Microbes and Infection, 2, 2000, 1865–1874
© 2000 Éditions scientifiques et médicales Elsevier SAS. All rights reserved
S1286457900013447/REV

Review

Inside and outside of the trypanosome flagellum: a multifunctional organelle

Philippe Bastin*, Timothy J. Pullen, Flávia F. Moreira-Leite, Keith Gull

Department of Biochemistry, School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, UK

ABSTRACT – Amongst the earliest eukaryotes, trypanosomes have developed conventional organelles but sometimes with extreme features rarely seen in other organisms. This is the case of the flagellum, containing conventional and unique structures whose role in infectivity is still enigmatic.
© 2000 Éditions scientifiques et médicales Elsevier SAS

trypanosomes / flagellum / cytoskeleton / axoneme / paraflagellar rod / microtubule

1. Introduction

Parasites of the Kinetoplastida order are responsible for a wide variety of diseases affecting humans, animals and plants. In medical terms, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* cause sleeping sickness in central Africa, *Trypanosoma cruzi* is responsible for Chagas' disease in South America and several *Leishmania* species are the causative agents of leishmaniasis, which can vary from the relatively benign cutaneous infection to the lethal visceral form [1]. In total, about 30 million people are infected and half a billion are at risk. There is no vaccine against any of these diseases and the few effective drugs often display highly toxic side effects.

Trypanosomes and leishmania are digenetic parasites, meaning that they alternate between two different hosts: a mammal and an insect vector. Differentiation prior to contamination of the next host is critical, given the abrupt change of their environment. African trypanosomes proliferate as extracellular parasites in the bloodstream of their mammalian host, from where they can be picked up by a tsetse fly during a bloodmeal. Differentiation-competent trypanosomes [2] survive and proliferate in the gut, then move in the peritrophic space of the midgut. After differentiation, trypanosomes migrate towards the salivary glands, where they attach to the epithelium before maturing into the infective form again [3]. In contrast, *T. cruzi* and *Leishmania* species need to invade cells and replicate as intracellular parasites in their mammalian hosts. Other species of kinetoplastids that have received attention are *Phytomonas*, infecting plants, and monogenetic parasites of insects such as *Crithidia* and *Herpetomonas* species.

Kinetoplastids are eukaryotes and hence exhibit conventional features such as the presence of a nucleus delimited by a nuclear membrane and of organelles such as the endoplasmic reticulum, the Golgi apparatus, the endo/exocytosis system, the mitochondrion, etc [4]. However, many of these organelles exhibit specific and sometimes extreme features often found only in kinetoplastids. A typical example is the mitochondrion, which shows 'traditional' characteristics such as the presence of the outer and inner membranes, tubular cristae, etc. However, there is only a single mitochondrion per cell, it is extremely large and elongated, and its whole DNA content is condensed in a sub-structure called the kinetoplast. In addition, mitochondrial gene expression is subjected to extensive RNA editing [5]. This mitochondrial genome is so big that it can be seen by fluorescence light microscopy (*figure 1*). This implies some accurate mechanisms for successful organelle duplication and segregation during the cell cycle. Trypanosomes have solved this for the mitochondrion by linking kinetoplast replication with that of an element of the cytoskeleton, the basal body of the flagellum [6, 7].

In this review, we examine the trypanosome flagellum, which shares common features with most flagella, such as the presence of a classic axoneme, but also exhibits a rare extra-axonemal structure, the paraflagellar rod (PFR) [8]. Trypanosomatids possess a single flagellum that exits from a flagellar pocket (*figure 2*), a specialised invagination of the plasma membrane where the entirety of endocytosis/exocytosis traffic takes place [9]. In *Trypanosoma* spp. the flagellum is attached along the cell body for most of its length, with the exception of the distal tip (*figure 1A, B*). The site of attachment defines a specialised region of the flagellum and of the cell body called the flagellum attach-

* Correspondence and reprints: Laboratoire de biophysique, Muséum national d'histoire naturelle, Inserm U 201 – CNRS-UMR 8646, 43, rue Cuvier, 75231 Paris cedex 5.
E-mail address: pbastin@mnhn.fr (P. Bastin).

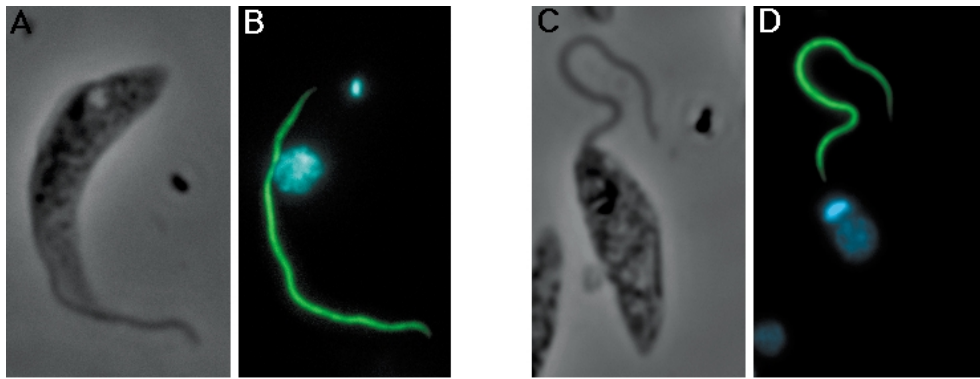

Figure 1. *T. brucei* (procyclic form) (**A**, **B**) and *L. mexicana* (promastigote form) (**C**, **D**) as viewed by phase contrast microscopy. Cells were fixed in methanol and processed for immunofluorescence with the anti-PFR antibody L13D6 [35], shown in green, that exclusively labels the flagellum. The nucleus (large signal in the middle of the cell) and the kinetoplast (spot at the base of the flagellum) were stained with the DNA-intercalating dye, DAPI (blue).

ment zone (FAZ). This is not the case of other trypanosomatids such as *Leishmania* species, which present a free flagellum (*figure 1C, D*). The *T. brucei* flagellum can be purified by detergent extraction of the cytoskeleton, followed by high-salt treatment. In these conditions, only the axoneme, the basal body and connected kinetoplast, the paraflagellar rod, and the four microtubules associated with the FAZ remain (*figure 3*).

