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A Trypanosome Structure Involved in Transmitting Cytoplasmic Information During Cell Division

Flávia F. Moreira-Leite, Trevor Sherwin,* Linda Kohl,† Keith Gull‡

African trypanosomes are protozoan parasites that cause sleeping sickness in humans through a tsetse fly vector. The procyclic form of *Trypanosoma brucei* has a single, attached flagellum that describes a helical path along the cell from posterior to anterior. During division, a specific flagellum-flagellum connection is elaborated between the new and old flagellum. This connector was present only during cell duplication and was found to be involved in the replication of the helical cell pattern and polarity. This finding implicates the concept of cytotaxis in cell morphogenesis in trypanosomes.

A procyclic (tsetse form) trypanosome has a single flagellum that emerges from an invagination of the plasma membrane termed the “flagellar pocket,” close to the posterior end of the cell. The flagellar pocket is the sole site for membrane traffic to and from the cell surface. The flagellum is attached to the cell body throughout most of its length and follows a left-handed helical path toward the anterior end. Along the region of attachment, known as the flagellum attachment zone (FAZ) (1, 2), the membranes of the flagellum and cell body are intimately apposed. Inside the cell, this region of apposed membranes is associated with two internal cytoskeletal structures—the FAZ filament and a subset of four specialized microtubules—both of which run from the basal body to the anterior end of the cell, underlying the flagellum. The flagellar cytoskeleton is cross-linked to the cell body cytoskeleton through the FAZ, and the arrangement of these structures confers the typical polarity and helicity of a trypanosome cell. During cell duplication, unique mechanisms such as the interaction between the flagellum and the mitochondrial DNA are involved in the rigorous replication of this cell pattern and polarity (3).

The emergence of a short new flagellum (Fig. 1A) from the flagellar pocket is one of the earliest events in cell duplication of *T. brucei*. Later, separation of the basal bodies (which subtend the proximal ends of the fla-

gella) produces a second flagellar pocket for the new flagellum (Fig. 1B) and mediates the segregation of the replicated mitochondrial DNA in the kinetoplasts (3). The new flagellum emerges closer to the posterior end of the cell and is always on the left side of the old flagellum (Fig. 1B) when the cell is viewed from its posterior end. Positioning of the new flagellum and FAZ defines a pattern that influences the helical arrangement of the cytoskeleton, the axis and polarity in cell division and cell shape, the position of membranous organelles, and the direction of motility of daughter cells (2, 4).

Examination of the cell cycle of *T. brucei* by scanning electron microscopy (SEM) (5)

revealed that, although the proximal ends of the flagella moved apart during division, the distal tip of the new flagellum remained associated with the old flagellum throughout cell duplication (Fig. 1, B and C). The distal tip of the new flagellum always contacted the lateral aspect of the old flagellum, and always from the left when the cell was viewed from the posterior end.

We were prompted to investigate this phenomenon further because it indicated the existence of a specifically localized flagellum-flagellum association during duplication. This would implicate the position and orientation of the old flagellum in directing the morphogenesis of the new flagellum and, consequently, of the internal cytoskeleton.

When we observed negatively stained cytoskeletons (detergent-extracted cells) of dividing trypanosomes by transmission electron microscopy (TEM) (5), we noted the presence of a characteristic structure at the distal tip of the new flagellum (Fig. 2). We termed this structure the “flagella connector” (FC) because it appeared to link the two flagella. The FC had a roughly triangular profile (Fig. 2, B to E) and was present only at the distal tip of the new flagellum. This corresponded exactly to the position where, as determined by SEM, the new flagellum was associated with the old (Fig. 1). Thus, it could be that the FC mediates the physical attachment of flagella and that this attachment is detergent-resistant. The FC has a lamellar core (Fig. 2, C to E), which may represent segments of membrane rendered detergent-resistant by proteins responsible for

