, kinetoplast; N, nucleus. Bar, 10 μm.

flagellar pocket in the absence of the new flagellar axoneme because it forms the normal lateral FAZ, membrane to membrane, macula adherens junctions with the plasma membrane (Fig. 4B,C) as described by Vickerman (Vickerman, 1969). Scanning electron microscopy of these cells gave an even clearer impression of the sleeve (Fig. 4D-I). In Fig. 4D,E a stub of a new flagellum has formed and then the sleeve of the empty new flagellum membrane is clearly seen. It is attached to the cell body and follows the same left-handed helix that would be expected for the new flagellum. The sleeve tracks between the exit of the new flagellar pocket and the lateral aspect of the old flagellum where the FC connects it to the old flagellum. A similar situation is seen in Fig. 4F-I. It is important to note that these micrographs clearly show that the basal bodies and flagellar pockets have segregated in the absence of full extension of the new flagellar axoneme but apparent extension of the internal FAZ filament, as evidenced by macula adherens (Fig. 4B,C).

Finally, to confirm that this sleeve is indeed the new flagellar membrane pulled out of the flagellar pocket by the FC we provided further evidence from four diverse approaches. First, we know that the new flagellum is present on the left-hand side of the old in cross section when the trypanosome is viewed from the posterior end. In electron micrographs polarity can be unambiguously determined because of the axial polarity of the outer microtubule doublets and associated dynein arms. This configuration was of course present in the uninduced *Tb*CHE2 mutant (Fig. 4J). However, when we viewed cross sections of the induced *Tb*CHE2 RNAi cell line we could detect the new empty flagellar sleeve at the left-hand side of the old flagellum (Fig. 4K). Second, we stained the induced cells with the AB1 monoclonal that detects the FC (Briggs et al., 2004) and showed that although no new axoneme had formed, there was an AB1 signal on the old flagellum indicating that the FC can move and pull the new flagellar membrane out of the flagellar pocket in the form of the sleeve (data not shown). Third, we then examined detergent-extracted cytoskeletons to visualise the FC directly. In such preparations the detergent removes the flagellar membranes along with all other membranes. However, we know that the FC is a detergent-resistant structure (Moreira-Leite et al., 2001) and Fig. 5A shows that indeed in a cell displaying only a stub of a new flagellum, the FC is present and has moved up the old flagellum in the absence of a new flagellum axoneme. Fourth, we conjectured that if our