The flagellum could accomplish several functions. Firstly, it is involved in cell motility: trypanosomes and leishmania are actively motile cells, swimming at average speeds of 10–30 µm per second in the culture medium [10, 11]. A striking feature is the fact that they swim with their flagellum leading, i.e. the flagellum drags the cell behind it. This is related to the way wave forms are initiated: from tip to base, and not from base to tip as seen in the majority of flagellated organisms. Secondly, in the case of parasites such as *T. cruzi* or *Leishmania* spp., the flagellum might be needed to reach and invade host cells. The intracellular stage of these parasites (amastigote) presents a very short flagellum, limited to the flagellar pocket. Thirdly, the flagellum is clearly involved in attachment of the parasite to host surfaces. When kinetoplastids transit through one or several parts of the digestive tract of their insect vector, they attach to some tissues, presumably to avoid being cleared before maturation. Attachment takes place via extensive development and ramification of the anterior end of the flagellum, accompanied by accumulation of large amounts of intraflagellar material. In addition, plaques resembling hemidesmosomes seem to anchor the flagellar membrane to the host tissues [12–14]. Finally, the flagellum could be involved in signalling and act as an environment sensor.

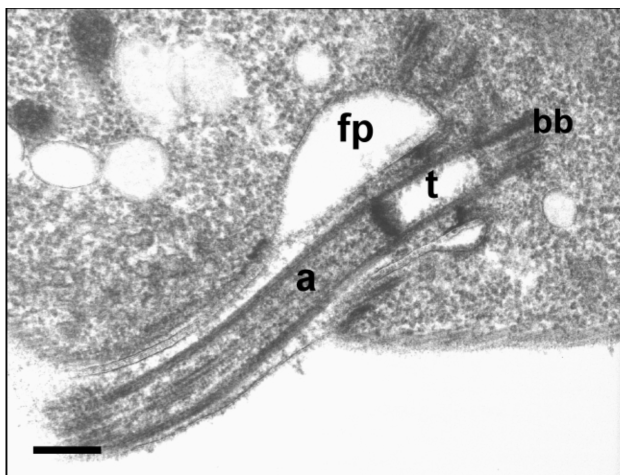

Figure 2. Longitudinal section of the flagellar pocket (fp) region of *T. brucei* showing the emergence of the flagellum from the cell body. The membranes of the cell body, flagellar pocket and flagellum represent different domains of a continuous bilayer. bb, mature basal body, a, axoneme. Scale bar, 200 nm.

The understanding of trypanosome biology has progressed rapidly over recent years thanks to the tremendous development accomplished in reverse genetics [15–18]. Questions we would not have dared to address 10 years ago can now be answered. In addition, the genomes of *T. brucei*, *T. cruzi* and *Leishmania* spp. are under scrutiny and millions of bases have already been sequenced and are directly available via the internet [19, 20]. This combination of reverse genetics and molecular information will no doubt provide more exciting discoveries from the intriguing trypanosomes, for both parasitic and scientific aspects.

2. The flagellar membrane

The *T. brucei* plasma membrane can be defined as consisting of three contiguous but distinct domains: the pellicular membrane, the flagellar membrane, and the flagellar pocket membrane [21] (*figure 2*). All three are covered by a densely packed variable surface glycoprotein coat when parasites infect the bloodstream or during the metacyclic stage in the salivary glands of the tsetse fly [22], or by a procyclin coat in the other insect vector

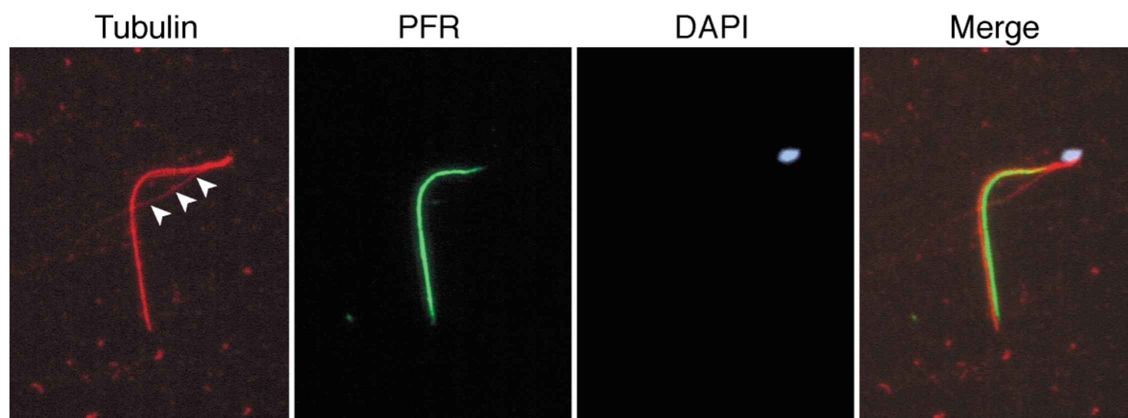

Figure 3. Trypanosome flagella were purified by detergent extraction of their cytoskeleton, followed by high-salt treatment [33], fixed and processed by immunofluorescence using the monoclonal antibodies TAT-1 [66] (recognising tubulin and hence the axoneme, shown in red) and ROD-1 [66] (recognising the PFR, shown in green). The preparation was also stained with DAPI (blue) identifying the mitochondrial DNA that remains tightly associated to the basal body of the flagellum. The four microtubules associated with the FAZ are indicated with arrowheads.

stages. However, the density of the glycoprotein coat appears different on the flagellar pocket membrane. Given its high specialisation as the only place of endocytosis and exocytosis, the flagellar pocket membrane is the major site where cell surface receptors are encountered, such as transferrin and LDL receptors [9]. Since all integral membrane components must first be transported to the flagellar pocket, the mechanisms of targeting to different domains must act here. This is raising interesting questions about mechanisms responsible for differential sorting of cell surface proteins.

The three domains of the plasma membrane differ significantly in both lipid and protein composition. Treatment of trypanosomes with the antibiotic filipin, which binds to β-hydroxysterols, forms complexes when viewed in freeze-fracture micrographs. The flagellar membrane of both procyclic and bloodstream *T. brucei* contains a higher density of filipin-sterol complexes than the pellicular membrane [23]. Similar results have been observed in *T. cruzi* [24] and *Leishmania mexicana* [25]. Since sterols make membranes more rigid and less deformable, Vickerman and Tetley [26] proposed this might be required for the flagellum, which lacks the underlying microtubule corset which supports the pellicular membrane. Freeze-fracture micrographs also revealed that intramembranous particles, thought to be integral membrane proteins, are less abundant in the flagellar membrane [26]. This difference is confirmed by the specific localisation of individual proteins to different domains of the plasma membrane.

At least three types of protein have been shown to be localised only to the flagellar membrane: adenylate cyclases encoded by the expression site-associated gene, *ESAG4* and related genes, *GRESAG 4.1*, *4.2* and *4.3* in *T. brucei* [27], a flagellar calcium-binding protein (FCaBP) in *T. cruzi* [28] and one isoform of a glucose transporter in *Leishmania enriettii* [29]. The latter is a particularly striking example of differential targeting: isoform 1 (ISO1) is localised to the flagellar membrane, whereas isoform 2 (ISO2) is found on the pellicular membrane. The two proteins only differ by their cytosolic amino-terminal end. The fact that both isoforms are found in the flagellar pocket demonstrates that sorting occurs after they reach the plasma membrane. While the above all highlight the functional differences between the membrane domains, they have also provided tools to investigate the mechanisms that form and maintain these differences.