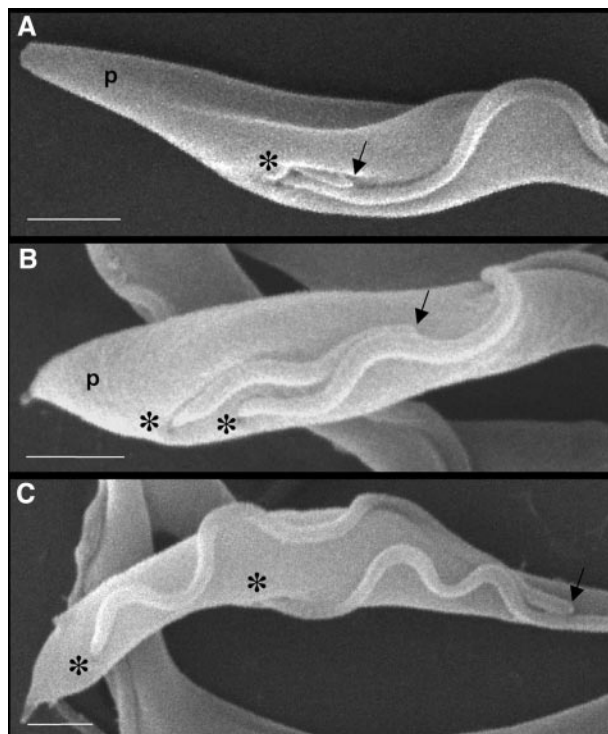


Fig. 1. The distal tip of the new flagellum remains associated with the old flagellum during cell duplication of *T. brucei*, even as the proximal ends of both flagella move apart. (A to C) SEM micrographs of trypanosomes in different stages of the cell cycle with new flagella of different lengths: late G_1/S (A), G_2 (B), and postmitotic (C) phases. Arrows point to the tip of the new flagellum; asterisks mark the region of the flagellar pocket and the basal body (p, posterior end of the cell). Scale bars, 1 μm .

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the attachment. The *T. brucei* flagellum contains, in addition to the axoneme, a massive paracrystalline structure—the paraflagellar rod (PFR) (6). Examination of cytoskeletons showed that the FC connected the distal tip of the axoneme of the new flagellum to the lateral aspect of the axoneme, but not the

PFR, of the old flagellum (Figs. 2 and 3).

We used two approaches to test whether the FC was indeed performing the flagellum-flagellum attachment independent of other internal cell body components. First, we treated whole-mount cytoskeletons with 60 mM Ca^{2+} (5), which depolymerizes the sub-

pellicular microtubules, leaving the axonemal microtubules and the PFR intact (7). The FC not only survived such treatment, but its substructure became even more clearly distinguishable (Fig. 3A). The FC appeared to have three main subcomponents: a “fuzzy” component at the extreme distal tip of the new flagellum, a short link structure, and a plate-like structure with a lamellar core that “sits” directly on the microtubules of the axoneme of the old flagellum (Fig. 3A).

The morphology of the connector, as seen in whole cytoskeletons and Ca^{2+} -flagella preparations, supports the idea of a junctional complex between two membrane domains, linked (directly or indirectly) to the internal axonemal microtubules. Because the FC changed position in the old flagellum as the new flagellum extended, the junctional complex must be moving along the old axoneme toward its distal tip, a motility that could be powered by its association with kinesin-type motor proteins.

The second approach used to test the localized “tip-to-side” flagellum-flagellum attachment hypothesis was to produce trypanosomes where flagellum–cell body attachment was disrupted. Trypanosomes lacking flagellum–cell body attachment were made by inducible double-stranded RNA interference (RNAi) (8–10) of the gene encoding Fla1 (11), a surface membrane glycoprotein vital for flagellum–cell body attachment in *T. brucei* (12) and the related human parasite *T. cruzi* (13). Inducible RNAi of Fla1 was produced by inserting a 1-kb segment of its coding sequence in the pZJM vector (14) and stably transfecting procyclic trypanosomes (15, 16).