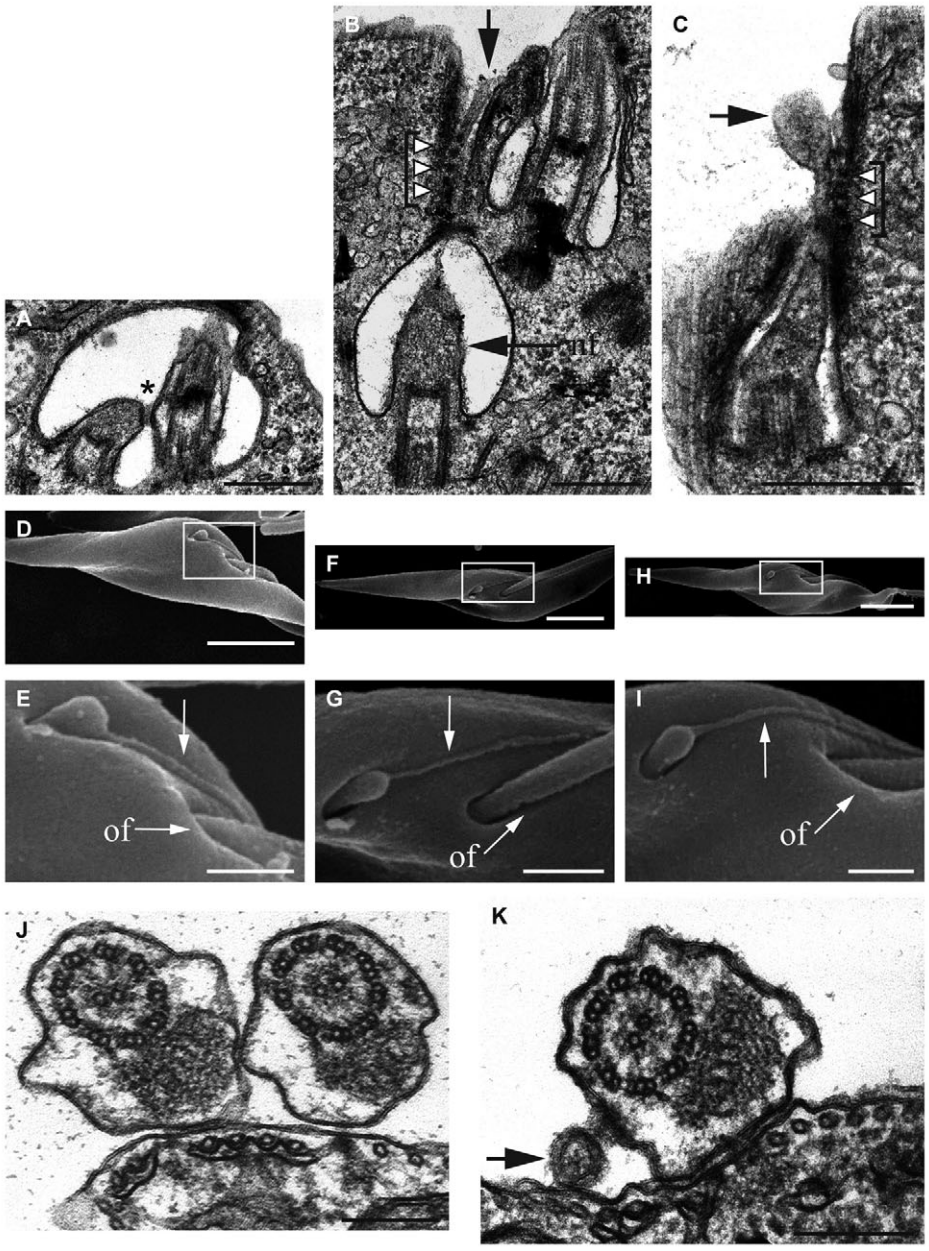

Fig. 4. Formation and movement of the FC in the absence axonemal assembly. (A) A thin-section electron micrograph shows formation of a FC (asterisk) in the flagellar pocket of a *Tb*CHE2 RNAi-induced cell. (B,C) Thin-section electron micrographs revealing the extrusion of flagellar membrane (arrows) beyond the flagellar pocket of *Tb*CHE2 RNAi-induced cells and the formation of macula adherens junctions within the FAZ (white arrowheads within a square bracket). nf, new flagellum. (D-I) Exit of a flagellar stub from the flagellar pocket and the left-handed helical twist of the flagellar sleeve in *Tb*CHE2 RNAi-induced cells as revealed by scanning electron microscopy. The boxed regions of D,F,H are shown at higher magnification in E,G, and I, respectively. of, old flagellum; arrows point to the flagellar sleeve. (J) The elongating new flagellum is positioned on the left-hand side of the old flagellum in cells with two flagella. (K) Transverse section through a *Tb*CHE2 RNAi-induced cell reveals the presence of a flagellar sleeve (arrow) to the left-hand side of the old flagellum and is attached by a FC. Bars, 500 nm (A,B,E,G,I,J); 2 μm (D,F,H); 200 nm (C,K).

interpretation of the extended empty new flagellar membrane sleeve is correct and not merely a sub-plasma membrane 'impression' of attachments in the cell body, we should not see such a structure in a cell line where the new flagellar axoneme is made, is surrounded by its membrane but is not attached to the cell body. The FLA1 mutant presents this phenotype (Moreira-Leite et al., 2001). We therefore examined induced RNAi *Fla1* mutants; Fig. 5B,C shows that the new flagellum has formed but is detached whereas the old flagellum remains attached to the cell body. As expected, there was no sleeve structure observed on the cell body linking the new flagellar pocket and the old flagellum.

Discussion

Basal body segregation and the FC

We have previously shown that there are two phases in basal body migration and separation when compared with new flagellum elongation (Robinson et al., 1995). Other evidence strongly supported our concept that flagellum extension, length and tip position, transduced through to the FAZ cytoskeleton, are major factors determining the initiation point and polarity of the cleavage plane (Briggs et al., 2004; Kohl et al., 2003; Moreira-Leite et al., 2001). Here we have extended this analysis to reveal that the FC exhibits a very particular behaviour that involves a stop point at a discrete point on the old flagellum. Moreover, this point correlates well with the inflection point that separates the two phases of basal body migration described above (Robinson et al., 1995). In essence the FC at the tip of the new flagellum ceases its movement along the old flagellum but remains attached; however new flagellum extension continues and this phase is coincident with the major movement apart from the basal bodies and/or