The sequence responsible for the differential targeting of ISO1 and ISO2 is found in their amino-terminal region, and has been characterised by reverse genetics [30, 31]. The first 30 amino acids are necessary for the correct targeting of ISO1, and the first 35 amino acids of ISO1 are sufficient to re-target ISO2 to the flagellum. This targeting sequence has been found to work in several *Leishmania* species and may even be more widely conserved. Closer analysis revealed that amino acids 20–35 are sufficient to partially target proteins to the flagellum. Although no single amino acid within this region is essential for flagellar targeting, individual mutations within a cluster of five contiguous amino acids, RTGTT, altered the degree of flagellar localisation.

The flagellar FCaBP of *T. cruzi* appears to be localised to the flagellum by a different pathway. Whereas ISO1 is an integral membrane protein targeted to the flagellar membrane via the flagellar pocket, FCaBP is attached to the plasma membrane by two acyl modifications. A myristoylation and a palmitoylation at the amino-terminus are responsible both for its attachment to the membrane and for its flagellar localisation [32]. Specific alanine substitutions which prevent the two acyl modifications inhibit association with the flagellar membrane, and result in localisation of the mutant protein throughout the cell. One possible explanation for the observed localisation is that the acyl modifications may only be able to interact with

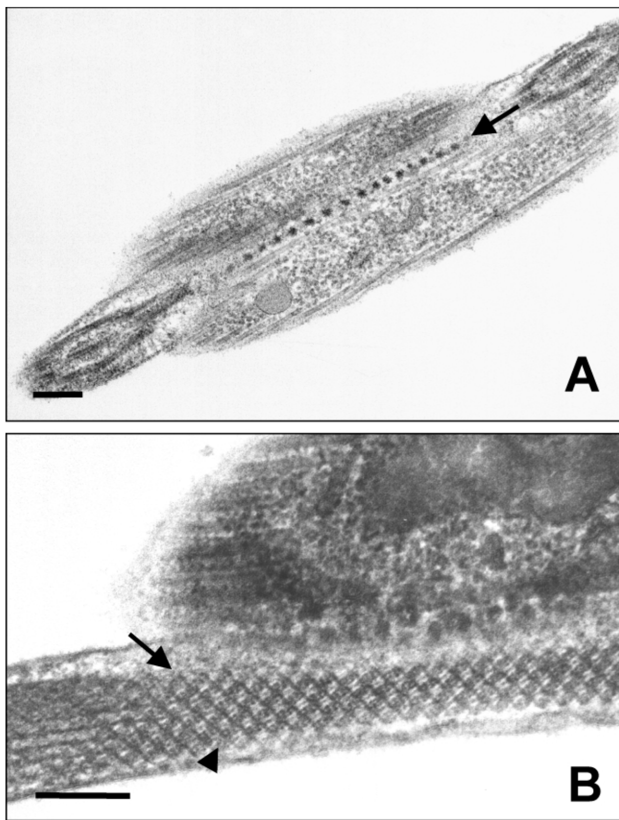

Figure 4. Longitudinal views of the flagellum attachment zone (**A**) and the paraflagellar rod (**B**). The FAZ is seen as a row of punctate structures (**A**, arrow) intercalated between microtubules of the subpellicular corset. In the PFR, plates of thin (**B**, arrow) and thick (**B**, arrowhead) filaments cross each other at a defined angle in the proximal and distal domains. Scale bar, 200 nm.

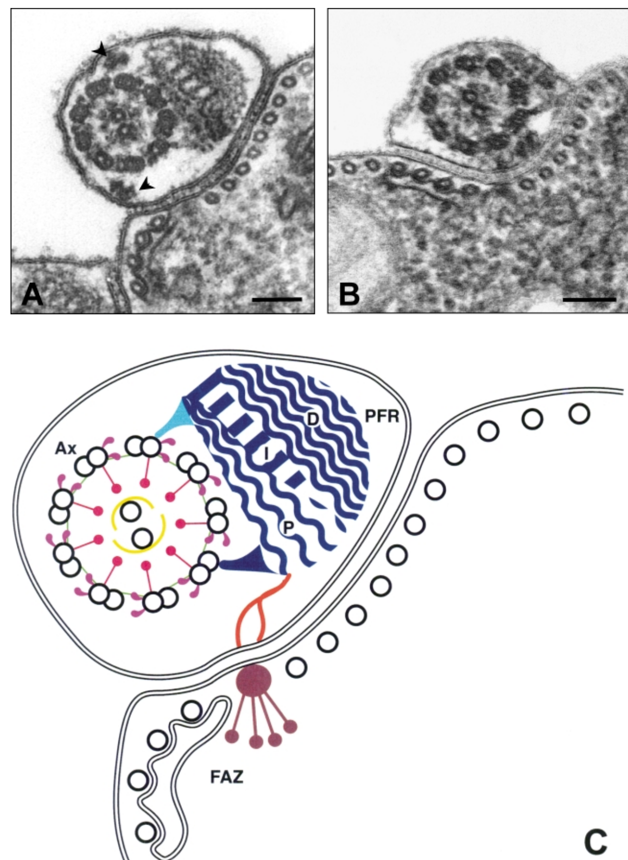

Figure 5. Transverse section of the flagellum of wild-type *T. brucei* (**A**) and a paralysed *snl-2* mutant (**B**) in the region of attachment to the cell body. Scale bar, 100 nm. The basic components of the flagellum are indicated in the diagram (**C**). While a well-developed PFR is present in the wild type (**A**), only a rudimentary structure is present in the paralysed mutant (**B**). Intraflagellar particles, possibly related to rafts described in other systems [79], are seen in some cross-sections (arrowheads on **A**). The axoneme (Ax) is made of the classic 9+2 structure with dynein arms (magenta), nexin links (green), radial spokes (red) and the central sheath (yellow). The PFR (blue) can be divided into three domains: proximal (p); intermediate (i); and distal (d), defined by their position relative to the axoneme. Filaments are connecting the proximal domain of the PFR to doublet 4 (light blue) and 7 (dark blue), and to the FAZ region (orange). This one is made of a cytoplasmic filament (brown), intercalated in a gap between two microtubules of the subpellicular corset, and of four specific microtubules associated with the smooth endoplasmic reticulum.

the particular lipid composition of the flagellar membrane described above.