After growing a nonsynchronous culture of stably transfected cells for 24 hours in the presence of tetracycline (to induce RNAi of Fla1), we specifically observed the subpopulation of cells in late G_2 or mitosis, because in these cells it was easy to discern both old and new flagella within the same cell (17). Our previous work (where cells in this stage of the cell cycle were particularly informative) showed that inducible RNAi in trypanosomes can act to ablate a flagellum protein in the new flagellum while leaving the old flagellum unaffected (10). Induction of RNAi in Fla1 produced trypanosomes with a new flagellum forming a loop detached from the cell body for most of its length, but remaining tethered to the old flagellum by the tip FC (Fig. 3B). In these cells, the old flagellum remained attached to the cell body and the ultrastructure of the FC appeared unaltered (Fig. 3B). An indication of the strength of FC attachment came from the observation of live induced cells by differential interference contrast microscopy. Films of these cells (18, 19) show that the loop of the new flagellum beats vigorously and freely while the distal tip

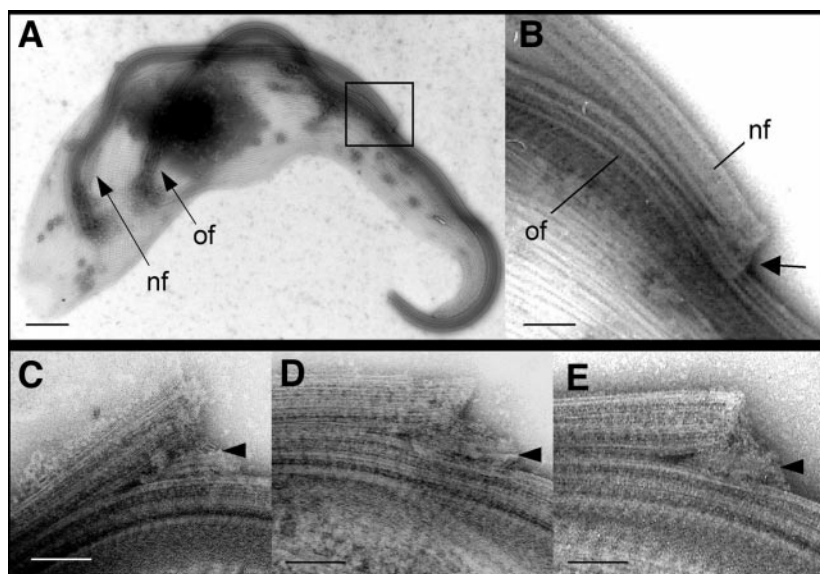
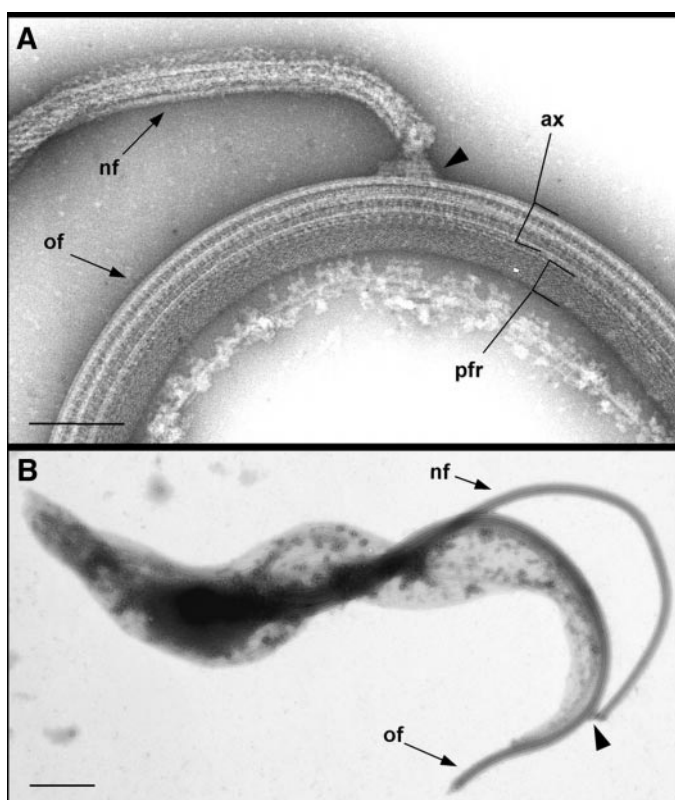


