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The flagellum of trypanosomes

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Keywords: flagellum, trypanosome, cytoskeleton, morphogenesis, polarity, cell size, cytokinesis, motility, RNA interference

Abstract

Eukaryotic cilia and flagella are cytoskeletal organelles that are remarkably conserved from protists to mammals. Their basic unit is the axoneme, a well-defined cylindrical structure composed of microtubules and up to 250 associated proteins. These complex organelles are assembled by a dynamic process, called intraflagellar transport. Flagella and cilia perform diverse motility and sensitivity functions in many different organisms. Trypanosomes are flagellated protozoa, responsible for various tropical diseases such as sleeping sickness or Chagas disease. In this review, we first describe general knowledge on the flagellum: its occurrence in the living world, its molecular composition and its mode of assembly, with special emphasis on the exciting developments that followed the discovery of intraflagellar transport. We then present recent progress regarding the characteristics of the trypanosome flagellum, highlighting the original contributions brought by this organism. The most striking phenomenon is the involvement of the flagellum in several aspects of the trypanosome cell cycle including cell morphogenesis, basal bodies migration and cytokinesis.

Key words: Flagellum, Cilia, Axoneme, Intraflagellar Transport, Cytoskeleton, Motility, Morphogenesis, Trypanosome

I. Introduction

Eukaryotic cells are characterised by the presence of multiple organelles that are specialised to accomplish particular functions. Some organelles are ubiquitous and their functions are conserved in most living species. In contrast, cilia and flagella are encountered in specific groups of organisms, and although displaying a remarkable structural conservation, they are able to play many different roles from one organism to the other, and even within the same organism. When one comes to think about cilia and flagella, motility springs to mind. Other functions have been highlighted over the years, including sensitivity, morphogenesis and attachment to substrates.

Trypanosomes are intriguing protozoa belonging to the Kinetoplastida order that diverged early from the main eukaryotic lineage, and are better known for the tropical diseases they are creating. However, they are also turning out to be of exceptional interest to study flagella. They possess a single flagellum and recent works revealed the multi-functional aspects of this organelle in a single-cell organism. In the first part of this review, we shall describe generalities regarding cilia and flagella, examining their occurrence throughout the living world, their morphology and molecular composition, their modes of assembly and their functions. In the second part, we shall focus on the specificities of the trypanosome flagellum and the recent contributions it brought to the knowledge of these often under-looked organelles.

II. Cilia and Flagella

A. Occurrence in the living world

Eukaryotic cilia and flagella are cell appendages, usually surrounded by membrane that are protruding in the external medium. A lot of protozoa exhibit various numbers of motile cilia or flagella such as in Diplomonads (*Giardia*), Euglenozoa (*Euglena*, *Trypanosoma*), Parabasalids (*Trichomonas*), Ciliates (*Paramecium*, *Tetrahymena*) or Dinoflagellates (*Oxyrrhis*, *Gonyaulax*). In Apicomplexa such as *Plasmodium*, flagella, when present, are restricted to the male gamete stages. In contrast, flagella are not encountered in fungi (with the exception of Chytridiomycetes), nor in Microsporidia.

Cilia and flagella are present in most multicellular animal organisms. Their composition, as well as their number, length and functions can vary from one cell type to the other within the same organism. In humans, spermatozoa exhibit a single, long, flagellum required for motility and fertility (Inaba, 2003). Ciliated cells, with up to 200 cilia are found in the respiratory tract, in the brain ventricles or in the oviduct where they are responsible for mucus, cephalorachidian liquid or egg movement respectively. An immotile cilium is found in light-sensitive retinal cells where it is involved in photopigment trafficking (Marszalek et al., 2000). A combination of motile and sensory cilia present at the surface of cells of the embryonic node act together in the establishment of left-right asymmetry (McGrath and Brueckner, 2003). Finally, the vast majority of human cells exhibit a primary cilium, whose sensory functions have been unveiled recently (Wheatley, 2004). In

contrast to this extensive diversity, nematodes do not have motile cilia or flagella and only possess sensory cilia localised to a precise subset of neurons (Perkins et al., 1986). Nematode sperm is aflagellated and exhibits an amoeboid-like motility. Ciliated neurons are encountered in insects, with the additional presence of flagellated spermatozoa.

In plants, flagella are found in unicellular species such as the green alga *Chlamydomonas* and in multicellular species such as *Volvox* (Silflow and Lefebvre, 2001). However in plants developing on land, these structures tend to be restricted to gametes, like in bryophytes, ferns or gymnosperms. Flagella are not present in higher plants which use seeds for fertilisation. Finally, many species of bacteria also possess flagella, but of totally different structure and composition (Aldridge and Hughes, 2002).

B. Flagellum composition

1. The axoneme

a. Definition and general aspects

The central core defining eukaryotic cilia and flagella is the axoneme. This highly-organised cylindrical structure is composed of 9 peripheral doublets of microtubules surrounding a central pair, and of a number of appendages (Fig. 1) (reviews in Dutcher, 1995; Silflow and Lefebvre, 2001; El Zein et al., 2003). The 9 doublets are arranged at regular intervals and constitute a hollow cylinder that extends throughout the flagellum. In peripheral doublets, the 'A' microtubule is composed of the 13 protofilaments typical of microtubules whereas the 'B'

microtubule contains only 11 and is fused to the 'A'. This organisation is probably responsible for the higher stability of flagellar microtubules compared to most of their cytoplasmic counterparts. Both microtubules of the central pair contain 13 protofilaments. Central pair microtubules are not in direct contact but are connected to each other by a thin filament or bridge.

Doublets are attached to each other via nexin links. The A tubule of peripheral doublets carries two side projections called outer and inner dynein arms (Fig. 1), containing a variable number of dynein motors involved in flagellum motility (DiBella and King, 2001). A third projection is orientated towards the central pair and termed radial spoke. The central pair microtubules are not identical and can be discriminated both at the structural and biochemical level (Porter and Sale, 2000). The C1 microtubule carries 2 long and 2 short projections whereas 3 projections have been identified on the C2 microtubule (Mitchell and Sale, 1999). This remarkable organisation is conserved in most motile eukaryotic cilia and flagella, from protists to mammals (Fig.1). In contrast, non-motile cilia exhibit the 9 peripheral doublets but usually lack the central pair, dynein arms and radial spokes (Pazour and Witman, 2003).

b. Unusual axoneme types

The "9+2" canonical structure is a hallmark of motile cilia and flagella. However, rules are bound to have exceptions and some interesting ones will be briefly summarised here. In mammals, cells of the embryonic node possess a single cilium with a "9+0" structure, i.e. missing the central pair, that is nevertheless motile (Nonaka et al., 1998) and plays a crucial role in the establishment of left-right

asymmetry (McGrath and Brueckner, 2003) (see II.D). Motile 9+0 axonemes have also been detected in gametes of several species during evolution including protists, flat worms, annelids, and eels (review in (Goldstein and Schr vel, 1982)). More striking exceptions to the 9+2 rule include the presence of supernumerary doublets in insect spermatozoa (12+0 and even 14+0, Baccetti et al., 1973). Even more spectacular structures were reported in the flagella of male gametes from several gregarines, apicomplexan parasites of invertebrates and urochordates. Axonemes of *Lecudina tuzetae* present a "6+0" structure (Schr vel and Besse, 1975) and those of *Diplauxis hatti* consist of 3 doublet microtubules (Prensier et al., 1980). In both cases, classic organisation of A and B tubules was observed. The presence of structures resembling dynein arms has only been observed in *Lecudina tuzetae*. Remarkably, both types of flagella are motile and capable of beating, although with a reduced beat frequency (Prensier et al., 1980; Goldstein and Schr vel, 1982). In these species, male and female gametes develop in the same cyst, and it has been hypothesised that flagellar motility contributes to extensive gametes mixing, ensuring optimal conditions for fertilisation.

c. Molecular composition

The axoneme is composed of a large number of proteins, as could have been expected from such a sophisticated organisation. Bi-dimensional gel electrophoresis analysis of purified axonemes from the green alga *Chlamydomonas* indicates the presence of up to 250 polypeptides (Piperno et al., 1977; Dutcher, 1995). A significant number of axonemal proteins has been identified by genetic and biochemical characterisation of mutants in this protist that can be grown in the

laboratory and that has facile forward genetics (Dutcher, 1995; Silflow and Lefebvre, 2001). Many different types of mutants have been obtained and classified according to defects in flagellum presence, length or motility. Recent genome comparison showed that the majority of these proteins is highly conserved in flagellated species but absent from non-flagellated species (Avidor-Reiss et al., 2004; Kohl et al., 2004; Li et al., 2004).

As structural components of microtubules, α - and β -tubulin are obviously the most abundant flagellar proteins. Flagellar α -tubulin is acetylated on the highly conserved lysine 40, a hallmark of stable microtubules (L'Hernault and Rosenbaum, 1985; Piperno and Fuller, 1985; Schneider et al., 1987). Flagellar microtubules are also extensively glutamylated (Edde et al., 1990; Schneider et al., 1997; Kann et al., 2003) and frequently poly-glycylated (Bre et al., 1996). The exact function(s) of these post-translational modifications is still unclear, although a putative role for glycylation in central pair assembly has been proposed in *Tetrahymena* (Thazhath et al., 2002).

After tubulins, the most prominent class of protein belong to the dynein family. Dyneins are microtubule-associated molecular motors whose activity is responsible for flagellum motility (review in DiBella and King, 2001). Axonemal dyneins are protein complexes composed of 1-3 dynein heavy chains and of a variable number of intermediate and light chains. Dynein heavy chains are the only ones to possess intrinsic ATPase and motor activity. They are large proteins (>500 kDa) containing typical signatures such as nucleotide binding domains (also known as P-loops), an amino-terminal stem involved in interaction with light or intermediate chains, and a microtubule-binding region. Association of different intermediate and light chains with the dynein heavy chain regulates their activity and determines their location and

specificity of action. Intermediate and light chains are characterised by the presence of protein-protein interaction motifs and do not have motor activity.

In *Chlamydomonas*, extensive biochemical and genetic comparison revealed the molecular composition of both outer and inner dynein arms (DiBella and King, 2001). All outer dynein arms are made of a complex containing 3 distinct heavy chains, 2 intermediate and 8 light chains. The dynein particle is bound to the A tubule of peripheral doublets *via* the docking complex (Takada et al., 2002). Mutations in α - or β -dynein heavy chains inhibit outer arm assembly and have severe consequences on motility. In contrast to outer dynein arms, inner dynein arms appear much more complex, as dynein composition varies from one doublet to the other, with at least 7 distinct heavy chains. Most inner arms possess dynein complexes made of a single heavy chain, associated with unique intermediate/light chains.

Mutations in the human genes encoding protein constituents of dyneins are associated with primary cilia dyskinesia, a rare genetic condition marked by axonemal abnormalities of respiratory cilia and sperm tails. Patients suffering from that genetic condition exhibit bronchiectasis, sinusitis and male sterility. In some cases, it is associated with *situs inversus*, i.e. an inversion of the main internal body organs, and is then called Kartagener syndrome (Afzelius, 1976). So far, mutations have been identified in 3 genes, all encoding proteins components of the outer dynein arm. The first one was the dynein intermediate chain 1, a protein containing WD-40 repeats (a domain involved in protein-protein interactions) that is required for outer arm assembly (Pennarun et al., 1999)(Fig. 1). Recently, two mutations in two

separate dynein heavy chains have also been identified (Bartoloni et al., 2002; Olbrich et al., 2002).

Radial spokes are T-shaped projections originating from the A tubule of each peripheral doublet and directed toward the central pair. When viewed in longitudinal section, radial spokes are repeated at 96 nm intervals, exactly in phase with inner dynein arms (Porter and Sale, 2000). They are composed of at least 22 proteins, whose central component is RSP3, a protein required for targeting and anchoring the whole spoke to the peripheral microtubules (Diener et al., 1993). Mutations in RSP3 lead to failure in radial spoke assembly and paralysed flagella (Williams et al., 1989).

The projections associated to the central pair contain at least 23 polypeptides (Dutcher et al., 1984). Four of them have been identified from *Chlamydomonas* mutants exhibiting severe paralysis phenotypes, confirming the importance of the central pair complex for flagellum motility. PF16 is an armadillo domain-containing protein associated to one of the main projections of the C1 microtubule (Smith and Lefebvre, 1996). It is supposed to participate to central pair stability as electron microscope examination of demembrated axonemes from *Chlamydomonas PF16* mutants reveals absence of predominantly that microtubule, although it is still present in non-treated flagella (Dutcher et al., 1984). PF20 is a WD-40 repeat-containing protein localised to the bridge connecting the C1 and C2 microtubules (Smith and Lefebvre, 1997). PF16 and PF20 homologues are found in mammals (Sapiro et al., 2000; Pennarun et al., 2002; Zhang et al., 2002) and their inactivation in mouse leads to severe consequences on sperm motility (Sapiro et al., 2002; Zhang et al., 2004). PF6 is a large Alanine-Proline rich protein involved in C1 microtubule stability and also required for flagellum motility in *Chlamydomonas* (Rupp et al., 2001). However

this gene is poorly conserved and difficult to identify with certainty in other species. Finally, the PF15 gene encodes the p80 subunit of katanin, a microtubule-severing enzyme (Dymek et al., 2004). A survey of cilia from patients suffering from cilia-related diseases reveals that defects of the central pair complex are associated to the most severe clinical cases (Tamalet et al., 2001).

Axoneme movement is best explained by the microtubule sliding model. When purified cilia or flagella are treated with a mild detergent to remove membranes, the axonemes will resume beating (reactivate) in the presence of Mg^{2+} and ATP. Comparison of the tips of resting versus bent cilia showed that the doublet microtubules can slide past one another (Satir, 1968). This sliding-microtubule hypothesis was directly demonstrated in experiments where isolated flagellar axonemes were treated with mild proteolysis, which break the linkages that normally limit microtubule sliding. Following addition of Mg^{2+} and ATP, the doublet microtubules of such digested axonemes slide along each other. Active microtubule sliding is coupled to dynein hydrolysis of ATP and is unidirectional (Sale and Satir, 1977), with the dynein arms of one microtubule doublet interacting briefly with the adjacent microtubule doublet. Since axonemal microtubules are anchored to the cell *via* the basal bodies, microtubule sliding causes the axoneme to bend. However, all of the dynein arms cannot be simultaneously active and an asymmetry is required to allow the complex axonemal bending movements. This is provided by the central pair, the only axonemal structure to display an obvious asymmetry (projections are not identical; Mitchell and Sale, 1999). In many organisms, the central pair twists during flagellum bending and could therefore interact transiently with specific microtubule doublets, thereby allowing activation only at that moment. This model is

supported by detailed analysis of several *Chlamydomonas* central pair or radial spoke mutants, as well as by recent data showing that the orientation of the central apparatus defines the position of active microtubule sliding (Wargo and Smith, 2003). Finally, several lines of evidence indicate that the activity of dynein is regulated by a specific complex, the dynein regulatory complex, localised in close proximity of the inner dynein arm on the A tubule (Piperno et al., 1992; Hutchings et al., 2002; Rupp and Porter, 2003).