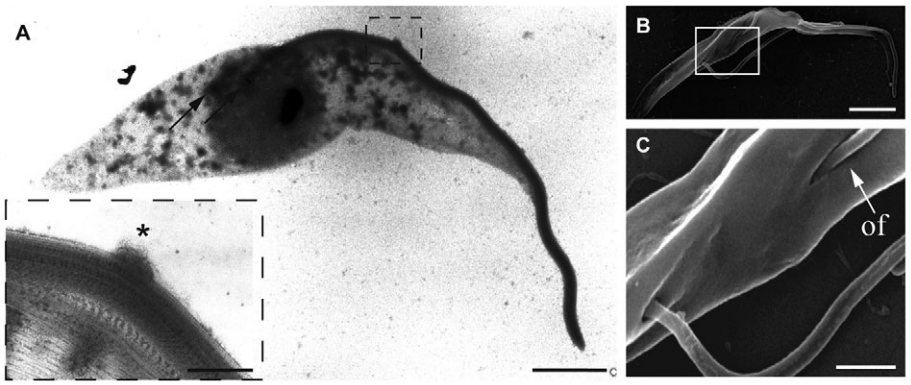

Fig. 5. Detection of the FC on the old flagellum of cells not elongating a new flagellum and the flagellar sleeve is not an artefact of *Tb*CHE2 RNAi. (A) Negatively stained whole-mount cytoskeleton showing a partial FC (asterisk in the inset) part way down the old flagellum (of) in a *Tb*CHE2 RNAi-induced cell with two mature basal bodies (arrows). (B,C) The flagellar sleeve cannot be seen by scanning electron microscopy in a mutant that elongates a detached flagellum. The boxed region of B is shown at higher magnification in C. Bars 2 μm (A,B); 500 nm (C and inset in A).

kinetoplasts, suggesting the possibility of a causal relationship between these two events.

Our demonstration of a stop point suggests a model whereby force exerted by flagellum elongation, resisted at the distal end by a halted FC, could be transduced into a posterior poleward movement of the new flagellum basal body and concomitant segregation of kinetoplasts. Many RNAi mutants analysed in our laboratory and others (e.g. Dawe et al., 2005; Kohl et al., 2003) have revealed a reduced posterior kinetoplast and/or basal body movement phenotype. The phenotype is often displayed as a kinetoplast that fails to move adequately to the posterior position and hence one of the daughter cells appears more like an epimastigote trypanosome (kinetoplast anterior to the nucleus) than the normal trypomastigote (kinetoplast posterior to the nucleus). However, motility of the flagellum per se does not appear to cause mis-positioning of the new basal body and kinetoplast because procyclic PFR RNAi mutants are relatively normal in this capacity (Bastin et al., 2000). The effect is seen most clearly with some mutants that result in axonemal defects (e.g. PACRG) (Dawe et al., 2005) or flagellum detachment (e.g. FLA1) (Moreira-Leite et al., 2001).

There are, however, other issues that need to be accommodated in our model of basal body separation. For instance, in a procyclic trypanosome at the stage when the FC reaches the stop point the new flagellum is already laterally 'zipped' to the cell body by the macula adherens. Unless the macula adherens can slide in the membrane, this model would require a balance of FAZ extension and macula adherens formation. Could it be that the FAZ is an elastic structural extension, or that once halted at this stop point, its concomitant assembly (at either proximal and/or distal end) results in new basal body posterior directed motion? The relationship between the morphogenetic events of the FC stop point and basal body separation will only be addressed by specific inhibition of FC function once we have information on the molecular components of the FC.

The stop point

The stop point is a fascinating concept and it is intriguing to consider how the FC is halted. So far we have been unable to find any electron microscopic evidence for a 'structural buffer' in the old flagellum. This raises the question of whether the stop point represents a physical structure or merely reflects a temporal coordination of cytoskeleton and organelle rearrangements. However, as the FC can move past this normal stop point in RNAi mutant cell lines and/or following treatment with the protein phosphatase inhibitor okadaic acid (E.C. and P.G.M., unpublished observations) it is unlikely that a physical barrier exists to prevent FC migration along the old axoneme. It should be noted that in both these instances basal body separation is compromised, thus reinforcing our suggestion that a strict correlation exists between FC migration and basal body movement.

Our descriptions using the MPM2 anti-phosphoprotein monoclonal antibody provide new insight to the FC. In particular, our discovery of the FC remnant in the old flagellum suggests that at cytokinesis the interstitial material between the new and old flagellar membrane (typified by the AB1 antigen) is removed but that a remnant of the FC remains inside the old flagellum. This suggests that FC disassembly is not completed until the beginning of the next cell cycle; MPM2 provides a means to distinguish between cells with new and old flagella and confirms the asymmetry of the division product. Phosphorylation of proteins inside both new and old flagella provides an attractive mechanism for control of FC movement and disassembly, as is the case with desmosomes. We are currently investigating the MPM2-reactive proteins of the cytoskeleton.