3. The flagellum attachment zone (FAZ)

The trypanosome flagellum is attached to the cell body along most of its length (*figure 1*). At this site, the flagellum and the cell body membranes are in tight contact and flanked by original structures, which have been defined as the FAZ. The most obvious feature is the presence of a cytoplasmic, electron-dense filament, located in a gap between two microtubules of the subpellicular corset (*figures 4A, 5*). A longitudinal view of the FAZ displays a row of punctate structures intercalated in the subpellicular corset of microtubules (*figure 4A*). To the left of this filament, when the cell is viewed towards the anterior end, there is a group of four microtubules always very closely associated to a profile of endoplasmic reticulum (*figure 5*). These 4 microtubules are very stable and seem to be anchored close to the base of the flagellum, at the expected position of the flagellar pocket [33] (*figure 3*). On the flagellum side of the FAZ, filamentous structures connect the proximal domain of the paraflagellar rod (see below) to the region of the membrane associated with the FAZ [33] (*figure 5*).

A few proteins have been localised to the FAZ in *T. brucei* and *T. cruzi* (reviewed in [34]). Recently, two monoclonal antibodies were shown to stain the FAZ by immunofluorescence and to recognise a doublet of high-molecular-weight proteins [35]. The corresponding gene encodes a protein containing repetitive elements and which shares homology with *T. cruzi* proteins (Kohl L., Bastin P., Gull K., unpublished).

It is not obvious why trypanosomes anchor their flagellum alongside the cell body. Interestingly, a *T. cruzi* mutant

where the *GP72* gene (encoding a surface glycoprotein) was deleted exhibited a free flagellum. The mutant grew normally but its motility was altered, with parasites tending to sink towards the bottom of the culture flask [36]. Apart from being detached, the overall morphology of the flagellum did not appear to be greatly modified. The survival of the mutant in the insect host was severely reduced, but it was difficult to assess whether this is due to the absence of gp72 or to the detached flagellum [37]. The *T. brucei* homologue, *FLA-1*, could not be deleted, suggesting it might be essential in this organism [38]. At present, there is no direct evidence that the components of the FAZ are actually involved in flagellum attachment. The FAZ may also play a central role in cell division, possibly for the determination of the axis of cytokinesis [39].

4. The axoneme

The axoneme of the flagellum of trypanosomes displays the canonical 9+2 structure that is remarkably well conserved throughout eukaryotic evolution. It exhibits the nine peripheral microtubule doublets and two central single microtubules with its associated structures (*figure 5*). Morphological analysis identifies the conventional components of the axoneme, apparently similar to other axonemes, such as the inner and outer dynein arms, the radial spokes, connecting the peripheral doublets to the central pair, the nexin links, associated with peripheral doublets, and the projections of and connections between the central pair of microtubules. However, the biochemistry of these components has not been studied in trypanosomes. Trypanosomatids present the usual 'flagellum-type' beating consisting of characteristic waves, starting from tip to base. Modulation of flagellar beating by Ca^{2+}, typical in other eukaryotes has been reported in *Crithidia oncopelti* [40, 41]. In addition, a number of Ca^{2+}-binding proteins have been localised to the trypanosome flagellum [28, 42]. The physiological function, if any, of the Ca^{2+}-binding properties of these proteins is unknown, but it is tempting to speculate that they could have a role in modulating axonemal function.

5. The paraflagellar rod (PFR)

In addition to the conventional axoneme, a second large structure can be seen within the trypanosome flagellum: the PFR, also called the paraxial rod or the paraxonemal rod (*figures 4B, 5*) [43]. It is present from the point where the flagellum exits the flagellar pocket and runs alongside the axoneme right to the distal tip. Longitudinal sections reveal an elegant organisation of filaments, crossing each other at defined angles (*figure 4B*). Cross-sections reveal that the *T. brucei* PFR has a crescent shape and a diameter of ~150 nm, quite similar to that of the axoneme. On such sections one can discriminate three domains defined by their position relative to the axoneme [44]: a short proximal domain, an intermediate domain and a more developed distal domain (*figure 5*). The proximal and distal domains have a very similar structure, with several 'plates' stacked parallel to each other, whereas the intermediate domain is composed of filaments linking the other two domains. The PFR is physically connected to the axoneme via fibres attaching the proximal domain to the microtubule doublets 4 through 7. This connection is extremely strong and resistant to a variety of treatments, making PFR purification difficult [45]. In addition, other filaments connect the proximal domain of the PFR to the FAZ (*figure 5*).

A PFR has so far only been seen in three groups of protists: the kinetoplastids, the euglenoids and the dinoflagellates [46]. It is therefore not restricted to parasitic species and has not been seen in multicellular organisms, nor in other protists such as the genus *Chlamydomonas*. The overall organisation of the PFR, with the three domains, has been described in other trypanosomatids [44]. However, PFR diameter can vary between species: from 150 nm in *T. brucei* and *Crithidia fasciculata* to 250–300 nm in *Leishmania*, *Herpetomonas* or *Phytomonas* species. [44]. As a result, the flagellum of the latter organisms presents a more elliptical shape. Some *Crithidia* species do not possess a PFR structure in their flagellum, and apparently behave normally, although detailed comparison remains to be carried out (discussed in [43]).

Kinetoplastids are divided into two subgroups: the trypanosomatids, exclusively parasitic organisms including the species listed above, and the bodonids, containing both parasitic and free-living members. The latter are characterised by the presence of two flagella, exiting from the pocket at the anterior end of the cell. One flagellum (leading or anterior) beats actively, dragging the cell behind it, whereas the other flagellum (recurrent or posterior), is often attached to the cell body, exhibiting a slower but planar movement, maybe to guide the cell forward. In the absence of beating of the anterior flagellum, the recurrent flagellum will push the cell forward or backward in a gliding motion [47]. Interestingly, only the recurrent flagellum exhibits the complex, 3-domain, PFR structure described above [48, 49]. The anterior flagellum presents a less developed PFR with a more tubular aspect, but that also exhibits a lattice-like structure when viewed in longitudinal sections. Hence, the PFR is larger in the recurrent flagellum than in the anterior one, a feature explaining the difference in diameter between the two flagella. Similar observations have been made in euglenoids, where a PFR with the tripartite structure is present in the slow-moving ventral (posterior or recurrent) flagellum, and a less well-defined and rather tubular structure flanks the axoneme in the dorsal (anterior) flagellum [49].

Extra-axonemal structures sharing morphological similarities with the PFR have been reported in some other protists, such as *Tritrichomonas* [50] or *Giardia* species [51]. Biochemical, immunological and genetic comparison of these structures with the PFR of kinetoplastid should be quite interesting. Finally, different extra-axonemal structures have been described in other organisms, in particular in the sperm flagellum (reviewed in [52,53]) but none of them are apparently related to the PFR.

For the last three decades scientists have tried to understand the composition and the function of the paraflagellar rod. Pioneering biochemical studies in *Euglena* spp. [54]

Table I. Nomenclature of *PFR* genes.