As the sequencing of several genomes of eukaryotic organisms has been completed, it is now possible to perform a global analysis of encoded flagellar proteins occurrence and conservation (Avidor-Reiss et al., 2004; Kohl et al., 2004; Li et al., 2004). Results can be put in perspective with flagellum presence and behaviour. A first level of information comes from comparison of genomes from flagellated organisms with those of non-flagellated organisms such as fungi (*S. cerevisiae*, *Schizosaccharomyces pombe* or *Neurospora crassa*), microsporidia (*Encephalitozoon culicis*) or high plants (*Arabidopsis thaliana*, *Oriza sativa*). Another level of refinement is provided by analysis of genomes from nematode species (*Caenorhabditis elegans* and *C. briggsae*) that possess only non-motile cilia. Therefore, genes coding for proteins involved in basic structural elements and in their assembly are expected to be conserved whereas those required for motility will be missing from nematodes. This was indeed found to be the case for axonemal dyneins (Vale, 2003) and for radial spoke and central pair associated proteins (Kohl et al., 2004).

2. Extra-axonemal structures

The simplest flagella, such as those of *Chlamydomonas* or cilia in human epithelial respiratory cells, are composed of an axoneme surrounded by the membrane. However, supplementary structures (or extra-axonemal structures) can be found in flagella in many instances, in particular in protists or sperm cells. An example of such a structure is the paraflagellar rod (also called paraxial or paraxonemal rod), a lattice-like structure running along the length of the axoneme in Euglenozoa, Dinoflagellates or Parabasalids (Cachon et al., 1988; Brugerolle, 1991; Bastin et al., 1996)(Fig. 1). In the dinoflagellate *Ceratium*, the diameter of the PFR can reach 560 nm, i.e., almost 4-fold that of the axoneme (Maruyama, 1982). Although these structures have been well analysed at the ultra-structural level (see for example Farina et al., 1986), little is known about their molecular composition and even less about their function within the flagellum.

Sperm flagella show great diversity regarding the presence of extraxonemal elements (Baccetti, 1986; Fouquet and Kann, 1994; Escalier, 2003). The flagellum of human spermatozoa has a large diameter at its base and progressively becomes thinner. Ultra-structural analysis demonstrates the existence of three different portions from base to tip: the principal piece, the midpiece and the endpiece (Inaba, 2003). In the principal piece, the axoneme is surrounded by a set of 9 “outer dense fibres”, (somehow replicating the 9 doublets of the axoneme), that itself is wrapped by mitochondria that are providing energy for dynein activity. In the midpiece, mitochondria and outer denser fibres are no longer present but a fibrous sheath now surrounds the axoneme. Finally, the axoneme is the only recognisable structure in the endpiece.

C. Flagellum assembly

Flagella are highly sophisticated organelles with a very precise organisation, which raises the question of their construction. They are built from a basal body, a centriolar-like structure made of 9 triplets of microtubules, that is used as a template for the assembly of the axonemal peripheral microtubule doublets (Beisson and Wright, 2003). These result from the extension of existing A and B tubules of the basal body whereas the central pair microtubules need to be nucleated *de novo* from the tip of the transition zone (a distal area of the basal body composed of peripheral doublets but still without the central pair), a process dependent on γ -tubulin (McKean et al., 2003). The flagellum usually extends on a cellular protrusion, thereby defining a separate compartment from the rest of the cytoplasm. The matrix of the flagellum does not contain ribosomes, implying that flagellar proteins need to be synthesised in the main cytoplasm and next imported in the flagellum. This transfer step might be reduced if RNAs coding for flagellar proteins are localised at proximity of the base of the flagellum. This is the case of the amoeboid-flagellate *Naegleria* where RNA for flagellar proteins were found to be concentrated in the basal body area prior to flagellum assembly (Han et al., 1997). The separation between the matrix of the flagellum and the rest of the cytoplasm raises the question of protein targeting to the flagellum and has been postulated to act as a “flagellar pore complex” (Deane et al., 2001; Rosenbaum and Witman, 2002). A physical structure, made of fibres stretching between the basal body triplets and the base of the flagellum membrane, seems to separate these two compartments.

1. Identification of intraflagellar transport and its role in flagellum formation

Flagellum construction takes place *via* assembly of new subunits at the distal end of the growing axoneme (Rosenbaum et al., 1969; Witman, 1975; Johnson and Rosenbaum, 1992; Piperno et al., 1996). Similarly, some extra-axonemal structures follow the same mode of assembly (Bastin et al., 1999a). As mentioned above, proteins are synthesised in the main cytoplasm body and imported in the flagellum compartment. This means that as the axoneme elongates, the distance between the site of flagellar protein production and the point of protein assembly increases considerably, raising the question of access to the construction site. This conundrum has been solved by the discovery of a motility system present within the flagellum (and independent from flagellum motility itself) called intraflagellar transport or IFT (review in Rosenbaum and Witman, 2002). IFT was first observed as the movement of small particles within paralysed flagella of *Chlamydomonas* (Kozminski et al., 1993). These particles travel either from the base to the tip of the flagellum (anterograde transport) or from the tip to the base (retrograde transport) with respective specific velocities of 2.0 and 3.5 μm per second. Their arrangement indicated that they correspond to specific electron-dense structures found in the flagellum, in close association with the B tubule of the peripheral doublets and with the flagellum membrane (Ringo, 1967).

Study of the temperature-sensitive *fla-10* *Chlamydomonas* mutant strain was instrumental in the understanding of intraflagellar transport. When grown at the restrictive temperature, cells from this mutant fail to assemble their flagella (Walther et al., 1994). Moreover, if *fla-10* flagellated algae are transferred from the permissive

to the restrictive temperature, their flagella become shorter and shorter until they are completely resorbed, suggesting that the mutated gene is involved in both assembly and maintenance of the organelle. The gene mutated codes for one subunit of heterotrimeric kinesin II, a motor protein complex localised to the small particles visualised by electron microscopy (Kozminski et al., 1995). It is composed of two heavy chains with motor and microtubule-binding activity that associate to each other via coiled-coil domains, and of one associated subunit, probably involved in specific binding to cargo (Cole et al., 1993). This led to the hypothesis that IFT could be the mechanism responsible for transporting a cargo containing axoneme precursors to the tip of the flagellum for assembly.

Kinesin II is a polar motor, that travels on microtubules from the minus end to the plus end, and is responsible for anterograde motility. However, IFT is observed in both directions and another motor is therefore required to explain the retrograde movement. This is powered by a specific type of cytoplasmic dynein, as demonstrated by the analysis of insertion mutants in *Chlamydomonas* (Cole et al., 1998; Pazour et al., 1998; Pazour et al., 1999; Porter et al., 1999). This dynein heavy chain is clearly different from the axonemal counterparts and has often been classified as cytoplasmic. However, it can be discriminated from the conventional cytoplasmic dynein heavy chain by a specific insertion (Pazour et al., 1999). Inactivation of this dynein leads to the production of tiny, disorganised, flagella filled with electron-dense material resembling IFT particles. These experiments indicate that both anterograde and retrograde transports are necessary for flagellum formation.

Purification of the IFT particles (also referred to as IFT 'rafts') was achieved after biochemical fractionation of *Chlamydomonas* flagella on sucrose gradients and revealed the presence of at least 16 different polypeptides (Cole et al., 1998). Several of these proteins have now been identified (Piperno and Mead, 1997; Cole et al., 1998) (Pazour et al., 2000; Brazelton et al., 2001; Deane et al., 2001) and are conserved in most flagellated species (Cole, 2003; Avidor-Reiss et al., 2004; Kohl et al., 2004). In agreement with their presence in a protein complex, their main characteristics is an abundance of domains involved in protein-protein interaction such as WD40, coiled-coils and tetratricopeptide repeats (Cole, 2003). Immunolocalisation experiments showed that these proteins are concentrated around the basal bodies and present as discrete spots along the length of the flagellum (Cole et al., 1998; Deane et al., 2001). Inactivation of a single IFT gene is sufficient to inhibit flagellum assembly, indicating that the integrity of the particle is required for efficient IFT (Pazour et al., 2000; Brazelton et al., 2001; Deane et al., 2001; Haycraft et al., 2001; Han et al., 2003; Kohl et al., 2003). The link between IFT movement and these motor proteins was definitely demonstrated by coupling to the green fluorescent protein (GFP) followed by fluorescence monitoring in *C. elegans* ciliated neurons (Orozco et al., 1999). GFP::osm-6 (IFT52) or GFP::KIF3 (kinesin II sub-unit) were localised at the base and along sensory cilia and both anterograde and retrograde movements of fluorescent particles were observed.

2. Functional conservation of IFT in cilia and flagella assembly

As expected from the central role of the IFT machinery in flagellum assembly, orthologues of genes coding for proteins of the heterotrimeric kinesin II, the IFT

cytoplasmic dynein and the IFT particles have been identified in the genome of all flagellated species analysed to date, with the exception of *Plasmodium* species (Kohl et al., 2004) (see below). The role of IFT-related proteins in flagellum assembly has been demonstrated in *Chlamydomonas*, and subsequently in other protists such as the ciliate *Tetrahymena* (Brown et al., 1999b) or the kinetoplastid *Trypanosoma brucei* (Kohl et al., 2003), and in multicellular organisms such as sea urchin (Morris and Scholey, 1997), *C. elegans* (Swoboda et al., 2000), *Drosophila melanogaster* (Han et al., 2003; Sarpal et al., 2003) or *Mus musculus* (Pazour et al., 2000). IFT was found to be equally essential for the formation of both motile flagella (Kozminski et al., 1995; Morris and Scholey, 1997; Brown et al., 1999b; Pazour et al., 2000; Han et al., 2003) and sensory cilia (Perkins et al., 1986; Marszalek et al., 2000; Swoboda et al., 2000; Han et al., 2003; Sarpal et al., 2003).

Two interesting exceptions to the central role of IFT in flagellum assembly have been observed. First, not a single orthologue of *IFT* genes could be identified in the complete genome of several *Plasmodium* species available (Avidor-Reiss et al., 2004; Kohl et al., 2004). This is likely due to the particular mode of flagellum formation taking place in these species. Indeed, flagella are assembled within the cytoplasm (Sinden et al., 1976) and not on cellular protrusions as observed in most situations. This means that flagellar proteins synthesised in the cytoplasm are freely available around the basal body and can be added directly to this template without the need for a transport system. As a consequence, axoneme assembly is much faster in *Plasmodium* than in species using IFT for their construction: 2-10 μm per minute instead of 2-10 μm per hour in *Chlamydomonas* or *Crithidia* (Russell and Gull, 1984; Lefebvre and Rosenbaum, 1986). Assembled flagella are then expelled

rapidly through a specific basket localised underneath the plasma membrane, ensuring membrane wrapping of the axoneme and the associated cytoplasm (Sinden et al., 1976). A similar situation is encountered during spermatogenesis in *Drosophila*: axonemes are assembled in a syncytium, hence would not require a specific transport system. This interpretation is confirmed by inactivation of the *Drosophila IFT88* homologue (also called *nomp-B*), or of the kinesin II associated protein KAP64, which prevents sensory cilia assembly but does not interfere with formation of the sperm flagellum that is completely normal (Han et al., 2003). This explanation is compatible with the formation of sometimes extremely long flagella in some *Drosophila* species (up to 6 cm in *D. hyundia*). Indeed, IFT would be much too slow to transport proteins to the tip of such flagella that are more than 10,000 times longer than *Chlamydomonas* flagella.

3. Model for the control of assembly of cilia and flagella

The discovery of IFT, its molecular machinery and its essential role in flagellum assembly is summarised in an interesting model (Cole et al., 1998; Iomini et al., 2001). IFT particles are assembled from the pool of IFT proteins present around the basal bodies and are loaded with proteins precursors of the axoneme, as well as with flagellar membrane-associated proteins. The particle is then imported in the flagellum and moved toward its tip by the action of the heterotrimeric kinesin II complex. Once at the tip, the particle is re-arranged and flagellar precursors are unloaded to be assembled at the growing end of the axoneme. The remodelled particle may contain proteins that are disassembled and removed from the axoneme, as well as flagellar membrane proteins. Chaperones like HSP70 (Bloch and

Johnson, 1995) are likely to be involved in these processes. At this stage, a shift in motor is carried out so that dynein now gets into action and transports the re-arranged complex toward the base of the flagellum, moving in direction of the minus end of the peripheral microtubules. At the base, the particle is disassembled and the cycle can re-iterate (Iomini et al., 2001). This model explains the data reported above: in the absence of kinesin II activity, flagella are not assembled as precursors cannot be delivered to the assembly site whereas in the absence of dynein activity, IFT particles can reach the tip of the flagellum but can not be recycled, therefore accumulating there and being depleted from their cytoplasmic pool (Porter et al., 1999). Their absence would prevent further flagellum assembly. Similarly, the integrity of the IFT particle is likely to be required for efficient transport, as observed in mutants of proteins composing the IFT particle. This system is active during flagellum formation and remains necessary for flagellum maintenance (see below).