Fig. 2. A characteristic detergent-resistant structure, the flagella connector (FC), is present at the tip of the new flagellum and contacts the old flagellum. (A) Negatively stained cytoskeleton of a trypanosome undergoing cell duplication (nf, new flagellum; of, old flagellum). (B) Higher magnification of the boxed area in (A), showing the FC at the tip of the new flagellum, linking the tip of the axoneme of this flagellum with that of the old flagellum. (C to E) Images of the FC in different cells, showing the central lamellar core (arrowheads), possibly representing membrane remnants. Scale bars, 1 μm (A), 200 nm (B to E).

Fig. 3. Evidence for the existence of flagellum-flagellum attachment at the FC. (A) CaCl_2 treatment removes the subpellicular microtubules, but does not affect the axoneme (ax) or the paraflagellar rod (pfr) (other labels as Fig. 2). Attachment at the FC also resists CaCl_2 treatment (arrowhead). (B) Induction of RNAi of Fla1 for 24 hours produces a phenotype where the new flagellum (but not the old) describes a loop, being detached from the cell body but still attached to the old flagellum by its tip via the FC (arrowhead). Scale bars, 200 nm (A), 1 μm (B).



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remains firmly attached to the old flagellum. Thus, FC attachment must have been of considerable strength because it was not disrupted by the vigorous movement of the new flagellum loop. The Fla1-RNAi phenotype showed that the nature of FC attachment is likely to be different from that of lateral flagellum–cell body attachment. The FC must disappear before the completion of cytokinesis, and preliminary data indicate that the connection between the old and new flagella disappears at, or soon after, the start of cytokinesis.

The FC is a unique structure that, during cell duplication, physically links a forming cell component with the preexisting version. It is unlikely that the old flagellum is providing a template for the formation of the new flagellar axoneme, because this has not been observed in other flagellates. Rather, a direct consequence of the “tip-tethering” is that the old flagellum restricts and directs the positioning and orientation of the new flagellum in relation to the topography of the cell body. The exact configuration of the attachment we have described guides and maintains the relative posterior position, circumferential position, and helical track of the new flagellum. This process is likely to be essential for defining the arrangement of internal cytoskeletal structures that are linked to the attached flagellum, such as the FAZ and the subpellicular microtubules.

There is evidence that the arrangement of these structures is intimately associated with the definition of cell shape and division plane (2, 4, 20). We have performed immunofluorescence microscopy on Fla1-RNAi cells to detect the internal FAZ organization. These experiments showed that when the flagellum was detached, the internal FAZ structures were not formed properly. It appears, therefore, that the cytotoxic cues of the FC are subsequently translated to define the lateral attachment process, which in turn influences the positioning and polarity of the internal FAZ structures so important for cell morphogenesis and division. The use of the attached flagellum in providing information for the morphogenesis of trypanosomatid parasites

seems to be at its most extreme in the trypanomastigote form (21, 22).

This reproduction of the existing helical pattern in such a distinctive manner implicates the concept of cytotaxis, defined by Sonneborn (23) as “ordering and arranging of new cell structure under the influence of pre-existing cell structure” in cell morphogenesis in trypanosomes. Since the early illustration of cytotaxis in *Paramecium* by Beisson and Sonneborn (24), a few other examples have come from other ciliates (25), and more recently the phenomenon has been implicated in maintaining the bipolar pattern of budding in yeast (26). Our results not only show a clear example of Sonneborn’s concept of cytotaxis, but also provide, in the form of the FC, a definition of the structure and mechanism that executes the phenomenon in this instance. Future knowledge of the composition of the FC will enable the molecular mechanisms involved to be elucidated.

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