Species	*PFR1*	*PFR2*
T. brucei [56, 57, 59]	*PFRC*	*PFRA*
L. mexicana [60, 75]	*PFR1*	*PFR2*
T. cruzi [58, 62]	*PAR3*	*PAR2*
E. gracilis [61]	n.d.	*PR-40*

n.d. not determined

and in *C. fasciculata* [45] identified a doublet of proteins subsequently found in all other organisms with a PFR: PFR1 (M_r 70–80 K) and PFR2 (M_r 65–72 K) ([43] and references therein). The first monoclonal antibody raised against *Euglena* PFR recognised both PFR1 and PFR2 by immunoblotting and cross-reacted with all *Trypanosoma* species tested, including *T. brucei* [55]. Similar results were obtained with different mono- and polyclonal antibodies, and further reports of band-specific antibodies suggested that PFR1 and PFR2 were not identical [35, 56–58]. By immunofluorescence, anti-PFR proteins exclusively recognised the flagellum (*figure 1*).

Such antibodies were used to clone the *PFR* genes from expression libraries, first from *T. brucei* [56, 57, 59], then from *L. mexicana* [60] and *Euglena gracilis* [61]. The *T. cruzi* homologues were discovered after direct amino acid sequencing of purified PFR proteins [58, 62]. Unfortunately, for historical reasons, the *PFR* genes were given different names (*table I*). For simplicity, we will refer to *PFR1* for the gene encoding the slowest migrating protein (top band) and to *PFR2* for the fastest one (bottom band). As expected from the immunological cross-reactivity described above, the sequences of *T. brucei* PFR1 and PFR2 display extensive identity throughout their length (60%), and quite remarkably, without a single insertion/deletion [56, 59]. PFR1 and PFR2 are well conserved between species and only the ends show significant diversity. When compared with proteins from other organisms, PFR1/2 form a unique family. Low identities are found with different regions of myosins, probably because of the abundance of predicted coil-coiled regions in both families [61] (our unpublished observations). There is no identity at all between PFR1/2 proteins and the myosin motor domains. Because of their unique localisation to the flagellum, PFR1/2 proteins represent an ideal model to study flagellar targeting and assembly [63]. Possible flagellum targeting motives have been identified but since these data have recently been reviewed [64], they will not be discussed here.

Given its complex structure, it is highly unlikely that the PFR would be composed of only two proteins. By comparison, the axoneme of *Chlamydomonas* sp. is made up of at least 250 proteins [65]. A few, albeit less abundant, PFR proteins have been identified. Three different antigens were localised to distinct regions of the PFR of *T. brucei*: (1) in the distal domain of the PFR [66, 67]; (2) throughout the PFR; and (3) on the connections between the PFR and the axoneme [68]. All three antigens are large proteins and only partial sequence is available. They all contain repetitive regions but do not share identity between themselves and have no counterpart in the sequence databases. In addition to these unknown antigens, calmodulin has been localised to the PFR [69, 70], as well as some calflagins (flagellar calcium-binding proteins) [11, 42]. Direct purification of the PFR structure from *Herpetomonas megaseliae* revealed enrichment of 4–6 protein bands of molecular mass of between 122 and 188 kDa [71]. Finally, direct amino acid sequencing of purified PFR proteins in *T. cruzi* identified two other constituents of unknown function: PAR1 and PAR4, which share a very low degree of identity with PFR1 and PFR2 [62]. We have recently identified homologues of PAR 1 and PAR4 in the *T. brucei* sequence databases (our unpublished observations). It is a possibility that screening of the trypanosomes and *Leishmania* sequence databases will allow the identification of divergent *PFR* genes, as was discovered recently for tubulins [72].

The remarkable development of reverse genetics in *T. brucei* and in *Leishmania* spp. has made it possible to study the function of the major *PFR* genes. Double gene knock-out of the *PFR2* genes in *L. mexicana* did not affect PFR1 expression but led to the assembly of only a rudimentary PFR, restricted to a structure resembling the proximal domain. The mutant cells displayed reduced swimming motility (from 25–30 to 5 μm/s) accompanied by a dramatic reduction in both frequency and amplitude of flagellar beating [10]. Molecular ablation of PFR2 in *T. brucei* by an RNA interference strategy [11, 73, 74] produced an even more dramatic phenotype: trypanosomes stopped swimming and sedimented at the bottom of the culture flask. The flagella of these *snl* mutants exhibited a greatly reduced beating pattern, with irregular frequency. When examined by electron microscopy, only a rudimentary PFR could be seen, again resembling the proximal domain (*figure 5B*). This structure was still properly connected to the axoneme and to the FAZ region.

Another striking feature of trypanosome *snl* mutants was the presence of a large dilation of the flagellum tip, or 'blob' [11, 73]. During the trypanosome cell cycle, the old flagellum remains in place whilst the new one is constructed, always at the posterior end of the cell [33]. PFR construction in the new flagellum starts at around 0.52 of the unit cell cycle and closely follows the flagellar axoneme. In biflagellated *snl* trypanosomes, a blob was only detected on the growing flagellum, where its size increased with the length of the new flagellum, but a blob was never seen at the tip of the old flagellum. Hence, after cell division, only one daughter cell carried a blob. Strikingly, this material was moved back towards the cell body before the trypanosome started to assemble a new flagellum. It was demonstrated that this blob contained non-assembled PFR precursors, mostly PFR1 but also calflagins and minor amounts of the antigen recognised by ROD-1 [11, 73]. We suggested that in the absence of PFR2, a normal PFR cannot be assembled, but several PFR precursors are still transported at the distal tip of the flagellum, where assembly normally occurs [63]. These would progressively accumulate at the distal tip as the flagellum elongates to generate the large blob. A similar blob of PFR1 material has subsequently been observed by Maga et al. [75] on *Leishmania* PFR2 null mutants. PFR1 null mutants and PFR1/PFR2 double null mutants have been

generated in *L. mexicana* by combined knock-outs, and both exhibit similar phenotypes: in the absence of PFR1 (or both PFR1 and PFR2), virtually no PFR at all could be assembled [75]. However, some material was present in the flagellum, alongside axonemal microtubule doublets 4 to 7, i.e. the expected position of the PFR. This was removed by detergent extraction, with the exception of a distinct structure usually attached to microtubule doublet 6. However, these mutants could not be discriminated from the original PFR2 null mutant in terms of motility and behaviour [75].

In culture, all these mutant parasites were viable and did not display abnormalities other than the flagellar and motility defects described above. The paralysed *snl* mutants showed a consistently slightly slower growth rate in vitro, whereas no particular delay has been reported for the *Leishmania* mutants. These parasite mutants offer the exciting possibility of assessing the role of both motility and PFR in infectivity. Preliminary results already suggest that the *Leishmania* PFR2 null mutant was poorly effective in infecting sandflies (the vector for leishmaniasis), arguing for an important role of motility in the parasite life cycle [76].