Two sets of data indicate that IFT indeed transports axonemal precursors in the flagellum of *Chlamydomonas*. The first evidence came from the analysis of the fusion of gametes from wild-type and *ida4* (gene coding for the inner dynein arm light chain p28) mutant strains. In these conditions, the wild-type protein is able to gain access to the *ida4* flagella and to complement their mutant phenotype (Piperno et al., 1996). Immunofluorescence analysis with anti-p28 antibodies showed that this rescue first takes place at the distal tip of the mutant flagella and progressively extends in direction of the base of the flagellum. However, if the same fusion experiment is carried out in a complete *fla-10* genetic background, addition of p28 at the distal tip does not happen, revealing that active FLA10 kinesin is required for transport of that inner dynein arm component. These results show that (1) proteins

are incorporated in assembled flagella and (2) that this transport is dependent of IFT activity. It should be noted that in parallel experiments, another component of the axoneme, the intermediate dynein chain IC69 (DNAI1/ODA6) of the outer dynein arm, could rescue the mutant flagella independently of functional kinesin II (Piperno et al., 1996).

Second, immuno-precipitation experiments recently demonstrated a physical interaction between IFT proteins and several axonemal components, including radial spoke proteins and dynein arms (Qin et al., 2004). These interactions take place in the “membrane/matrix” fraction, supporting the idea that the IFT machinery indeed transports axonemal proteins. In most cases, radial spoke and dynein arms were already found as protein complexes in the cytoplasm, i.e. before their import in the flagellum (Fok et al., 1994; Fowkes and Mitchell, 1998; Qin et al., 2004).

IFT particles are in close proximity of the flagellar membrane, that appears constricted in their vicinity (Kozminski et al., 1995). IFT is therefore a good candidate for protein targeting specifically to the flagellar membrane. A striking example is found in retinal cells, where an immotile cilium separates the main cell body from the outer segment, a specialised region of the cell made of stacks of membrane that possesses a high density of photosensitive pigments such as rodopsin (in rods) or opsin (in cones). As it is the case for flagella, the outer segment is completely devoid of ribosomes, meaning that all its protein constituents are synthesised in the main portion of the cytoplasm and need to transit through the cilium. Given the high concentration of photopigments in the outer segment, these proteins have to face a massive density gradient to reach their location. Moreover, these proteins are subjected to an intense turn-over (~2,000 opsin molecules transit through the cilium

per minute). IFT actively transports these transmembrane proteins along the connecting cilium and delivers them to the outer segment. In the return trip, IFT brings inactivated opsin or rodopsin back to the main cell body where they are degraded. This was nicely demonstrated by specific knock-out of kinesin II exclusively in the cones using the cre-lox technology (Marszalek et al., 2000). In the absence of kinesin II, the connecting cilium progressively disappeared and opsin was redistributed throughout the surface of the cell.

The current model has several interesting implications, some of them have been tested experimentally. First, IFT could control protein entry in the flagellum compartment. This function is supported by the presence of a high concentration of IFT proteins around the basal body area, i.e. at the root of the flagellum, that could act as a selective filter (Cole et al., 1998; Deane et al., 2001). Moreover, immunogold labelling revealed that the IFT52 protein is localised to the transition fibers, especially in the area where these fibres are in close proximity with the flagellar membrane, a position often defining the boundary between the main cytoplasm and the matrix of the flagellum (Deane et al., 2001).

Second, IFT may regulate the production of flagellar proteins. Several data demonstrate that flagellar gene expression is tightly controlled (Guttman and Gorovsky, 1979; Lefebvre et al., 1980). In *Chlamydomonas*, deflagellation results in up-regulation of flagellar gene transcription (Lefebvre et al., 1980). It is intriguing to note that no accumulation of axonemal precursors has been reported in mutants that fail to assemble their flagella. Moreover, IFT172, a conserved component of the IFT particles, shows sequence identity with proteins interacting with transcription factors of the LIM family (Cole, 2003). It is tempting to propose a model whereby the

machinery controlling flagellum assembly would also control flagellum protein production.

Another function of IFT is the control of flagellum length. Since the IFT machinery is required for flagellar protein transport and for flagellum assembly, it is a first-choice candidate to control the amount of what goes in versus what goes out and hence determine the size of the organelle. This has been demonstrated in *Chlamydomonas* by a series of elegant experiments. First, metabolic labelling revealed that proteins of fully-assembled flagella are subject to significant turn-over (Song and Dentler, 2001). Second, fusion between wild-type gametes and their counterparts from a strain expressing an epitope-tagged α -tubulin, followed by immuno-fluorescence analysis with an anti-tag antibody, revealed the incorporation of tagged tubulin at the distal tip of the wild-type flagella. This process is dependent on IFT. Third, incubation of the mutant *fla-10* at temperatures intermediate between permissive (normal flagellum length) and restrictive (no flagellum at all) leads to progressive reduction in the amount of functional kinesin II and is accompanied by the production flagella of intermediate sizes (Marshall and Rosenbaum, 2001). This result confirmed that flagella are dynamic structures that can be remodelled once flagellum assembly is terminated (turn-over had been reported previously: Rosenbaum et al., 1969; Stephens, 1994; Stephens, 1997; Bastin et al., 1999a; Song and Dentler, 2001). Another series of experiments performed in *Trypanosoma brucei* showed that diminishing the amount of IFT88 or of the IFT dynein heavy chain lead to the assembly of flagella shorter than normal (Kohl et al., 2003)(see below).

D. Flagellum functions

1. Motility-related functions

The most obvious functions of cilia and flagella are related to motility: these organelles beat, sometimes at high frequency, and are responsible for cell propulsion and/or for displacing external fluids or materials. This is particularly true for protists living in aqueous media where flagella motility is essential for nutrition, predator or obstacle avoidance, and progeny dispersion. In the case of photosynthetic protists, swimming towards optimal sources of light is another obvious function. In evolutionary terms, the emergence of flagella is very ancient as the first eukaryotes have been postulated to be flagellated (Cavalier-Smith, 1982).

As organisms grew bigger, cilia and flagella developed more sophisticated functions: they are used for both locomotion and nutrition in ciliates such as *Tetrahymena* that uses cilia movement to bring food particles to the oral apparatus. Inhibition of cilia formation in *Tetrahymena* kinesin II double knock-out mutants leads to cell death, presumably due to starvation, as incubation in rich media allows survival (Brown et al., 1999b).

In many unicellular and multicellular organisms, one or both gametes rely on flagella motility for mating. Flagella can act beyond motility as in *Chlamydomonas* where flagellar adhesion is a crucial step preceding gamete fusion.

2. Sensory functions

As flagella protrude from the cell, they are candidates to perform sensory functions. A bunch of recent data has demonstrated that primary cilia of animal cells

are enriched in signalling molecules (Pazour and Witman, 2003; Wheatley, 2004). Inhibition of primary cilia assembly leads to defects in kidney functions, resulting in polycystic kidney disease (Pazour et al., 2000).

An exquisite combination of motility and sensory functions has been recently highlighted for the establishment of left-right asymmetry in mouse development (McGrath and Brueckner, 2003). The embryonic node is a short triangular structure present at the ventral face of the embryo at the gastrula stage. It is made of monociliated cells that contain a 9+0 axoneme. Nevertheless, some outer and inner dynein arms can be recognised (Takeda et al., 1999) and these cilia are actually capable of rotary motion (Nonaka et al., 1998). This is powered by a dynein motor, the left-right dynein heavy chain (Ird) (Supp et al., 1997). Inactivation of this dynein leads to cilia paralysis, but also to defects in the establishment of left-right asymmetry that appears random (Supp et al., 1997). However, flagella from spermatozoa and cilia from respiratory epithelial cells are still beating properly, showing that Ird dynein is not essential for motility of 9+2 axonemes (Supp et al., 1999). The Ird dynein, also termed DNAH11 (Bartoloni et al., 2002) is found in several vertebrate species, from fish to mammals (Essner et al., 2002). What is the role of this rotary motility ? Actually, it provokes a leftward movement of the perinodal fluid, an event that would break embryonic symmetry. This is confirmed by exposing live mouse embryos to artificial flow: a rapid rightward flow (opposite to the normal leftward one) results in complete inversion of the left-right asymmetry (Nonaka et al., 2002). Remarkably, the Ird dynein is found in only a centrally located subset of nodal. In contrast, the cation channel polycystin-2, also central in the establishment of left-right asymmetry, is located on the cilium of all nodal cells. Cilia possessing the Ird dynein initiate a

leftward nodal flow, that is going to bend cilia present of that side of the embryo (McGrath et al., 2003). This triggers an asymmetric calcium signalling only on the left side of the node that will ultimately be responsible for the expression of markers required for specific organ formation. This process would be responsible for the internal left-right asymmetry of organs observed in mammals.

3. Other functions

In addition to these motility and sensory functions, the separation of flagella from the rest of the cell body allows for a compartmentalisation function, that is found in different types of neurons, from worms to mammals (Rosenbaum and Witman, 2002). Novel roles for the flagellum in cell morphogenesis, shape, polarity, cytokinesis and division have been recently reported in *Trypanosoma* (Kohl et al., 2003), suggesting that flagella could be even more essential players in eukaryotic development than initially thought. These data will be reviewed in details in the section below.

III. The trypanosome flagellum

A. General characteristics of trypanosomes

Trypanosomes are protozoa belonging to the Kinetoplastida order, that is defined by the presence of a single, large, mitochondrion called the kinetoplast. This organelle is characterised by a high concentration of condensed circular DNA, visible by light microscopy. Members of the order are divided into two families : Bodonidae

(biflagellated) and Trypanosomatidae (monoflagellated). Species belonging to the latter are almost exclusively parasitic and exhibit an amazing range of hosts. Parasites of mammals, reptiles, frogs, fishes, birds, insects, snails and plants have been reported (Vickerman, 1976a). They include several parasites of significant human and veterinary importance and have therefore been the subject of considerable investigation. Human pathogens comprise *Trypanosoma brucei gambiense* and *T. b. rhodesiense* (African trypanosomes), responsible for sleeping sickness in central Africa, *Trypanosoma cruzi* (American trypanosomes), the agent of Chagas disease and several *Leishmania* species, leading to various types of leishmaniasis in South and Central America, Asia, Africa and Southern Europe. These three groups of parasites are digenic, i.e., they complete their life cycle *via* passage through two different hosts.

1. *Trypanosoma brucei*

T. brucei infects mammalian blood and is transmitted *via* the bite of a tsetse fly where they undergo a rather complex development in the midgut and the salivary glands. The most extensively studied stage of the parasites is derived from the peritrophic space of the midgut of the insect vector, and is called the “procyclic” stage (Fig. 2). It can be easily cultivated in the laboratory and genetically manipulated. The cell is 20-25 μm long and 3-5 μm wide and exhibits a helical shape. It possesses a single flagellum that is attached to the cell body and follows its helical path, with the exception of its distal tip. The axoneme is the elongation of the basal body, the latter being flanked by an immature pro-basal body. The cell does not possess the conventional centrosome and nuclear mitosis takes place without centrioles or

spindle pole bodies. The flagellum emerges from the flagellar pocket, a plasma membrane invagination of about $1 \mu\text{m}^3$, situated in a position posterior to the nucleus (Overath and Engstler, 2004). Such an arrangement of the kinetoplast relative to the nucleus defines the trypomastigote stage (Fig. 2)(Hoare and Wallace, 1966).

Trypanosomes swim with their flagellum leading, defining a narrow anterior end and a larger posterior end (Fig. 2). Electron microscopy analysis of the flagellum reveals the presence of a conventional axoneme, but also of an unusual extra-axonemal structure termed the paraflagellar rod or PFR (Fig. 1). Underneath the plasma membrane lies a subpellicular corset of microtubules that retains cell shape after detergent extraction (Sherwin and Gull, 1989a). This corset is only interrupted at the base of the flagellum by the flagellar pocket. This specialised area is the only site of endocytosis and exocytosis (Morgan et al., 2002a; Morgan et al., 2002b) for the trypanosome as the presence of the microtubule corset physically prevents membrane pinching or fusion anywhere else.

As trypanosomes migrate between different tissues of the tsetse fly, and from the insect vector to a mammalian host, they activate specific differentiation programmes to adapt to each environment encountered (Matthews et al., 2004). These are accompanied by numerous molecular, biochemical and morphological changes. In the next paragraph, we shall describe only the morphological changes as they are tightly associated to the flagellum (Vickerman, 1985; Vickerman et al., 1988; Van Den Abbeele et al., 1999).

Trypanosomes present in the blood meal traverse the peritrophic membrane, a specialised membrane separating the gut epithelium from the lumen. Procyclic trypanosomes actively proliferate in this peritrophic space. After several days,

trypanosomes differentiate and become extremely long (up to 40 μm , about twice their size at the procyclic stage) and narrow, with an apparently concomitant increase in the flagellum length (Van Den Abbeele et al., 1999). These highly motile cells do not replicate, traverse the peritrophic membrane and migrate *via* the oesophagus, proboscis lumen and hypopharynx towards the salivary glands. Here, trypanosomes undergo a new step of differentiation and start to multiply again. The flagellum now emerges from a position *anterior* to the nucleus (epimastigote form) and is only attached to the cell body for about one third of its length. The remaining part of the flagellum is used to anchor the trypanosomes to microvilli of epithelial cells. Cells remain attached for the whole replication and division process. After several rounds of cell division, the length of the flagellum anchored to host tissues is progressively reduced and trypanosomes again adopt the trypomastigote form (flagellum emerges from a position *posterior* to the nucleus). These parasites are released in the saliva and correspond to the non-proliferating metacyclic stage, that is infectious for mammalian hosts. During a blood meal, the fly injects saliva that contains anti-coagulants and a few metacyclic trypanosomes that will persist in the chancre for several days before invading the bloodstream. There, trypanosomes develop as extracellular parasites and two stages can be recognised: the proliferating, long slender form and the non-proliferating short stumpy form. The latter is non-dividing and competent for transmission in the tsetse fly, thereby completing the parasite cycle. All bloodstream stages display the trypomastigote form. Therefore, all stages are flagellated but flagellum length and positioning are variable according to the life cycle.

2. *Trypanosoma cruzi*

T. cruzi, the aetiological agent of Chagas disease is transmitted to humans via bugs. *T. cruzi* proliferates in the insect midgut, and exhibits the epimastigote form (Fig. 3). It attaches to the gut epithelium, mostly in the rectum (Kollien et al., 1998). Some parasites stop replicating, adopt the trypomastigote form and get detached from the epithelium. During a blood meal, the bug defecates, releasing parasites that can penetrate host tissues through minor cuts in the skin. Once within the mammalian host, the trypomastigote stage of *T. cruzi* is able to invade a range of host cells (Tan and Andrews, 2002) and to differentiate into proliferating, small, non-motile, spherical cells that possess a tiny flagellum barely exiting from the flagellar pocket (de Souza, 1984). This is called the amastigote form. At the end of the proliferating stage, trypanosomes differentiate and elongate to adopt the epimastigote and then the trypomastigote form. Such parasites are released in the bloodstream after lysis of the host cell and can either invade other host cells and re-iterate the cycle described above or they can be ingested by the insect and transform eventually in multiplying epimastigotes (Kollien and Schaub, 2000). In contrast to *T. brucei*, bloodstream trypomastigote stages of *T. cruzi* do not multiply.