The present demonstration of a relationship between the extending new flagellum, a stop point for connector movement and the segregation of the basal bodies and/or kinetoplasts raises an issue of whether there is also a 'licensing' of movement separate to the basal bodies at this point in the cell cycle. The basal body cycle in trypanosomes has been described in detail. When the new flagellum extends, the new and old basal body or probasal body complexes are close together. Presumably there is then a licensing event that allows their segregation. Although physical separation of duplicated basal bodies is clearly crucial to trypanosome morphogenesis, the licensing mechanisms controlling this event remain poorly understood. By analogy to centrosome disjunction in mammalian cells it is reasonable to assume that a balance between kinase and phosphatase activities will regulate basal body cohesion and separation (Meraldi and Nigg, 2001). NIMA-related kinases are central to this model and several studies have identified members of this protein kinase family as pivotal to centrosome maturation and cohesion (Prigent et al., 2005). Indeed, the Robinson laboratory have recently described a basal body located NIMA-related kinase (*Tb*NRKC) that appears to have a role in controlling *T. brucei* basal body separation (Pradel et al., 2006). This may well be

the first biochemical insight into molecular mechanisms controlling basal body segregation in trypanosomes. A large number of such kinases are present in the *T. brucei* genome (Parsons et al., 2005), and it is therefore likely that we still have much to learn about basal body disjunction and the coordination of this event with other morphogenetic processes.

FC movement and motor activity

How does the FC move? Our test of various possible locations for a FC motor, rehearsed above, revealed that the FC moves independently of construction of the new flagellum axoneme. Thus, an active process independent of new axoneme construction is sufficient to move the FC out of the flagellar pocket, dragging the sleeve of new flagellum membrane with it. Our previous work has shown that the FC forms and moves as normal in the paralysed PFR RNAi mutants showing that motility of the flagella per se is not a requirement for FC movement (Bastin et al., 2000). Therefore, it appears that the engine for the FC motor is present, at least in part, in the old flagellum. However, because the flagellum is attached along the length of the cell body, we cannot completely exclude an argument for a FAZ-dependent contribution to templated motor activity once the FC has exited the flagellar pocket. At present there is no discernable connection of the FC to the FAZ, but we need to acknowledge this as a formal possibility. Future structural and component analyses of the FC will resolve this.

One might reasonably ask whether our results provide evidence that FC movement is an IFT-independent process and therefore constitutes a novel motility function within a eukaryotic flagellum. We argue that our data are suggestive of this possibility, but here also there is a small caveat that must be considered. Although the phenotypes of IFT RNAi mutants in trypanosomes show that this process plays a major part in flagellum construction, its ablation has no significant effect on the old flagellum (this study) (Kohl et al., 2003), (J. A. D. Briggs and K.G., unpublished), raising crucial questions regarding IFT-mediated protein turnover in the old flagellum. However, incorporation of new protein into the old flagellum can clearly occur in wild-type trypanosomes, as exemplified by incorporation of epitope-tagged PFR2 protein (Bastin et al., 1999). Thus, there is a formal possibility that compartmentalisation of IFT machinery within the old flagellum represents a closed system, in which IFT motor proteins, IFT particles and molecular cargo simply recycle within the old flagellum compartment. Within such a hypothetical privileged compartment, RNAi-mediated loss of IFT may not have an immediate impact.

The FC and flagellar membrane formation

One of the major experimental findings from our studies is that a flagellar membrane is formed even in the absence of a new flagellum axoneme. This observation raises intriguing questions regarding the molecular composition of this membrane, and more specifically whether or not flagellar membrane proteins can be inserted into this sleeve structure. Can flagellar membrane proteins be transported into a flagellum in which IFT is clearly absent? The answer to this question is undoubtedly yes, because non-IFT-dependent flagellum transport mechanisms clearly exist; as evidenced by the accumulation of the membrane protein polycystin-2 at the distal tip of cilia in IFT-defective kidney epithelial cells (Pazour et al., 2002). Non-IFT-dependent transport has also been reported in *Leishmania major*; a trypanosomatid parasite closely related to *T. brucei*. Experiments by the McConville laboratory (Tull et al., 2004) have demonstrated that SMP-1, a small myristoylated flagellum membrane protein, is targeted and retained in flagellar membranes that lack an axoneme and PFR. How are these membrane proteins transported into the flagellum compartment in the absence of IFT? Studies in *Chlamydomonas* implicate the flagellum matrix and suggest that a Ca^{2+}-dependent mechanism operates to control directed flow of flagellar membrane proteins (Bloodgood and Salomonsky, 1994). However, much still remains to be learnt about this transport pathway and the *T. brucei Tb*CHE2 IFT mutant reported here may provide a model system to study this process.