6. Flagellar assembly and intraflagellar transport (IFT)

When trypanosomes build their flagellum, they must coordinate the assembly of three joint cytoskeletal structures: the axoneme, the PFR and the FAZ [35]. Both PFR and axoneme precursors have to be transported to the distal tip of the growing flagellum, where they are incorporated in their respective structure [63, 77]. The growing end of the four microtubules of the FAZ is likely to be towards the anterior end of the cell, i.e. the same growth orientation as that of the two intraflagellar structures [39]. It is not yet known how the precursors of the FAZ filaments are added but it is quite likely that assembly polarity will be similar to the other flagellum-associated structures.

Axonemal assembly and maintenance could follow the same pattern exhibited by other organisms. One possible candidate for transport of precursors to the tip of the flagellum is intraflagellar transport (IFT). Initially described as the bidirectional movement of particles, called 'rafts', below the flagellar membrane in *Chlamydomonas* sp. [78], IFT was later implicated in flagellar growth and maintenance [79]. In chlamydomonas, a heterotrimeric kinesin, termed FLA10, is thought to be the motor of anterograde (plus-end-directed, base to tip) IFT [80], possibly transporting components to be incorporated in the axoneme. The opposite movement, retrograde IFT (minus-end-directed, tip to base), appears to be mediated by a specific cytoplasmic dynein heavy chain (DHC 1b) in association with at least one light chain (LC8) [79]. However, direct correlation between IFT particles, already purified and biochemically characterised, and axonemal precursors is yet to be made. Also, IFT is likely to be involved in flagellar membrane maintenance and function [79]. There is evidence of involvement of IFT particles and motors in ciliogenesis in a variety of organisms apart from chlamydomonas [79]. IFT has not been described in trypanosomes, but particles possibly related to rafts can easily be seen between the axoneme and the flagellar membrane in cross-sections (*figure 5* [33]). In *snl* mutants, the movements of the blob during the cell cycle described above illustrate intraflagellar anterograde and retrograde movement of PFR material [11, 64].

7. Conclusions

In addition to the presence of an apparently conventional axoneme, the trypanosome flagellum exhibits intriguing features such as the specialised flagellar pocket at its base, the enigmatic PFR and the no less intriguing FAZ. It will be fascinating to understand how the cell coordinates the assembly of these joint cytoskeletal structures inside the flagellum, inside the cell body and on the specialised region of the cell surface. Despite advances in understanding the biochemical composition of the flagellum and the function of some of its constituents, several enigmatic questions remain unanswered. How does the PFR contribute to flagellum motility? Why do some organisms need a PFR, whereas simple flagella with only an axoneme appear perfectly functional? What is the exact role of the FAZ? Why is the flagellum attached to the cell body? In parasitic terms, the importance of the specific features for successful infection of a host or of an insect vector should be determined, as well as their putative potential as therapeutic targets [81]. The rapid progress made on genome sequencing projects, combined with the progress of reverse genetics will hopefully help us to address these fundamental questions.

Acknowledgments

Work in the authors' laboratory is supported by a Wellcome Programme and Equipment Grant and by the BBSRC.

References

[1] Molyneux D.H., Ashford R.W., The biology of *Trypanosoma* and *Leishmania*, parasites of man and domestic animals, Taylor and Francis, London, 1983.

[2] Matthews K.R., Developments in the differentiation of *Trypanosoma brucei*, Parasitol. Today 15 (1999) 76–80.

[3] Vickerman K., Developmental cycles and biology of pathogenic trypanosomes, Br. Med. Bull. 41 (1985) 105–114.

[4] Clayton C., Hausler T., Blattner J., Protein trafficking in kinetoplastid protozoa, Microbiol. Rev. 59 (1995) 325–344.

[5] Benne R., Van den Burg J., Brakenhoff J.P., Sloof P., Van Boom J.H., Tromp M.C., Major transcript of the frame-shifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA, Cell 46 (1986) 819–826.

[6] Robinson D.R., Gull K., Basal body movements as a mechanism for mitochondrial genome segregation in the trypanosome cell cycle, Nature 352 (1991) 731–733.

[7] Robinson D.R., Gull K., The configuration of DNA replication sites within the *Trypanosoma brucei* kinetoplast, J. Cell Biol. 126 (1994) 641–648.

[8] Vickerman K., The mechanisms of cyclical development in trypanosomes of the *Trypanosoma brucei* subgroup: a hypothesis based on ultrastructural observations, Trans. R. Soc. Trop. Med. Hyg. 56 (1962) 487–495.

[9] Overath P., Stierhof Y.D., Wiese M., Endocytosis and secretion in trypanosomatid parasites-a tumultuous traffic in a pocket, Trends Cell Biol. 7 (1997) 27–33.

[10] Santrich C., Moore L., Sherwin T., Bastin P., Brokaw C., Gull K., LeBowitz J.H., A motility function for the paraflagellar rod of *Leishmania* parasites revealed by PFR-2 gene knockouts, Mol. Biochem. Parasitol. 90 (1997) 95–109.

[11] Bastin P., Pullen T.J., Sherwin T., Gull K., Protein transport and flagellum assembly dynamics revealed by analysis of the paralysed trypanosome mutant *snl*-1, J. Cell Sci. 112 (1999) 3769–3777.

[12] Killick-Kendrick R., Molyneux D.H., Ashford R.W., *Leishmania* in phlebotomid sandflies. I. Modifications of the flagellum associated with attachment to the mid-gut and oesophageal valve of the sandfly, Proc. R. Soc. Lond. B Biol. Sci. 187 (1974) 409–419.

[13] Killick-Kendrick R., Molyneux D.H., Ashford R.W., Ultrastructural observations on the attachment of *Leishmania* in the sandfly, Trans. R. Soc. Trop. Med. Hyg. 68 (1974) 269–281.

[14] Tetley L., Vickerman K., Differentiation in *Trypanosoma brucei*: host-parasite cell junctions and their persistence during acquisition of the variable antigen coat, J. Cell Sci. 74 (1985) 1–19.

[15] ten Asbroek A.L., Ouellette M., Borst P., Targeted insertion of the neomycin phosphotransferase gene into the tubulin gene cluster of *Trypanosoma brucei*, Nature 348 (1990) 174–175.

[16] Lee M.G., Van der Ploeg L.H.T., Homologous recombination and stable transfection in the parasitic protozoan *Trypanosoma brucei*, Science 250 (1990) 1583–1587.

[17] Eid J., Sollner-Webb B., Stable integrative transformation of *Trypanosoma brucei* that occurs exclusively by homologous recombination, Proc. Natl. Acad. Sci. USA 88 (1991) 2118–2121.