3. *Leishmania* species

Major *Leishmania* species include *L. donovani*, responsible for visceral diseases, and *L. mexicana* or *L. major*, involved in cutaneous and muco-cutaneous infections. *Leishmania* are transmitted by sandflies where they proliferate in the digestive tract. The principal morphological difference between *Leishmania* and *Trypanosoma* lies in the anterior positioning of the flagellum and the fact that it is only

attached for a very short distance to the edge of the flagellar pocket, the rest of the flagellum being completely free from the cell body (Fig. 3). This is called the promastigote form. In the mammalian host, *Leishmania* is captured by macrophages via phagocytosis and differentiates in the amastigote stage that proliferates in the food vacuole (Alexander et al., 1999).

B. Trypanosomes as models to study the flagellum

Trypanosomes are turning out to be an excellent model to study flagellum biology (Bastin et al., 1999a; Godsel and Engman, 1999; Ersfeld and Gull, 2001; Kohl et al., 2003; McKean et al., 2003). The most amenable species is certainly *T. brucei*, for the following reasons. First, trypanosomes are among the few flagellated organisms that can be propagated easily in culture where they can reach densities up to 15-20 millions cells per ml, with a relatively rapid doubling time of 8-12 hours. Second, flagella are present throughout the cell cycle: when the cell grows a new flagellum, the old flagellum is not disassembled, thereby providing the opportunity to compare a mature flagellum with a flagellum being assembled in the *same* cell (Bastin et al., 1999a). Third, the trypanosome genome is fully sequenced (El-Sayed et al., 2003; Hall et al., 2003)(see also <http://www.genedb.org/genedb/trypan/index.jsp>). As it does not contain introns, gene identification and cloning is straightforward. Fourth, and probably the most important in terms of functional studies, potent and flexible tools for reverse genetics are available (Clayton, 1999; Motyka and Englund, 2004). Trypanosomes can be transfected by electroporation and the prevalence of homologous recombination

allows for precise targeted insertion of expression vectors, a great advantage for gene deletion (knock-out) approaches (Clayton, 1999). The development of tightly regulated tetracycline-inducible expression systems has been instrumental for the multiple facets of functional studies (Wirtz and Clayton, 1995; Wirtz et al., 1999). It has already been exploited successfully to monitor protein expression and assembly in cytoskeletal structures (Bastin et al., 1999a). Inducible expression of double-stranded RNA allows for conditional RNA interference (RNAi), an extremely powerful and flexible tool to study gene function (Motyka and Englund, 2004). Trypanosomes were among the first organisms where RNAi was discovered (Bastin et al., 1998; Ngô and Bouck, 1998). RNAi can be efficiently generated by transient transfection of double-stranded (ds) RNA (Ngô et al., 1998), or by stable transformation with vectors expressing dsRNA under the control of tet-inducible promoter(s) (Bastin et al., 2000a; Bringaud et al., 2000; LaCount et al., 2000; Shi et al., 2000; Wang et al., 2000; Wang and Englund, 2001). Gene silencing by RNAi is efficient and specific (Durand-Dubief et al., 2003) and its generation by inducible expression of double-stranded RNA allows for kinetic analysis of phenotype resulting from protein loss, even in the case of essential genes (see for example: Wang and Englund, 2001; Allen et al., 2003; Kohl et al., 2003; Garcia-Salcedo et al., 2004). After the onset of RNAi, the target RNA is rapidly destroyed but the protein only disappears according to its own turnover (Bastin et al., 2000a). As RNAi is reversible, production of dsRNA can be stopped and protein re-expression can be observed (Bastin et al., 2000a). This is particularly useful when studying proteins that are components of cytoskeletal structures that need to be assembled, such as flagella (Bastin et al., 2000a). Moreover, the design of a *T. brucei* RNAi library opens up new possibilities in terms

of forward genetics providing an efficient screening method is available (Morris et al., 2002). Unfortunately, RNAi does not appear to be functional in *T. cruzi*, nor in *Leishmania*, despite extensive investigations (Robinson and Beverley, 2003; DaRocha et al., 2004), therefore limiting the possibilities for functional studies in these species. This is presumably due to the lack of some essential components of the RNAi pathway (Durand-Dubief and Bastin, 2003; Shi et al., 2004). Nevertheless, gene deletion is feasible providing the gene of interest is not essential (Cooper et al., 1993; Santrich et al., 1997). In the following sections, we shall focus on the flagellum of *T. brucei*, including data from the other species when available.

C. Composition of the trypanosome flagellum

Electron microscopy analysis of the trypanosome flagellum revealed the presence of an apparently conventional axoneme and of electron dense particles resembling IFT particles (Fig. 1), but also of other associated elements: the paraflagellar rod (PFR), the flagellum attachment zone (FAZ) and the flagella connector (FC) (Vickerman, 1962; Angelopoulos, 1970; Sherwin and Gull, 1989a; Bastin et al., 2000b; Moreira-Leite et al., 2001). These structures will be described in detail in the sections below. Initial information about their molecular composition has been acquired after protein purification or after screening of expression libraries with specific antibodies (Schlaeppli et al., 1989; Birkett et al., 1992). Determination of the genome sequence of *T. brucei* but also of *L. major* and *T. cruzi* now offers the possibility to identify flagellar genes by homology searching with data available from other organisms (Kohl et al., 2004).

1. The axoneme

The axoneme presents the typical structure with 9 peripheral doublets of microtubules surrounding a central pair of singlet microtubules (Fig. 1)(Vickerman, 1976b). As in many other organisms, it is made from a basal body, constituted of typical triplets of microtubules and prolonged by a transition zone (both do not possess the central pair). The 9 peripheral doublets are flanked by inner and outer dynein arms, and the radial spokes can be recognised. Projections irradiating from the central pair microtubules are also present, although they have not been characterised in detail so far. These data confirm the high structural degree of conservation of the axoneme in trypanosomes.

Homologues of most genes encoding components of the axoneme identified in *Chlamydomonas*, sea urchin or mouse are found in the *T. brucei* genome (Avidor-Reiss et al., 2004; Kohl et al., 2004). The main components of microtubules, α - and β -tubulins, are of course present and exhibit the usual post-translational modifications such as acetylation (Schneider et al., 1987) and glutamylation (Schneider et al., 1997). The carboxy terminal end of α -tubulin is tyrosinated on newly formed microtubules of the axoneme, located at the distal tip of the growing flagellum (Sherwin et al., 1987; Sherwin and Gull, 1989b). In contrast, glycylation of α - and β -tubulins, a post-translational modification required for cilia motility in other species (Million et al., 1999; Thazhath et al., 2002) is not present in trypanosomes (for a detailed review of these modifications, see Gull, 1999). Less-abundant tubulins

associated to centrioles or basal bodies such as γ -, δ - and ϵ -tubulin are also found in trypanosomes, with an original member, ζ -tubulin, specific to the Trypanosomatids (Vaughan et al., 2000; McKean et al., 2001).

At least 13 dynein heavy chains are present in the *T. brucei* genome, 10 of them are likely to be of the axonemal type, with 2 other heavy chains involved in IFT and the conventional cytoplasmic type. Most intermediate and light dynein chains are also present. The deduced proteins exhibit 32-48 % identity with orthologues from *Chlamydomonas*. Similarly, typical proteins belonging to the radial spoke family or to projections of the central pair microtubules are present and show high degree of conservation in trypanosomes (Kohl et al., 2004). A rare exception is the *PF6* gene, coding for a protein associated with the central pair apparatus in *Chlamydomonas* (Rupp et al., 2001). However, this gene is poorly conserved and its identification in other organisms is difficult. Our laboratory has cloned numerous axonemal genes and determined their functions by inducible RNAi silencing in *T. brucei*. Results confirmed the expected functions of these proteins in flagellum beating (Kohl et al., 2004). These data are important as they validate the use of the trypanosome as a model to study flagella and flagella-related diseases.

One axonemal gene, trypanin, has been first identified in *T. brucei*. It is coding for a component of the dynein-regulatory complex and plays an important role in the control of flagellum beating and of cell motility (Hill et al., 2000; Hutchings et al., 2002; Rupp and Porter, 2003). Its inactivation by RNAi leads to perturbation of trypanosome movement and although the flagellum is still motile, cells cannot move forward and appear to tumble (Hutchings et al., 2002).

In *T. cruzi* and *Leishmania*, the amastigote stage possesses a very short, immotile, flagellum, barely exiting the flagellar pocket. It is strongly constricted at the neck of the pocket, where the flagellum diameter is significantly reduced: from 250 nm to 140 nm (Alexander, 1978). The flagellum often displays a 9+0 configuration, with frequent mis-arrangement of doublet microtubules (Alexander, 1978). Interestingly, *IFT* and *PFR* gene expression is strongly down-regulated upon differentiation of promastigote to amastigote stage (Mishra et al., 2003).

2. The paraflagellar rod (PFR)

Examination of sections through the trypanosome flagellum revealed the presence of an extra-axonemal structure called the paraflagellar rod (PFR), also referred to as the paraxial rod or the paraxonemal rod (Vickerman, 1962). The PFR is composed of filaments finely organised and can be sub-divided in 3 regions called the proximal, intermediate and distal domains (Farina et al., 1986). This structure is detected alongside the axoneme once the flagellum exits from the flagellar pocket and runs till its tip. It is tightly connected to doublets 4 and 7 of the axoneme *via* specific filaments (Fig. 1). This connection is very stable as only trypsin treatment manages to separate the PFR from the axoneme (Russell et al., 1983). The position of the flagellum relative to the cell body is precisely defined, with the PFR always seen in closer proximity to the cell body. This is clearly demonstrated by immunofluorescence staining of trypanosomes with anti-tubulin antibodies, that reveals a dark zone between the intense cell body staining (due to the subpellicular microtubules of the cell corset) and the flagellum staining (due to the axonemal

microtubules) (Woods et al., 1989). This area corresponds to the presence of the PFR.

The PFR has so far only been identified in Kinetoplastids and Euglenoids, with possibly a related structure in dinoflagellates (reviews in Cachon et al., 1988; Bastin et al., 1996; Maga and LeBowitz, 1999; Bastin et al., 2000b). The PFR does not show ultrastructural resemblance to other extra-axonemal structures that have been identified in protists or in spermatozoa flagella.

Two major proteins, termed PFRA and PFRC in *T. brucei* have been purified from the paraflagellar rod of trypanosomes, and the corresponding genes have been identified (Schlaeppli et al., 1989; Deflorin et al., 1994). Orthologues have only been found in related kinetoplastids (*Trypanosoma*, *Leishmania*) (Beard et al., 1992; Moore et al., 1996; Fouts et al., 1998; Maga et al., 1999) or in *Euglena gracilis* (Ngô et al., 1998), but are absent from the genomes of all other eukaryotic species examined to date (Kohl et al., 2004). The composition of the PFR in dinoflagellates is still unknown, so it is not possible at this stage to evaluate whether the same type of protein is involved in its composition. Immunological comparison with monoclonal antibodies raised against the *Euglena* PFR proteins (Gallo and Schrevel, 1985) failed to identify any cross-reactivity (P. Huitorel, unpublished observations). PFRA and PFRC are coiled-coil rich proteins and contain a putative calmodulin-binding domain (Schlaeppli et al., 1989). However, no other specific signature could be clearly recognised. We have identified a putative third member of this family, called *PFRE*, in the *T. brucei* genome. The protein shares 20-25 % identity with PFRA and PFRC and is conserved in *Leishmania* and *T. cruzi* (Branche & Bastin, unpublished data).

Comparison of bi-dimensional gels of flagella purified from normal and mutant trypanosome strains deprived of a PFR suggests that this structure is composed of at least 20 other polypeptides (Pullen et al., 2004). This number is probably an underestimation as proteins of high molecular weight are excluded from this type of analysis. Due to their unique nature, the PFR components are highly immunogenic (Woods et al., 1989; Woodward et al., 1994; Kohl et al., 1999) and anti-PFR antibodies have frequently been reported in infected animals (Imboden et al., 1995). Screening expression libraries identified 3 separate novel genes, encoding proteins of high molecular weight containing repeated domains (Woodward et al., 1994; Imboden et al., 1995). It has been postulated that such an organisation might be involved in polymerisation or stabilisation of the PFR filaments.

A significant amount of calmodulin is localised throughout the PFR, as revealed by immuno-fluorescence and immuno-electron microscopy (Ruben and Patton, 1987; Ridgley et al., 2000). Calcium-binding proteins containing EF-hand domains have been found associated to the flagellum, probably both in the axoneme and the PFR (Wu et al., 1992; Bastin et al., 1999b). A proteomic approach revealed the presence of several adenylate kinases in the PFR, however their inactivation by RNAi does not create a visible phenotype (Pullen et al., 2004). Finally, a two-hybrid screen suggests that a calcium-stimulated adenylyl cyclase could interact with the major PFR proteins in *T. cruzi* (D'Angelo et al., 2002). However, *in vivo* association and co-localisation has yet to be demonstrated.