How does the FC form? It apparently forms very early within the flagellar pocket (Briggs et al., 2004), but data presented in this paper clearly demonstrate that it does not have a strict requirement for new axoneme formation. This appears to place more emphasis upon the flagellar membranes themselves and indeed there is evidence for possible interaction between the two flagellar membranes in the flagellar pocket. If this interpretation is correct, then a role for non-IFT-dependent membrane transport may also need to be considered as an integral part of FC formation. However, FC formation may also be driven by secretion of interstitial FC material, such as the AB1 antigen, into the flagellar pocket and both processes may be important for FC formation.

This work has provided intriguing new insights into the functioning of the FC transmembrane mobile junction. Moreover, we have provided novel views of mechanisms that may well underpin morphogenetic processes in the trypanosome cell cycle. The recent announcement of the *T. brucei* genome sequence (Berriman et al., 2005) and our proteomic analysis of flagellar and cytoskeletal fractions (Broadhead et al., 2006) are important in enabling future identification of molecular components of the FC. This search will be advanced by this description of the reactivity of reagents such as MPM2. Once we have a view of components, the causal and functional dissection of the phenomena described here will be interesting.

Materials and Methods

Cell lines

Procyclic *T. brucei* cells (427 strain and its 29-13 derivative) were maintained at 27°C in SDM-79 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum as described previously (Brun and Schonenberger, 1979). To generate the *Tb*CHE2 inducible RNA interference (RNAi) mutant the primer combination 5′-*CTCGAG*CTGTAGAGGGGAAGGGAAAT-3′ and 5′-AAGCTTCTACCAAAG-TGACGCAGAAG-3′ (*Xho*I site italicised; *Hind*III site underlined) was used for PCR amplification from genomic DNA of a 740 bp region of the *T. brucei CHE2* orthologue. The resulting PCR amplicon was cloned into pGEM-T Easy (Promega), excised by digestion with *Xho*I and *Hind*III, and cloned into *Xho*I-*Hind*III-digested pZJM (Wang et al., 2000) to yield pZJM^{TbCHE2}. 20 μg *Not*I-digested pZJM^{TbCHE2} was used for transformation of the 29-13 cell line (Wirtz et al., 1999) and stable transformants were obtained using established protocols. *Tb*CHE2 RNAi mutants were maintained in the presence of the selectable agents hygromycin (50 μg ml^{-1}), G-418 (15 μg ml^{-1}), and phleomycin (3 μg ml^{-1}) until 48 hours before the start of an experiment. Inductions were initiated by addition of doxycycline to a final concentration of 1 μg ml^{-1}. Cell counts were determined using a CASY®1 cell counter (Schärfe Systems).

Immunolocalisation

Procyclic trypanosomes were settled onto glass slides and whole-mount cytoskeletons prepared by extraction with 0.5% NP-40 in 100 mM PIPES, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM EDTA. Cytoskeletons were fixed in 3.7%

paraformaldehyde for 10 minutes before antibody labelling with MPM2 (Upstate) and either ROD1, BBA4 (Woods et al., 1989) or AB1 (Briggs et al., 2004). MPM2 labelling was visualised by FITC-conjugated anti-mouse IgG (Sigma), and ROD1, BBA4 and AB1 labelling by TRITC-conjugated anti-mouse IgM (Chemicon) secondary antibodies. Cells were embedded in Vectashield containing 4,6-diamidino-2-phenylindole (Vector Laboratories). Slides were examined on a DeltaVision RT microscope, images captured on a Hamamatsu CCD camera and processed in softWoRx (Applied Precision) and Adobe Photoshop (Adobe). Cytological measurements taken from MPM2-labelled images were measured using softWoRx (Applied Precision).

Electron microscopy

Cells were harvested by centrifugation (800 ***g***, 10 minutes), fixed in 2% paraformaldehyde, 2% glutaraldehyde and 0.2% picric acid in 100 mM phosphate buffer (pH 7.2), post-fixed and processed for transmission electron microscopy as described previously (Sherwin and Gull, 1989). Scanning electron microscopy and the preparation of negatively stained whole mount cytoskeletons for transmission electron microscopy was also carried out using previously established methods (Moreira-Leite et al., 2001; Sherwin and Gull, 1989). Measurements of cell and flagella lengths and positions of organelles were made on electron micrographs of cytoskeletons (Sherwin and Gull, 1989) projected onto a screen.

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