[18] Wirtz E., Clayton C., Inducible gene expression in trypanosomes mediated by a prokaryotic repressor, Science 268 (1995) 1179–1183.

[19] Myler P.J., et al., *Leishmania major* Friedlin chromosome 1 has an unusual distribution of protein-coding genes, Proc. Natl. Acad. Sci. USA 96 (1999) 2902–2906.

[20] El-Sayed N.M., Hedge P., Quackenbush J., Melville S.E., Donelson J.E., The African trypanosome genome, Int. J. Parasitol. 30 (2000) 329–345.

[21] Balber A.E., The pellicle and the membrane of the flagellum, flagellar adhesion zone, and flagellar pocket: functionally discrete surface domains of the bloodstream form of African trypanosomes, Crit. Rev. Immunol. 10 (1990) 177–201.

[22] Cross G.A.M., Antigenic variation in trypanosomes: secrets surface slowly, Bioessays 18 (1996) 283–291.

[23] Tetley L., Freeze-fracture studies on the surface membranes of pleomorphic bloodstream and in vitro transformed procyclic *Trypanosoma brucei*, Acta Tropica 43 (1986) 307–317.

[24] De Souza W., Structural organization of the cell surface of pathogenic protozoa, Micron 26 (1995) 405–430.

[25] Tetley L., Coombs G.H., Vickerman K., The surface membrane of *Leishmania mexicana mexicana*: comparison of amastigote and promastigote using freeze-fracture cytochemistry, Z. Parasitenk. 72 (1986) 281–292.

[26] Vickerman K., Tetley L., in: Bloodgood R.A. (Ed.), Ciliary and Flagellar Membranes, Plenum, London, 1990, pp. 267–304.

[27] Paindavoine P., Rolin S., Van Assel S., Geuskens M., Jauniaux J.C., Dinsart C., Huet G., Pays E., A gene from the variant surface glycoprotein expression site encodes one of several transmembrane adenylate cyclases located on the flagellum *of Trypanosoma brucei*, Mol. Cell. Biol. 12 (1992) 1218–1225.

[28] Engman D.M., Krause K.H., Blumin J.H., Kim K.S., Kirchhoff L.V., Donelson J.E., A novel flagellar Ca^{2+}-binding protein in trypanosomes, J. Biol. Chem. 264 (1989) 18627–18631.

[29] Piper R.C., Xu X.W., Russell D.G., Little B.M., Landfear S.M., Differential targeting of 2 glucose transporters from *Leishmania enriettii* is mediated by an NH2-Terminal domain, J. Cell Biol. 128 (1995) 499–508.

[30] Snapp E.L., Landfear S.M., Cytoskeletal association is important for differential targeting of glucose transporter isoforms in *Leishmania*, J. Cell Biol. 139 (1997) 1775–1783.

[31] Snapp E.L., Landfear S.M., Characterization of a targeting motif for a flagellar membrane protein in *Leishmania enriettii*, J. Biol. Chem. 274 (1999) 29543–29548.

[32] Godsel L.M., Engman D.M., Flagellar protein localization mediated by a calcium-myristoyl/palmitoyl switch mechanism, EMBO J. 18 (1999) 2057–2065.

[33] Sherwin T., Gull K., The cell division cycle of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations, Philos. Trans. R. Soc. Lond. B Biol. Sci. 323 (1989) 573–588.

[34] Kohl L., Gull K., Molecular architecture of the trypanosome cytoskeleton, Mol. Biochem. Parasitol. 93 (1998) 1–9.

[35] Kohl L., Sherwin T., Gull K., Assembly of the paraflagellar rod and the flagellum attachment zone complex during the *Trypanosoma brucei* cell cycle, J. Eukaryot. Microbiol. 46 (1999) 105–109.

[36] Cooper R., de Jesus A.R., Cross G.A.M., Deletion of an immunodominant *Trypanosoma cruzi* surface glycoprotein disrupts flagellum-cell adhesion, J. Cell Biol. 122 (1993) 149–156.

[37] de Jesus A.R., Cooper R., Espinosa M., Gomes J.E., Garcia E.S., Paul S., Cross G.A.M., Gene deletion suggests a role for *Trypanosoma cruzi* surface glycoprotein GP72 in the insect and mammalian stages of the life cycle, J. Cell Sci. 106 (1993) 1023–1033.

[38] Nozaki T., Haynes P.A., Cross G.A.M., Characterization of the *Trypanosoma brucei* homologue of a *Trypanosoma cruzi* flagellum-adhesion glycoprotein, Mol. Biochem. Parasitol. 82 (1996) 245–255.

[39] Robinson D.R., Sherwin T., Ploubidou A., Byard E.H., Gull K., Microtubule polarity and dynamics in the control of organelle positioning, segregation, and cytokinesis in the trypanosome cell cycle, J. Cell Biol. 128 (1995) 1163–1172.

[40] Holwill M.E., McGregor J.L., Effects of calcium on flagellar movement in the trypanosome *Crithidia oncopelti*, J. Exp. Biol. 65 (1976) 229–242.

[41] Sugrue P., Hirons M.R., Adam J.U., Holwill M.E., Flagellar wave reversal in the kinetoplastid flagellate *Crithidia oncopelti*, Biol. Cell 63 (1988) 127–131.

[42] Wu Y., Deford J., Benjamin R., Lee M.G., Ruben L., The gene family of EF-hand calcium-binding proteins from the flagellum of *Trypanosoma brucei*, Biochem. J. 304 (1994) 833–841.

[43] Bastin P., Matthews K.R., Gull K., The paraflagellar rod of Kinetoplastida: solved and unsolved questions, Parasitol. Today 12 (1996) 302–307.

[44] Farina M., Attias M., Souto-Padron T., De Souza W., Further studies on the organization of the paraxial rod of Trypanosomatids, J. Protozool. 33 (1986) 552–557.

[45] Russell D.G., Newsam R.J., Palmer G.C., Gull K., Structural and biochemical characterisation of the paraflagellar rod of *Crithidia fasciculata*, Eur. J. Cell Biol. 30 (1983) 137–143.

[46] Cachon J., Cachon M., Cosson M.P., Cosson J., The paraflagellar rod: a structure in search of a function, Biol. Cell 63 (1988) 169–181.

[47] Sleigh M.A., Flagellar beat patterns and their possible evolution, Biosystems 14 (1981) 423–431.

[48] Brugerolle G., Lom J., Nohynkova E., Joyon L., Comparaison et évolution des structures cellulaires chez plusieurs especes de bodonidés et de cryptobiiés appartenant aux genres *Bodo*, *Cryptobia* et *Trypanoplasma* (Kinetoplastida, Mastigophora), Protistologica 15 (1979) 197–221.

[49] Walne P.L., Dawson N.S., A comparison of paraxonemal rods in the flagella of euglenoids and kinetoplastids, Arch. Protistenkd. 143 (1993) 177–194.