3. The flagellum attachment zone (FAZ)

Specific cytoskeletal structures following the attached flagellum of trypanosomes can also be recognised on the cell body side, and have been defined as the flagellum attachment zone (FAZ) (Kohl and Gull, 1998). Although FAZ structures are not part of the flagellum *per se*, they are intimately associated to this organelle and its functions, and therefore will be described here. As soon as the flagellum emerges from the flagellar pocket, it is bent and attached to the cell body. Flagellum and cell body membranes are in tight contact (Fig. 1). The FAZ is recognisable from the flagellar pocket area, follows the flagellum path and terminates at the anterior tip of the cell whereas the flagellum extends a couple of μm further (Fig. 2). The FAZ contains two distinct structures: the FAZ filament and a unique set of 4 microtubules. The FAZ filament is an electron-dense filament that lies in a gap between two microtubules of the subpellicular corset underneath the flagellum (Sherwin and Gull, 1989a). Longitudinal sections through the FAZ filament show a regular array of thick particles, like a rosary. It should be noted that substructures present in the flagellum seem to link the PFR to the membrane region closest to the FAZ filament (Sherwin and Gull, 1989a). Immunisation of mice with cytoskeletal preparations produced several monoclonal antibodies recognising components of the FAZ filament (Woods et al., 1989; Kohl et al., 1999). By immuno-staining, they exhibit a typical dot-like pattern, starting in proximity to the basal body/flagellar pocket area and finishing at the distal end of the cell body. FAZ proteins are specific to the cell body as no labelling could be visualised in the flagellum. Several genes encoding proteins of the FAZ filament have been identified in *T. brucei* or in *T. cruzi*. They are large proteins (>200 kDa) containing repeated domains (reviewed in (Kohl and Gull, 1998) that, like the large PFR proteins, could participate in protein/protein

interactions required for assembly of the filament. Interestingly, these are conserved in *Leishmania*, whose flagellum is only attached to a limited portion of the cell body, just aside of the flagella pocket (Alexander, 1978; Wiese et al., 2003).

A second recognisable structural element is a set of 4 microtubules, always positioned on the same side of the FAZ filament, and associated to the smooth endoplasmic reticulum (Angelopoulos, 1970; Sherwin and Gull, 1989a). The polarity of these microtubules (plus end at the anterior of the cell) is opposite to that of all the other subpellicular microtubules (plus end toward the posterior end), but is actually the same as for the axonemal microtubules in the flagellum (Robinson et al., 1995). In addition to the difference in polarity, these 4 microtubules have distinct biochemical characteristics when compared with their sub-pellicular counterparts as they are resistant to high salt treatment (Sherwin and Gull, 1989a). Immunological data indicate a couple of other differences: (1) an anti- β -tubulin monoclonal antibody recognises exclusively these 4 microtubules (Gallo et al., 1988) and (2) an anti- γ -tubulin polyclonal antibody produces a clearly more intense staining of this quartet (Scott et al., 1997).

4. Intraflagellar transport (IFT) particles

Electron-dense particles resembling IFT rafts have been described in trypanosomes (Sherwin and Gull, 1989a; Bastin et al., 2000b). Similar to those reported in *Chlamydomonas*, they are found between the outer doublet microtubules and in close proximity to the flagellum membrane (Fig. 1). They have not been seen in proximity to doublets 4-7, presumably due to the presence of the PFR. By mining the *T. brucei* genome, orthologues of all available *IFT* genes have been detected

(Kohl et al., 2003; Kohl et al., 2004). They include the motor proteins (heterotrimeric kinesin II and IFT dynein complex) as well as structural components of the IFT particle. Curiously, two different IFT dynein heavy chains (motor for retrograde transport) are present in Trypanosomatid genomes, instead of only one in all other species studied to date (Kohl et al., 2004)(Adhiambo and LeBowitz, unpublished data). Our laboratory has performed RNAi silencing of 15 candidate IFT genes that all turned out to be necessary for flagellum assembly (Kohl et al., 2003)(Benghanem, Kohl, Rusconi & Bastin, unpublished data).

5. The flagellum membrane

Although the flagellum membrane is in continuity with the cell body membrane, its composition is not identical. In trypanosomes, a third specialised region must be discriminated: the membrane of the flagellar pocket. As this is the only place for endocytosis and exocytosis, it is the unique site for addition or removal of membrane-associated proteins from flagellar or cell body origin (Morgan et al., 2002a; Overath and Engstler, 2004). Like the cell body membrane, the flagellum membrane is covered by major surface proteins that are involved in immune evasion in the bloodstream of mammals, (Cross, 1975; Balber, 1990) or in protection against the aggressive digestive medium in the midgut of the tsetse fly, (Ruepp et al., 1997).

The flagellum adhesion protein FLA1 in *T. brucei*, also called GP72 in *T. cruzi*, is a more specific protein. Initially identified by an immunological approach in *T. cruzi*, GP72 is a glycoprotein of unusual composition (Snary et al., 1981). GP72 and FLA1 localise mainly to the membrane of the flagellum in both *T. cruzi* and *T. brucei*, with a reduced abundance to the rest of the cell surface (de Jesus et al., 1993; Nozaki et

al., 1996). The protein has a typical amino-terminal signal sequence for entry in the endoplasmic reticulum as well as a carboxy-terminal transmembrane segment (Cooper et al., 1991; Nozaki et al., 1996). *GP72* gene knock-out in *T. cruzi* are viable but display an amazing phenotype: the flagellum is detached from the cell body and, although it beats actively, the mutant cells fail to swim properly and sink at the bottom of the culture flask (Cooper et al., 1993). A similar phenotype is observed after RNAi silencing of *FLA1* in *T. brucei*, but here it turns out to be lethal (Moreira-Leite et al., 2001; LaCount et al., 2002). These data demonstrate a crucial role for GP72/FLA1 in flagellum adhesion to the cell body, and also reveal differences in the importance of this attachment between *T. brucei* and *T. cruzi*. GP72 and FLA1 share only 44 % identity at the amino acid level, and although they are both involved in flagellum adhesion to the cell body, they might not function by exactly the same mechanisms. This was confirmed by expression of *T. cruzi* GP72 in wild-type *T. brucei* that leads to flagellum detachment, suggesting it interferes with proper FLA1 function (LaCount et al., 2002). Curiously, such cells appear viable. A homologue of GP72/FLA1 is present in the *Leishmania* data base (Kohl and Bastin, unpublished data), despite the limited adhesion of the flagellum to a region just outside the flagellar pocket (Santrich et al., 1997; Wiese et al., 2003). Homologues were not found in other organisms. Finally, it should be noted that two copies of *FLA2*, a *FLA1*-related gene have recently been identified in *T. brucei*. The FLA2 protein sequence shares ~60 % identity with FLA1 and is characterised by a 44-amino acid proline-rich insertion (LaCount et al., 2002).

The flagellum calcium binding protein (FCaBP) of *T. cruzi* is the only characterised protein specifically localised to the inner face of the flagellum

membrane (Engman et al., 1989) (Godsel and Engman, 1999). It is composed of 4 EF-hand calcium-binding domains (Engman et al., 1989). The protein is dually acylated by myristoylation of glycine at position 2 and by palmitoylation of cysteine at position 4 of the amino-terminal end of the protein. Alanine-replacement experiments nicely demonstrated that both modifications were required for flagellum membrane association in *T. cruzi*, a process dependent on calcium (Godsel and Engman, 1999). Two integral membrane proteins have been localised to the flagellum: an adenylate cyclase in *T. brucei* (Paindavoine et al., 1992) and a minor isoform of a glucose transporter that is specifically targeted to the flagellum membrane of *Leishmania enriettii* (Piper et al., 1995). Convincing explanations for this specific localisation remains to be established.

Few data are available on the lipid composition of the flagellum membrane. The antibiotic filipin, which binds β -hydroxysterol, forms complexes that are visible by freeze-fracture electron microscopy. These complexes are clearly enriched on the flagellum membrane of *T. brucei*, *T. cruzi* and *L. mexicana* (Tetley, 1986; Tetley et al., 1986; De souza, 1995). Recent biochemical and morphological data suggest that lipid rafts components (sphingolipids, sterols) are abundant in the flagellum membrane of *T. cruzi* and could be associated with proteins of the calflagin family. Detergent-extraction of *T. cruzi* at low temperature solubilises most of the surface membrane with the exception of small remnants that can be visualised by scanning electron microscopy. These elements are vastly over-represented on the surface of the flagellum and found as linear arrays distributed along the axoneme but not along the PFR (Tyler et al., unpublished data), a localisation reminiscent of the IFT particles that are also restricted to the axoneme. One must remember that in

Chlamydomonas, the flagellum membrane appears constricted when in proximity of IFT particles (Kozminski et al., 1995), suggesting that the IFT particle might clinch to the membrane. These data lead the authors to propose that lipid rafts could be clustered and transported by the intermediate of the IFT machinery, with possible implications for trafficking of flagellar membrane proteins (Tyler et al., unpublished data).

6. The flagella connector

A novel specific structure has been discovered recently at the distal tip of the assembling flagellum in the procyclic stage of *T. brucei*. This structure is visible after detergent-extraction of the cytoskeleton, and appears to tether the extremity of the new flagellum to the side of the old flagellum and therefore has been called the flagella connector (FC) (Moreira-Leite et al., 2001). This pyramidal structure is constituted of several layers with its large base (400 nm) associated to one side of the axoneme of the old flagellum and its tip connected to the extremity of the elongating microtubules in the new flagellum (Briggs et al., 2004). The FC is exclusively present when a new flagellum is being assembled, as early as before the flagellum exits from the flagellar pocket (Moreira-Leite et al., 2001; Kohl et al., 2003; Briggs et al., 2004). So far, it has only been identified in the procyclic stage of *T. brucei*. It is missing from the corresponding bloodstream stage and from the related *T. cruzi* and *Leishmania* species (Briggs et al., 2004). Strikingly, the FC is mobile as it progressively moves towards the anterior end of the old flagellum while the new flagellum elongates (Fig. 4). Antibodies recognising specifically the FC have been obtained, indicating its unique nature (Kohl et al., 2003; Briggs et al., 2004).

However, to date, no molecular components have been identified. To demonstrate the role of the FC in tethering the tip of the new flagellum to the old one, Moreira-Leita et al. (2001) used inducible RNAi silencing of *FLA-1*, the gene coding for the surface protein responsible for flagellum attachment to the cell body, in order to detach the new flagellum whilst the old one still remained associated to the cell body. In these conditions, the new flagellum is only anchored to the cell by its basal body, it exits from the flagellar pocket and it is then free from the main cell body with the striking exception of its distal tip that is held by the FC onto the old flagellum. This new flagellum beats vigorously, yet its tip remains connected, demonstrating the strength of the interaction mediated by the FC. Gull and colleagues proposed that this anchoring system would permit orientation of flagellum elongation that in turn would guide assembly of the FAZ components and somehow would allow to copy the existing cytoskeletal organisation, defining a kind of cytoplasmic heredity (Moreira-Leita et al., 2001).

D. Assembly of the trypanosome flagellum

The trypanosome flagellum is assembled from a basal body composed of 9 triplets of microtubules and its formation is strictly linked to the cell cycle (Sherwin and Gull, 1989a; Woodward and Gull, 1990; Kohl et al., 1999). The mature basal body is flanked by an immature, pro-basal body (Burton and Dusanic, 1968; Vickerman, 1976b). When the new flagellum is constructed, the existing flagellum remains in place, providing an ideal opportunity to compare the building structure versus its already assembled counterpart. This situation has been observed in many

protists such as *Entosiphon* (Brugerolle, 1992), *Giardia* (Ghosh et al., 2001) or *Trichomonas* (Noel et al., 2003) for example. However, it is not the case in other species such as sea urchin embryos or *Chlamydomonas* where cilia or flagella are disassembled at every cell cycle and basal bodies used as centrioles at the spindle poles, before nucleating new flagella formation (Johnson and Porter, 1968; Cavalier-Smith, 1974).

Several lines of evidence indicate that the trypanosome flagellum follows the conventional mode of IFT-dependent assembly described in *Chlamydomonas* (see II.C), with production of flagellar proteins in the cytoplasm, import in the flagellum compartment and transport to the distal tip for assembly (Bastin and Gull, 1999). Two specific aspects need to be taken into account: (1) two separate intra-flagellar structures need to be assembled (the axoneme and the PFR) and (2) the FAZ filament and 4 associated microtubules follow the flagellum path closely, suggesting their assembly is at least partially dependent on flagellum presence (Kohl et al., 2003).

1. Replication of the flagellar apparatus during the trypanosome cell cycle

Trypanosomes are highly polarised cells, that swim with their flagellum leading, defining the narrow anterior end and the wider posterior end. In the trypomastigote stage, the nucleus occupies a central position, and the flagellar pocket, the basal body and the mitochondrial genome are found at the posterior end of the cell. The subpellicular corset of microtubules is intimately linked to the typical helical aspect and retains cell shape after detergent-extraction (Sherwin and Gull, 1989a).

The flagellum occupies a central position in the trypanosome cell cycle. In the next paragraphs, we shall summarise data from extensive studies performed on the culture-adapted procyclic stage (Sherwin and Gull, 1989a; Woodward and Gull, 1990; Robinson et al., 1995; Ploubidou et al., 1999) and then highlight main differences with other *T. brucei* life cycle stages and with *T. cruzi* and *Leishmania* (Figs. 2-3). All these Trypanosomatids replicate by binary fission, without disassembling their cytoskeleton. Procyclic trypanosomes in the early phase of their cell cycle possess a basal body complex (a mature basal body subtending the flagellum and a pro-basal body), a single flagellum and its associated FAZ structures, a single kinetoplast and a single nucleus (Fig. 2). The first obvious sign of cell cycle progression is the maturation of the pro-basal body, followed by basal bodies duplication (Sherwin and Gull, 1989a), a process reminiscent of centrosome maturation and duplication in many other cell types (Bornens, 2002; Beisson and Wright, 2003). This duplication step is rapidly followed by mitochondrial genome replication (Sherwin and Gull, 1989a; Woodward and Gull, 1990). The basal bodies then migrate apart to separate by 5-6 μm (Robinson and Gull, 1991; Robinson et al., 1995; Kohl et al., 2003). This migration step is concomitant with that of the mitochondrial genome. This is explained by the fact that the mature basal body is tightly connected to the mitochondrial genome *via* specific filaments and is responsible for kinetoplast movement during cell cycle progression (Robinson and Gull, 1991; Ogbadoyi et al., 2003). The mature posterior basal body subtends the new flagellum that exits from the flagellar pocket and elongates towards the anterior end of the cell. A new FAZ filament and set of 4 microtubules is produced from the basal bodies/flagellar pocket area and follows flagellum elongation closely (Kohl et al., 1999; Kohl et al., 2003).

The tip of the new flagellum is in constant contact with the existing, old flagellum and is immediately attached to the cell body as soon as it exits from the flagellar pocket. An unusual cytoskeletal sub-structure, named the flagella connector (FC), is present at the distal end of the new flagellum and tethers it to the old flagellum (Moreira-Leite et al., 2001) (Fig. 4).

Basal body duplication is followed by nuclear S phase and mitosis (Sherwin and Gull, 1989a; Woodward and Gull, 1990). This is a closed mitosis that takes place within the nuclear membrane but without centrioles (Ogbadoyi et al., 2000). Chromosomes do not condense and the nucleolus is not dispersed but rather duplicates and segregates along the mitotic spindle (Vickerman and Preston, 1970; Durand-Dubief and Bastin, 2003). The most posterior nucleus migrates in the gap between the 2 basal bodies/kinetoplasts. Now that cells have duplicated nuclear and mitochondrial genomes, basal bodies, flagella and associated FAZ structures, cytokinesis is initiated from the anterior end of the trypanosome cell, apparently in close proximity to the anterior end of the FAZ (Robinson et al., 1995). It proceeds in a helical manner towards the posterior end, following the path of the new flagellum and produces two siblings, each inheriting a single set of these individual organelles (Fig. 2).

The cell cycle of bloodstream *T. brucei* is closely related but with two major differences. First, migration of duplicated basal bodies is much more limited and nuclei remain close to each other after mitosis (Vickerman, 1976b; Tyler et al., 2001; Briggs et al., 2004). Cell division is also initiated at the anterior end but physically separates first the nuclei and then the basal bodies/kinetoplasts. The second noticeable difference is the absence of the FC, despite the fact that the tip of the new

flagellum appears in vicinity of the old flagellum, although not in direct contact as observed in the procyclic stage. The FC cannot be detected at all, neither by electron microscopy observation, nor by immuno-staining of its components with anti-FC antibodies (Briggs et al., 2004).

Epimastigotes of *T. cruzi* and promastigotes of *Leishmania* follow a cell cycle that is more similar to the bloodstream stage of *T. brucei* (Fig. 3). Here too, basal bodies duplicate first and separate kinetoplast DNA. The duplicated complexes remain in very close proximity while nuclear mitosis and new flagellum outgrowth take place. Given the anterior position of the kinetoplast/basal bodies relative to the nucleus, cell division first separates basal bodies and kinetoplast DNA before nuclei. Flagellum elongation does not appear to be complete at this stage as the new flagellum is shorter than the old one. Finally, the tip of the new flagellum is clearly separate from the old flagellum and no FC has been detected (Briggs et al., 2004).

2. Protein targeting to the trypanosome flagellum

Due to the absence of ribosomes in the flagellum compartment, both PFR and axoneme precursors are synthesised in the cytoplasm and need to be imported. In *Chlamydomonas* and in *Tetrahymena*, proteins constituting dynein arms or radial spokes are pre-assembled in the cytoplasm to form a complex (Fok et al., 1994; Fowkes and Mitchell, 1998) that is transported in flagella by IFT (Qin et al., 2004). Import of axonemal proteins in the trypanosome flagellum has not been studied to date, but given the conservation of the IFT machinery, it sounds reasonable to assume that a similar process is present.

In contrast, import of PFR proteins in the cytoplasm has been studied in more

details and the situation turns out to be a bit different. Absence of PFRA does not prevent import and accumulation of PFRC in flagella (Bastin et al., 1999b) and reciprocally (Maga et al., 1999; Durand-Dubief et al., 2003). This suggests that each protein contains the information necessary for access to the flagellum. Deletion studies of PFRA in *T. brucei* identified a discrete sequence at the carboxy-terminal end of the protein that is essential, but not sufficient, for flagellum targeting. This 9-amino acid domain is conserved in PFRA/PFRC and PFR proteins from *T. cruzi*, *L. mexicana* and *Euglena gracilis* (Bastin et al., 1999a) as well as in other Euglenoid species such as *Distigma curvatum* and *Phacus smulkowskianus* (Talke, S., Ruppel, H.G. and Preisfeld, A., sequences available via PubMed). Interestingly, a related sequence is found in axonemal dynein heavy chain β of *Chlamydomonas*. A tripeptide histidine-leucine-alanine (HLA) located in the middle of this short domain is found in a unique insertion of an unusual actin-related protein associated to the axoneme (Ersfeld and Gull, 2001). Deletion of this tripeptide in PFRA and in the actin-related protein abolished flagellum localisation (Ersfeld and Gull, 2001). At this stage, it is not known whether this sequence works as a flagellum entry signal or as a docking system for another protein (or protein complex) that would itself be carrying the flagellum targeting signal. We have described above (see III.C5) how acylation was involved in targeting the flagellum calcium-binding protein to the internal face of the flagellum membrane (Godsel and Engman, 1999). There is no homology between the myristoylation and palmitoylation signals and the PFRA/actin-related protein domains.

3. Axoneme construction and the control of flagellum length

Assembly of the peripheral doublets in the trypanosome axoneme takes place by extension of the existing A and B tubules of the basal body. Addition of tubulin occurs at the distal tip, as illustrated by the use of a monoclonal antibody recognising tyrosinated α -tubulin, a marker of newly assembled microtubules. This showed a discrete gradient along the growing new flagellum, with the highest concentration at the distal tip (Sherwin and Gull, 1989b). The central pair microtubules are only nucleated from the top of the transition zone, in a process dependent on γ -tubulin (McKean et al., 2003). Although the polarity of these microtubules has not been experimentally demonstrated, it is very likely that their plus end is at the distal end of the flagellum.

As observed in other organisms, flagellum formation does not take place if IFT is inhibited (Kohl et al., 2003)(Benhanem, Rusconi, Kohl and Bastin, unpublished data). Inducible RNAi silencing of genes coding for several separate IFT proteins (IFT20, IFT52, IFT55, IFT88, IFT172, dynein heavy chains) was used to study the importance of IFT in flagellum formation. This approach is very powerful as it allows to monitor intermediate stages between wild-type phenotype and total absence of flagella. In *Chlamydomonas*, IFT proteins are mostly localised around the basal body area, with a small proportion actively involved in IFT within the flagellum itself (Cole et al., 1998; Deane et al., 2001). Triggering RNAi silencing targeting *IFT* genes in *T. brucei* leads to a progressive reduction in the pool of IFT proteins associated to the basal bodies. The first visible consequence is the formation of a shorter new flagellum, without obvious modification of the old flagellum. In the next cell cycles, trypanosomes produced shorter and shorter new flagella (Kohl et al., 2003). The length of the PFR and of the FAZ was reduced in

close proportion as that of the axoneme, indicating that IFT controls (directly or not) the assembly of all flagellar structures. These results support the proposed role of IFT in the control of flagellum length in *Chlamydomonas* (Marshall and Rosenbaum, 2001). Longer exposures to absence of IFT resulted in a second wave of phenotypic alterations, where no flagella could be grown at all, despite the presence of an apparently normal basal body (Kohl et al., 2003). Cells that grow a shorter new flagellum display defects in overall cell body size and those that completely fail to grow a new flagellum show numerous problems in morphogenesis and cytokinesis, eventually leading to cell death (see III.E).

Examination of *T. cruzi* and *L. major* genomes confirms the presence of homologues of most *IFT* genes (Kohl et al., 2004). Gene deletion of the IFT dynein heavy chain in *L. mexicana* severely impairs flagellum assembly (Adhiambo and Le Bowitz, unpublished data), confirming the functional conservation of IFT in flagellum formation in other Trypanosomatids. However, the *Leishmania* flagellum does not seem to be as vital as for survival as the knock-out cell line turns out to be viable.

In *Leishmania*, two proteins potentially involved in the control of flagellum length at the promastigote stage have been identified: Ld-ARL3A, a small G protein (Cuvillier et al., 2000) and LmxMKK, a mitogen-activated protein kinase (Wiese et al., 2003). Over-expression of a mutated Ld-ARL3A, that is blocked in the GTP-bound form, leads to drastic reduction of the flagellum length, that barely exits the flagellar pocket (Cuvillier et al., 2000). Gene deletion of *LmxMKK* produces very similar effects, reducing the length of the flagellum to 1.7 μm (Wiese et al., 2003). Tiny amounts of PFR material were still detected in *LmxMKK* $-/-$ mutants, and were sometimes assembled as a rudimentary structure. Both proteins are selectively

expressed in the promastigote stage of the parasite, but are not detected in the amastigote stage (that possesses a very short flagellum).

4. Paraflagellar rod construction

The PFR is only visible once the flagellum exits the flagellar pocket, i.e. $\sim 1 \mu\text{m}$ after the end of the transition zone. Nothing is known about the way the structure is nucleated at that precise position. PFR construction in the new flagellum always appears slower than that of the axoneme. Electron microscopy observation of whole mount cytoskeletons of cells with 2 flagella shows that both PFR and axoneme terminate at almost the same position at the extremity of the old flagellum whereas the new axoneme always extends further than the new PFR (Kohl, Sherwin & Gull, unpublished data). The PFR is made of several domains that are likely to be assembled on top of each other. This is visualised by comparison of old and new flagella after immuno-fluorescence using monoclonal antibodies against tubulin and a PFR protein located at the outer domain of the PFR (Woods et al., 1989; Woodward et al., 1994), that has been used as a marker for fully assembled PFR (Bastin et al., 1999b). Both signals terminate at about the same position in the old flagellum. However, in the new flagellum, PFR staining does not reach the distal tip (Benghanem & Bastin, unpublished data). These data suggest that PFR formation is axoneme-dependent and IFT-dependent. This idea is supported by the fact that the PFR never extends beyond the axoneme in the short flagella of IFT RNAi mutants and that a PFR is not assembled in the absence of axoneme (Kohl et al., 2003).

PFR formation is prevented upon silencing of one or the other major components (PFRA or PFRC), suggesting that like α - and β -tubulins that are both essential for microtubule formation, PFRA and PFRC are both required for assembly of the PFR structure (Bastin et al., 1998; Durand-Dubief et al., 2003). However, inhibition of PFR formation has no consequence on axoneme assembly that goes on unabated.

Several data show that the PFR proteins are imported and transported at the distal tip of the PFR structure for assembly, in a process that is similar to axoneme assembly. First, addition of new subunits takes place predominantly at the PFR distal tip, as shown by induction of expression of an epitope-tagged PFRA protein during flagellum assembly (Bastin et al., 1999a). Second, when RNAi targeting *PFRA* is triggered in a cell that has already started to assemble a new flagellum, the protein is still found in the proximal part of the flagellum but is missing from the distal tip (Bastin et al., 2000a). Third, the absence of PFRA not only prevents construction of a normal PFR structure but in addition leads to formation of a 'blob' of non-assembled PFRC protein, visible during flagellum assembly. A small amount of excessive material starts to be detected at the tip of short new flagella and becomes bigger and bigger as the flagellum elongates. This leads to a spectacular dilation of the distal tip of the flagellum that can be explained if, in the absence of PFRA, PFRC is still produced and imported in the flagellum where it is transported to the distal end for assembly. There, it cannot find its partner and as a consequence cannot be assembled in PFR filaments. The excessive material progressively accumulates as more PFRC protein is imported during flagellum elongation. After cell division, size of this blob is progressively reduced until it is not detected any more, suggesting

unassembled proteins have been removed and brought back to the cell body, presumably for degradation (Bastin et al., 1999b). Same results were obtained for PFRA after *PFRC* silencing (Durand-Dubief and Bastin, 2003). All these observations demonstrate the existence of both anterograde and retrograde transport of PFR proteins, in a manner analogous to IFT transport. It now remains to establish whether the same machinery is used for transport of axonemal and PFR precursors.

Gene knock-outs of the *PFRA* and/or *PFRC* orthologues in *L. mexicana* also lead to failure in assembling a full PFR structure and accumulation of unassembled PFR material, but do not interfere with axoneme construction (Santrich et al., 1997; Maga et al., 1999).

E. Flagellum functions in trypanosomes

Several functions can be assigned to the flagellum in trypanosome biology. Beyond the classic motility and sensitivity roles, this organelle also participates to attachment of the parasites to various types of host tissues and to cell morphogenesis and cytokinesis. These aspects vary between different species of Trypanosomatids, as well as during separate stages of their life cycle, and will be discussed in the next session.

1. Cell motility

The flagellum beats vigorously and plays an obvious role in trypanosome motility (reviewed in Hill, 2003). Trypanosomes swim with their flagellum leading and

waves are initiated from the distal tip, propagating towards the base (Walker, 1961). This is the case of all Kinetoplastids studied so far (Alexander and Burns, 1983; Sugrue et al., 1988). The axoneme participates in flagellum and trypanosome motility, as demonstrated by RNAi silencing experiments targeting genes coding for components of different sub-structures. It leads to modification, reduction or complete inhibition of flagellum beating and as a result, cells fail to swim properly or are paralysed and sink at the bottom of the culture flask. Typical examples include proteins associated with the central pair (PF16, PF20), components of the radial spoke (RSP3) or of dynein arms (dynein light, intermediate or heavy chains) (Kohl et al., 2001)(Kohl, Branche & Bastin, unpublished data). To our knowledge, functional studies of axoneme components has not been reported in *T. cruzi*, nor in *Leishmania*.

But the axoneme is not the only determinant of cell motility. Silencing of PFRA and/or PFRC, the two main components of this extra-axonemal structure, results in failure of PFR assembly and to flagellum and cell paralysis (Bastin et al., 1998; Bastin et al., 1999b; Bastin et al., 2000a) (Durand-Dubief et al., 2003). Gene knock-outs of PFRA and PFRC orthologues in *L. mexicana* produced the same phenotype (Santrich et al., 1997; Maga et al., 1999). How could the PFR contribute to flagellum motility ? One hypothesis is that the PFR structure acts as a strengthening fibre for the flagellum, allowing for more efficient axoneme beating. Two experimental observations of *Leishmania* mutant cells deprived of PFR indicate that their flagella have indeed distinct mechanical properties. First, wild-type flagella typically retain complex bends with acute angles when fixed, while flagella of mutant parasites without a PFR were fixed in smooth gentle curves (Santrich et al., 1997).

Second, wild-type flagella could be mechanically sheared off from cell bodies by douncing, but the flagella of PFR null mutants could not (Santrich et al., 1997).

Another hypothesis postulates that the PFR serves as a docking site for enzymes or other proteins involved in control of axoneme beating (Bastin and Gull, 1999; Pullen et al., 2004). The requirements for a PFR in cell motility is intriguing as flagella of similar length in many other species are composed solely of the axoneme wrapped by the membrane and are fully motile. Nevertheless, it is still not clear why do Trypanosomatids need that extra-structure to swim properly.