[50] Brugerolle G., Flagellar and cytoskeletal systems in amitochondrial flagellates- Archamoeba, Metamonada and Parabasala, Protoplasma 164 (1991) 70–90.

[51] Holberton D.V., Fine structure of the ventral disk apparatus and the mechanism of attachment in the flagellate *Giardia muris*, J. Cell Sci. 13 (1973) 11–41.

[52] Baccetti B., Evolutionary trends in sperm structure, Comp. Biochem. Physiol. A 85 (1986) 29–36.

[53] Fouquet J.P., Kann M.L., The cytoskeleton of mammalian spermatozoa, Biol. Cell 81 (1994) 89–93.

[54] Hyams J.S., The *Euglena* paraflagellar rod: structure, relationship to other flagellar components and preliminary biochemical characterization, J. Cell Sci. 55 (1982) 199–210.

[55] Gallo J.M., Schrevel J., Homologies between paraflagellar rod proteins from trypanosomes and euglenoids revealed by a monoclonal antibody, Eur. J. Cell Biol. 36 (1985) 163–168.

[56] Schlaeppi K., Deflorin J., Seebeck T., The major component of the paraflagellar rod of *Trypanosoma brucei* is a helical protein that is encoded by two identical, tandemly linked genes, J. Cell Biol. 109 (1989) 1695–1709.

[57] Birkett C.R., Parma A.E., Gerke-Bonet R., Woodward R., Gull K., Isolation of cDNA clones encoding proteins of complex structures: analysis of the *Trypanosoma brucei* cytoskeleton, Gene 110 (1992) 65–70.

[58] Beard C.A., Saborio J.L., Tewari D., Krieglstein K.G., Henschen A.H., Manning J.E., Evidence for two distinct major protein components, PAR 1 and PAR 2, in the paraflagellar rod of *Trypanosoma cruzi*. Complete nucleotide sequence of PAR, J. Biol. Chem. 267 (1992) 21656–21662.

[59] Deflorin J., Rudolf M., Seebeck T., The major components of the paraflagellar rod of *Trypanosoma brucei* are two similar, but distinct proteins which are encoded by two different gene loci, J. Biol. Chem. 269 (1994) 28745–28751.

[60] Moore L.L., Santrich C., LeBowitz J.H., Stage-specific expression of the *Leishmania mexicana* paraflagellar rod protein PFR-2, Mol. Biochem. Parasitol. 80 (1996) 125–135.

[61] Ngô H.M., Bouck G.B., Heterogeneity and a coiled coil prediction of trypanosomatid-like flagellar rod proteins in *Euglena*, J. Eukaryot. Microbiol. 45 (1998) 323–333.

[62] Fouts D.L., Stryker G.A., Gorski K.S., Miller M.J., Nguyen T.V., Wrightsman R.A., Manning J.E., Evidence for four distinct major protein components in the paraflagellar rod of *Trypanosoma cruzi*, J. Biol. Chem. 273 (1998) 21846–21855.

[63] Bastin P., MacRae T.H., Francis S.B., Matthews K.R., Gull K., Flagellar morphogenesis: protein targeting and assembly in the paraflagellar rod of trypanosomes, Mol. Cell. Biol. 19 (1999) 8191–8200.

[64] Bastin P., Gull K., Assembly and function of complex flagellar structures illustrated by the paraflagellar rod of trypanosomes, Protist 150 (1999) 113–123.

[65] Piperno G., Huang B., Luck D.J., Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardtii*, Proc. Natl. Acad. Sci. USA 74 (1977) 1600–1604.

[66] Woods A., Sherwin T., Sasse R., MacRae T.H., Baines A.J., Gull K., Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies, J. Cell Sci. 93 (1989) 491–500.

[67] Woodward R., Carden M.J., Gull K., Molecular characterisation of a novel, repetitive protein of the paraflagellar rod in *Trypanosoma brucei*, Mol. Biochem. Parasitol. 67 (1994) 31–39.

[68] Imboden M., Muller N., Hemphill A., Mattioli R., Seebeck T., Repetitive proteins from the flagellar cytoskeleton of African trypanosomes are diagnostically useful antigens, Parasitology 110 (1995) 249–258.

[69] Ruben L., Patton C.L., Calmodulin from *Trypanosoma brucei*: immunological analysis and genomic organization, Meth. Enzymol. 139 (1986) 262–276.

[70] Ridgley E., Webster P., Patton C., Ruben L., Calmodulin-binding properties of the paraflagellar rod complex from *Trypanosoma brucei*, Mol. Biochem. Parasitol. 109 (2000) 195–201.

[71] Moreira-Leite F.F., de Souza W., da Cunha-e-Silva N.L., Purification of the paraflagellar rod of the trypanosomatid *Herpetomonas megaseliae* and identification of some of its minor components, Mol. Biochem. Parasitol. 104 (1999) 131–140.

[72] Vaughan S., Attwood T., Navarro M., Scott V., McKean P., Gull K., New tubulins in protozoal parasites, Curr. Biol. 10 (2000) R258–R259.

[73] Bastin P., Sherwin T., Gull K., Paraflagellar rod is vital for trypanosome motility, Nature 391 (1998) 548.

[74] Bastin P., Ellis K., Kohl L., Gull K., Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system, J. Cell Sci. 113 (2000) 3321–3328.

[75] Maga J.A., Sherwin T., Francis S., Gull K., LeBowitz J.H., Genetic dissection of the *Leishmania* paraflagellar rod, a unique flagellar cytoskeleton structure, J. Cell Sci. 112 (1999) 2753–2763.

[76] Maga J.A., LeBowitz J.H., Unravelling the kinetoplastid paraflagellar rod, Trends Cell Biol. 9 (1999) 409–413.

[77] Sherwin T., Gull K., Visualization of detyrosination along single microtubules reveals novel mechanisms of assembly during cytoskeletal duplication in trypanosomes, Cell 57 (1989) 211–221.

[78] Kozminski K.G., Johnson K.A., Forscher P., Rosenbaum J.L., A motility in the eukaryotic flagellum unrelated to flagellar beating, Proc. Natl. Acad. Sci. USA 90 (1993) 5519–5523.

[79] Rosenbaum J.L., Cole D.G., Diener D.R., Intraflagellar transport: the eyes have it, J. Cell Biol. 144 (1999) 385–388.

[80] Kozminski K.G., Beech P.L., Rosenbaum J.L., The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane, J. Cell Biol. 131 (1995) 1517–1527.

[81] Wrightsman R.A., Miller M.J., Saborio J.L., Manning J.E., Pure paraflagellar rod protein protects mice against *Trypanosoma cruzi* infection, Infect. Immun. 63 (1995) 122–125.