Different types of extra-axonemal structures have been identified in flagella of spermatozoa (Escalier, 2003). Recent functional investigations demonstrate that the outer dense fibres and the fibrous sheath also bring an important contribution to flagellum movement in mice spermatozoa and possibly in men (Miki et al., 2002; Escalier et al., 2003). Like for the PFR inactivation reported above, the axoneme appeared unmodified. A possible explanation for the presence of these extra-axonemal structures in certain types of flagella could come from the analysis of motility requirements during the natural development of the species that bear them.

During their life cycle, trypanosomes develop in several very different environments such as the bloodstream of a mammal, the midgut or the salivary glands of the tsetse fly. They adapt to these variable conditions by activating specific programmes of differentiation (Matthews et al., 2004). These adaptations are accompanied by morphological changes involving the flagellum and its position relative to the cell body and the nucleus. In *T. brucei*, most stages adopt the trypomastigote form, i.e. the basal body is located posterior to the nucleus and the flagellum extends towards the anterior end of the cell (Fig. 2). It is wrapped around

the cell body with the same helical aspect. This arrangement means that flagellum movement generates a typical « corkscrew » motion, reminiscent of what is observed in bacterial spirochetes (Charon and Goldstein, 2002). Flagellum attachment is crucial for cell motility as demonstrated by observation of *GP72* knock-out in *T. cruzi* or of *FLA1* RNAi-silencing in *T. brucei* (Cooper et al., 1993; Moreira-Leite et al., 2001; LaCount et al., 2002). As described above, the flagellum of these cells is only anchored at the basal body but is not attached any more to the cell body as soon as the flagellum exits the flagellar pocket. Despite the fact that the flagellum is still beating actively (see movie in Moreira-Leite et al., 2001), these cells sink at the bottom of the culture flask and are not capable of significant forward motility.

An interesting parallelism can be drawn with the behaviour of a group of flagellated bacteria, the spirochetes. These prokaryotes exhibit a helical aspect and possess variable number of flagella that are localised within the periplasmic space. As a result, bacteria swim with a typical corkscrew motility, that would be more efficient to swim through viscous environments and permit spirochetes to find their way through tissues during infections (review in Charon and Goldstein, 2002). Absence of flagella leads to loss of the helical aspect and to cell paralysis (Motaleb et al., 2000). Remarkably, *Borrelia* is transmitted by ticks and several stages of their life cycle have intriguing similar aspects with *T. brucei*, such as transition through the midgut and the salivary glands (Schwan and Piesman, 2002).

After blood containing trypanosomes has been ingested by the tsetse fly, the parasites cross the peritrophic membrane, proliferate in the peritrophic space, then need to traverse the peritrophic membrane again to be able to migrate toward the salivary glands *via* a complex path through several organs (Vickerman et al., 1988;

Van Den Abbeele et al., 1999). In the mammalian stages, trypanosomes proliferate in the chancre and then propagate within the bloodstream. All these environments can be regarded as highly viscous and swimming through them certainly demands specific adaptations, as the corkscrew-type motility. It is remarkable to find similar adaptations in organisms as distant as spirochetes and trypanosomes to propagate through insect tissues.

The only developmental stage of *T. brucei* not to adopt the trypomastigote form is encountered when the parasites are found attached to the epithelium of salivary glands (Tetley and Vickerman, 1985). Here, trypanosomes exhibit the epimastigote form, where the basal body of the flagellum is found in a position anterior to the nucleus, with only about the initial third of the flagellum attached to the cell body (Tetley and Vickerman, 1985). Adhesion to host epithelium allows trypanosomes to complete differentiation in the so-called metacyclic stage that is fully infective for mammals. Premature release in the salivary glands would produce parasites unfit to survive in the mammalian bloodstream. The flagellum is used to physically anchor the trypanosome to the brush border of the salivary gland epithelium. The membrane of the flagellum expands considerably and penetrates between the host microvilli. These membrane outgrowths are in close contact with the epithelial cell membranes and attachment plates looking like hemidesmosomes are frequently observed (Tetley and Vickerman, 1985). Such tentacular outgrowths are only visible on the long free distal part of the flagellum, and not seen on the portion that is attached to the cell body. Differentiating to the epimastigote form at this stage could be justified by two reasons. Firstly, increasing the length of the free part of the flagellum optimises anchoring to the host salivary gland epithelium as it

provides a larger surface for adhesion. Secondly, swimming is no longer required now that the parasite needs to remain attached to avoid premature release in the saliva. Nevertheless, attached epimastigote trypanosomes remain motile as the flagellum is still beating actively. Because of flagellum anchoring to the epithelial cells, only the posterior end of the trypanosome is effectively motile. It has been proposed that this form of motility could contribute to circulate the surrounding medium and to allow sufficient nutrient access to the flagellar pocket, the only site of endocytosis (Tetley and Vickerman, 1985). One should remember that in *Tetrahymena*, absence of cilia leads to cell death by starvation, presumably because cilia and cell motility would be required to bring food to the oral apparatus that is also the main area for phagocytosis in ciliates (Brown et al., 1999b).

The analysis of the behaviour of mutant trypanosome strains impaired in flagellum and cell motility either in the bloodstream of a mammalian host or during tsetse fly infection promises to be exciting. The current limitation is due to frequent loss of infectivity and differentiation abilities upon prolonged laboratory cultures.

In contrast to *T. brucei* that spends most of its life cycle under the trypomastigote conformation, *T. cruzi* shows more diversity (de Souza, 1984; Kollien and Schaub, 2000; De souza, 2002). The multiplying epimastigote form is found in the gut of the insect vector where it anchors to the epithelium of the rectum. Like *T. brucei*, this adhesion step is mediated by outgrowths of the free flagellum. *T. cruzi* then transforms to the non-multiplying trypomastigote form. During a blood meal, trypanosomes are excreted from the insect vector and can infect a mammalian host after passage through skin cuts. In contrast to *T. brucei*, the trypomastigote stage of *T. cruzi* is non-dividing. Moreover, during the infection of a mammal, *T. cruzi* does

not proliferate as an extracellular parasite but instead needs to invade host cells. Again, a very efficient motility system is needed to perform these functions and probably justifies the adoption of the trypomastigote form and its associated corkscrew-type motion. Once in the host cell, *T. cruzi* differentiates in the so-called amastigote form, characterised by a much shorter size, an almost spherical shape and a very short flagellum, barely exiting the flagellar pocket. The tip of this flagellum often looks abnormal, with frequent mis-arrangement of axonemal microtubules and without a PFR (de Souza, 1984). Cells proliferate as intracellular parasites and do not swim. Similarly, *Leishmania* adopt the amastigote form once they replicate in macrophages and do not swim. *IFT* gene expression could also be down-regulated upon differentiation of promastigote to amastigote stage, suggesting that the short mis-organised flagellum (Alexander, 1978) is assembled in a manner independent of IFT (Mishra et al., 2003). At the end of intracellular proliferation, *T. cruzi* differentiate to adopt the non-dividing trypomastigote form again that exits the host cell upon lysis and access the bloodstream where they can migrate and infect other host cells. The trypomastigote form itself is not an absolute requirement for penetration of host cells as *GP72* null mutants whose flagellum is detached from the cell body are still able to invade *in vitro* macrophages or L6E9 cells, a non-phagocytotic rat skeletal muscle cell line (de Jesus et al., 1993). One could assume that the corkscrew type motility is mostly needed during transit in the bloodstream, as suggested in *GP72* null mutants that exhibit lower infectivity (Basombrio et al., 2002).

In *T. brucei*, novel functions for cell motility have recently been highlighted: it is critically involved in cell separation to complete cytokinesis and participates to basal body migration (see section III.E3).

2. Sensitivity

Due to their complex life cycle, trypanosomes need to detect in what environment they are and what differentiation programmes they need to active (or repress). Very little is known about factors that trigger differentiation processes. A temperature drop and the addition of cis-aconitate reproduces *in vitro* the transformation from the bloodstream to the procyclic stage (Czichos et al., 1986; Matthews et al., 1995). However, the mechanism of action remains obscure (Saas et al., 2000). Other differentiation steps cannot be reproduced in the laboratory. Cilia and flagella are known to perform sensory functions in different organisms (Pazour and Witman, 2003). Since trypanosomes swim with their flagellum leading, it is reasonable to assume some sensory functions. Prior to attachment to host tissues, the leading flagellum needs to detect an optimal substrate for adhesion. Specific surface proteins might be recruited to the flagellum membrane to facilitate and mediate the adhesion. In rare cases, incubation on certain types of plastic can reproduce *in vitro* parasite adhesion and lead to flagellum differentiation (Brooker, 1970; Beattie and Gull, 1997).

The separation of the flagellum compartment from the rest of the cell body, associated to the dynamic action of intraflagellar transport, allows for concentration of specific proteins, including those involved in signalling (Perkins et al., 1986; Signor et al., 1999; Marszalek et al., 2000) (review in Pazour and Witman, 2003). The trypanosome flagellum contains several calcium-binding proteins (Ruben and Patton, 1987; Bastin et al., 1999b; Godsel and Engman, 1999; Ridgley et al., 2000). Calcium is well-known for its various roles in signalling as well as in the control of motility. An

adenylate cyclase has also been specifically localised to the flagellum membrane in *T. brucei* (Paindavoine et al., 1992). In the amastigote stage of *T. cruzi* or *Leishmania*, the flagellum is very short and its slightly dilated tip narrowly exits from the flagellar pocket (Alexander, 1978; Wiese et al., 2003), perhaps suggesting sensory functions like those observed in the ciliated neurons of *C. elegans* (Perkins et al., 1986). Overall, a lot remains to be done to clarify what sensory functions could be performed by the trypanosome flagellum.

3. Flagellum functions in trypanosome morphogenesis and division

a. Control of cell size

The timing of flagellum replication and elongation is strictly linked to that of the cell cycle (see III.D1). To determine the possible involvement of the flagellum in cell cycle and cell morphogenesis in trypanosomes, its formation was blocked after inducible RNAi targeting separately various components of the IFT machinery known to be essential for flagellum assembly in other species. This methodology permits interfering with new flagellum formation without direct perturbation of the existing flagellum (Bastin et al., 2000a). Modification of flagellum assembly turned out to be dramatic for cell morphogenesis. At early stages of RNAi targeting IFT components, cells produced shorter flagella than normal. In these conditions, cells with shorter flagella possess a shorter FAZ complex (both the FAZ filament and the four associated microtubules were shorter). Strikingly, cells with a shorter flagellum are smaller than cells with a normal length flagellum (Fig. 5). This relationship is virtually linear, the smallest cells being the ones without flagella that exhibit a size of only 10 μm , instead of 20-25 μm for normal trypanosomes. These data reveal that the

flagellum controls cell size in trypanosomes, a central process in cell morphogenesis. How can the flagellum achieve such an amazing function ? A first, simple, hypothesis consist in saying that flagellum elongation controls cell body growth. To evaluate this possibility, the total cell body size was measured in bi-nucleated trypanosomes. These cells are about to divide and to give birth to two daughter cells. Measurements were carried out in normal trypanosomes (with a normal length new flagellum) and in cells growing a flagellum that was obviously too short. No difference in cell body elongation could be detected, demonstrating that flagellum length is not involved in cell body growth. A second hypothesis consist in assuming that the flagellum tip defines the initiation of cytokinesis, as suggested previously from morphological observations (Robinson et al., 1995). Since cell body size is normal, assuming that the flagellum tip defines the initiation of cytokinesis, a too short new flagellum should initiate cleavage from a position that is not anterior enough, hence producing a smaller cell with a short flagellum, and a longer cell with the normal-length, old flagellum. Detailed measurements of mother and daughter cells support this hypothesis (Kohl et al., 2003).

These amazing results demonstrate that flagellum length controls cell body size. We have described above the many adaptations performed by trypanosomes during their life cycle (see III.A). These changes are accompanied by extensive modulation of cell size and shape and interestingly the flagellum follows these changes tightly. The increase in cell size between the procyclic stage and the stage responsible for colonisation of the salivary glands (from 20-25 μm to 40 μm) is correlated with an increase in flagellum length (Van Den Abbeele et al., 1999). When trypanosomes are present in the bloodstream of their mammalian host, two stages

can be discriminated: the long slender, proliferating form, and the short stumpy, non-proliferating but differentiating form (from bloodstream to insect stage). The slender to stumpy differentiation program incorporates a round of cell division. Interestingly, when such dividing cells were examined just prior to cytokinesis, the average length of the new flagellum was shorter than the one measured from replicating slender cells at the same stage (21 μm instead of 25 μm) (Tyler et al., 2001). It is tempting to speculate that regulating the amount of functional IFT particles could be a system to control both flagellum length and cell size.

The amastigote form of *T. cruzi* possesses a very short flagellum barely extending beyond the flagellar pocket and its cell body size and shape resembles that of non-flagellated *T. brucei* mutants. In *Leishmania*, despite the fact that the flagellum is only attached through a tiny portion of its length, remarkable relationships between cell size and flagellum length have been noticed. First, differentiation of *L. braziliensis* from the promastigote stage to the short flagellated amastigote stage is concomitant with drastic reduction of cell body length and alteration in cell shape (Stinson et al., 1989). Second, in several *L. mexicana* mutants that assemble a very short flagellum, cell size appeared reduced (Cuvillier et al., 2000; Wiese et al., 2003). Third, “metacyclic” stages possess a longer flagellum and, although their cell body length is barely modified, they display a more elongated shape (Zakai et al., 1998).

b. Cytoskeletal control of cell cycle and morphogenesis

How can the tip of the flagellum initiate cell division? It sounds unlikely that the flagellum itself would be the active part of this process. We rather propose that the flagellum acts as a marker for the action of structures present in the cell body. In

many animal cells, an actino-myosin ring is deposited at the site of cell cleavage. The constriction of this structure is responsible for cell division (Scholey et al., 2003). No such structure can be recognised at the site of cell division in trypanosomes. In addition, no clear homologues of myosins can be found in the trypanosome genome and although actin is present, it does not appear essential for cell division at the procyclic stage (Shi et al., 2000; Garcia-Salcedo et al., 2004). A more likely structure to be involved in cell division is the FAZ complex. Indeed, this one is present within the cell body and terminates at the anterior end of the cell, the exact site of initiation of cell division. Moreover, cell division is helical and follows the path of the flagellum and therefore that of the FAZ complex that had been proposed as a candidate to guide cell cleavage (Robinson et al., 1995). This model receives strong support from the observation of cells that do not grow a new flagellum at all due to IFT inhibition (Kohl et al., 2003). These cells still possess the old flagellum, replicate their basal bodies but fail to produce a flagellum. In this situation, a new FAZ complex is still produced from the new basal body area and elongates towards the anterior end of the cell. However, it is much too short, reaching barely half of its normal mature length. Nevertheless, its extremity appears in close contact with the old FAZ. Immuno-staining with antibodies recognising the FAZ filament or the set of 4 microtubules revealed that both structures were present. Such cells divide from the anterior end of the FAZ despite the absence of the new flagellum, showing that this one is not directly responsible for cleavage.

Non-flagellated cells exhibit an aberrant spherical or nut-like shape (Kohl et al., 2003). They progressively lose their polarity as revealed by analysis of their endocytic network and by new microtubule behaviour. Endocytosis takes place in the

flagellar pocket and vesicles are normally trafficking intensely at the posterior area of the cell (Overath and Engstler, 2004). Immunofluorescence staining of non-flagellated cells with various markers of the endocytic compartments reveals their progressive dispersion throughout the cytoplasm (Kohl et al., 2003)(Kohl, Field & Bastin, unpublished observations). Incorporation of new tubulin subunits takes place primarily at the posterior end of normal flagellated trypanosomes. In non-flagellated cells, new tubulin subunits are either poorly incorporated without a defined polarity or added at two opposite poles (Kohl et al., 2003).

Non-flagellated cells are able to duplicate their basal bodies, however these do not migrate apart. Kinetoplast DNA cannot be separated efficiently and cells with a large DNA network sandwiched between two apposed basal bodies are frequently seen (Kohl et al., 2003). Nuclear mitosis takes place normally but nuclei remain in close proximity. Strikingly, the FAZ filament and 4 microtubules are not assembled any more in a recognisable structure and cytokinesis does not occur. Nevertheless, nuclear mitosis can re-iterate, producing multinucleated cells (Benaghanem, Kohl & Bastin, unpublished data).

c. The role of flagellum force in cell separation and basal body migration

The new flagellum is motile as soon as it emerges from the flagellar pocket, raising the question of a possible participation of motility in the morphogenesis processes. Our recent data reveal two novel essential functions for flagellum motility in the trypanosome cell cycle. First, we noticed that inhibition of new flagellum assembly in the various IFT RNAi mutants is accompanied by accumulation of large cellular aggregates in the culture flasks. Closer examination showed that cell

separation is slowed down or inhibited. At the end of the cytokinesis process in normal, wild-type, trypanosomes, future daughter cells are only attached by their posterior ends. Flagella are pointing towards opposite anterior ends and are actively beating. We propose that these opposite forces, added to the opposite helical movement, exert tensions on the midbody that contribute to tear daughter cells apart and permit final separation. This hypothesis is clearly demonstrated in bloodstream form trypanosomes upon introduction of *PFRA* dsRNA. Absence of PFRA protein in the new flagellum inhibits its motility, but does not interfere with flagellum formation, nuclear mitosis and initiation of cytokinesis. However, final cell cleavage was totally inhibited. Such duets of cells re-enter the cell cycle and produce two new flagella (still PFRA negative) and undergo nuclear mitosis (Buisson and Bastin, unpublished data). Trypanosomes at the procyclic stage seem to have slightly different requirements for motility in cell septation, as PFRA does not seem to be required (Bastin et al., 1998). However, silencing of axoneme central pair components PF16 or PF20 also lead to formation of multi-cellular aggregates. Shaking the cell culture significantly reduces the frequency of aggregates, confirming that physical forces are indeed involved. This could be due to the fact that the PF16 and PF20 mutants exhibit the strongest paralysis phenotype observed so far in trypanosomes. PFRA RNAi mutant cells are still able to twitch and to produce slow helical motility that could be sufficient for cell separation in the procyclic stage. Forces generated by motility is required for final cell septation in *Tetrahymena* and in *Dictyostelium* (Tuxworth et al., 1997; Brown et al., 1999a).

Examination of the *PF16* or *PF20* RNAi mutant at the procyclic stage reveal two other striking consequences: (1) basal bodies fail to migrate apart after

duplication and (2) the new flagellum detaches from the cell body, with the exception of its distal tip that remains anchored to the old flagellum at the FC. These cells assemble a FAZ filament that initiates from the basal body/flagellar pocket area but, like in IFT mutants, it appears too short and looks like a straight line terminating in proximity of the distal tip of the new flagellum at the FC position. This result further strengthens the role of the FC in tethering the tip of the new flagellum to the old one. This also confirms that the tip of the flagellum controls FAZ elongation (Kohl et al., 2003). As for non-flagellated cells, mitosis and cell body growth go on normally. When elongation of the new, paralysed, flagellum is complete, it is separated from the old, presumably by disassembly of the FC, and is now only anchored *via* the basal body. Cell cleavage takes place at the tip of the short new FAZ, producing a cell with a normally attached flagellum rooted at the posterior end of the nucleus, and a cell with a partially detached flagellum, but with its basal body *anterior* to the nucleus, actually resembling to the epimastigote stage. At this stage, it is difficult to predict whether this phenotype is a consequence of a severe reduction in flagellum motility or of specific modifications of the axoneme linked to alterations of the central pair.

An exciting explanation for these results is provided by the hypothesis that flagellum force acts on basal body segregation in the procyclic stage of *T. brucei*. The new flagellum is active as soon as it exits from the flagellar pocket and the FC is present even before that (Briggs et al., 2004). We propose that combined flagellum movement and elongation exerts a pressure on the FC that is pushed distally on the side of the old flagellum. However, due to the presence of the connector, a resistance is encountered and this leads to a reverse, posterior-orientated, force that

is transmitted along the new flagellum, leading to displacement of the basal body subtending the new flagellum towards the posterior end of the cell. In cells with a severely paralysed flagellum, efficient force cannot be generated and the new flagellum cannot push on the FC. Nevertheless, flagellum elongation still occurs and since it is anchored to the basal body at one end and to the FC on the other, the new flagellum gets detached from the cell body and basal bodies fail to migrate apart. This model is supported by observations of IFT mutants. Firstly, when cells with an old flagellum fail to assemble a new one, the extensive migration step of duplicated basal bodies is severely reduced (Kohl et al., 2003), as would be expected from the model. Since mitosis takes place normally, bi-nucleated cells are frequently observed with the poorly segregated basal bodies in between the nuclei, a DNA staining pattern very similar to that of *PF16* RNAi mutant cells. Secondly, in non-flagellated cells (without new and old flagella), basal body migration frequently does not take place at all (Kohl et al., 2003).

If this model is true, inhibition of flagellum attachment to the cell body (but not to the basal body or the FC) observed in *FLA1*-RNAi cells should have severe consequences on trypanosome morphogenesis and cell cycle. This was indeed found to be the case in three different studies: (1) early stages of *FLA1* silencing in procyclic trypanosomes prevents flagellum attachment only in the new flagellum (but not anchoring to the basal body nor to the FC) and is associated to poor segregation of basal bodies (see Fig. 3 in (Moreira-Leite et al., 2001)). (2) Silencing *FLA1* for longer periods was associated to defects in cytokinesis (LaCount et al., 2002), presumably due to anchoring of the FC along a detached old flagellum, therefore preventing normal FAZ formation. (3) *GP72/FLA1* knock-out in the dividing

epimastigote stage of *T. cruzi* leads to flagellum detachment but does not interfere with cell proliferation at that particular stage. However, these cells fail to differentiate in trypomastigote, a process that requires extensive migration of the basal body/kinetoplast complex on the other side of the nucleus to the posterior end of the cell.

Conclusions

The trypanosome flagellum is clearly a multi-functional organelle, being involved in motility, morphogenesis and parasite attachment to host tissues. The amenability of trypanosomes to genetic manipulation, combined to the multiple tools for cell biology examination, offers the opportunity to further dissect the sophisticated usage of the flagellum. In addition, trypanosomes provide an ideal model to study flagella functions and assembly in general.

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Figure legends

Fig. 1. Ultrastructure of human (A-B) and trypanosome (D) flagellum. Cross-section through human cilia from a healthy individual (A) or from a person suffering from primary cilia dyskinesia (B). Outer dynein arms are clearly visible in (A) but are completely missing in (B) due to mutations in the dynein intermediate chain DNAI1. (one of the 9 outer dynein arms is indicated by a red star in (A)). (C). Schematic representation of the axoneme: the 9 peripheral doublets of microtubules surround the central pair. Dark blue, outer dynein arms; light blue, inner dynein arms; magenta nexin links; green, radial spokes; yellow, central pair projections. (D) Cross section through the trypanosome flagellum. An extra-axonemal structure, the paraflagellar rod (PFR) is also visible, as well as intraflagellar transport (IFT) particles, indicated by arrowheads. (A,B), courtesy of Dr. Estelle Escudier, Paris and (D) reproduced from Bastin et al., 2000, with permission.

Fig. 2: Cell cycle of the culture-adapted procyclic stage of *T. brucei* that exhibits the trypomastigote form: wild-type (A-C) and of 48 hours-induced (TbDHC1b)RNAi, where IFT was inhibited upon silencing of the retrograde transport motor (D-F). Flagellum was labelled with an anti-PFRA antibody (green) whereas FAZ filament is shown as red lines, basal bodies as red spots, and DNA stained with DAPI (blue). Top panels, DIC merged with flagellum staining (green), bottom image, merged fluorescence. Yellow arrows indicate kinetoplast DNA position. After 48 hours of induction, trypanosomes cannot assemble a new flagellum but nevertheless

formation of a short new FAZ (tip indicated by the white arrowhead) is observed.

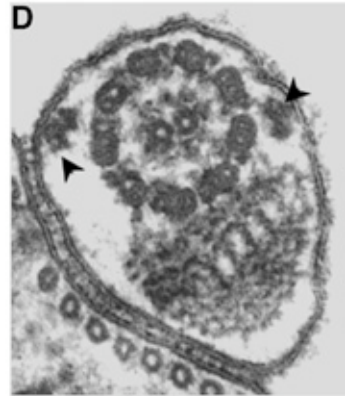
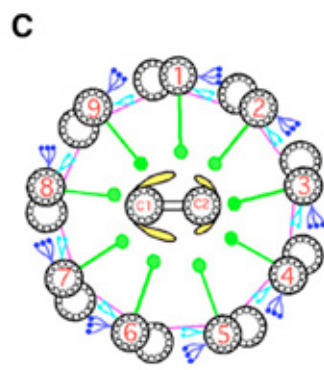
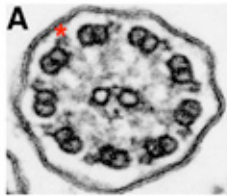
Reproduced from Kohl et al. (2003).

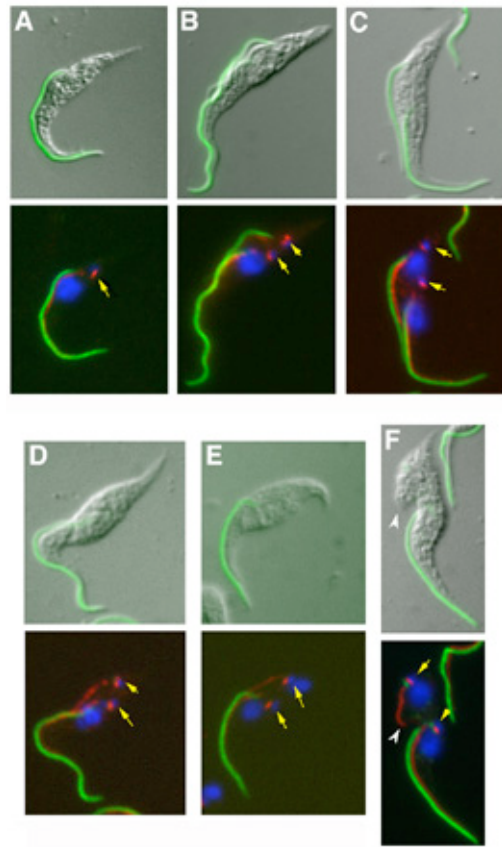
Fig. 3. Cell cycle of *T. cruzi* (epimastigote stage) and of *L. major* (promastigote stage). Cells were stained with DAPI (blue). Left panels, uni-flagellated cells, centre panels, bi-flagellated cell prior to nucleus mitosis, right panels, bi-flagellated cell after mitosis. Kinetoplast (K) and nucleus (N) positions are indicated. The white arrow points at the new flagellum.

Fig. 4. The flagella connector (FC) in wild-type trypanosomes. Cells were stained with an anti-FC antiserum. Left panels, phase contrast image merged with DAPI staining (blue) and anti-FC immuno-staining (green). Central panels, FC staining only. Right panels: magnification of the area surrounding the FC. Top, phase contrast image. Bottom, phase contrast image merged with FC staining (green). Stars, tip of the old flagellum, arrows, tip of the new flagellum. (A) The left cell has got only one flagellum, its tip is not stained. The right cell has an elongating new flagellum and its distal tip shows defined staining. In contrast, the distal tip of the old flagellum is not stained. Similar observations were made as the new flagellum elongated (B-D). As the new flagellum elongates, the FC migrates on the old flagellum.

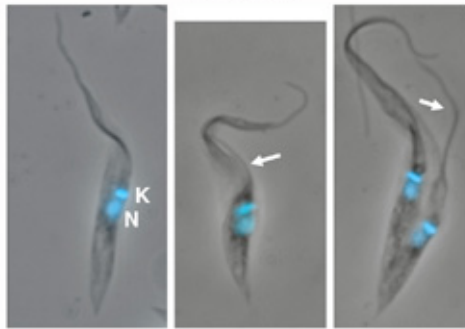
Fig. 5. Flagellum length governs cell body size in trypanosomes. Flagellum assembly has been perturbed by inducible RNAi silencing of the intraflagellar transport machinery. The image shows a mixture of trypanosome cells with normal flagella, or with flagella that are too short, or without flagellum. The flagellum is stained in green, the FAZ is shown as red lines, the basal bodies as red spots, and DNA is stained in

blue. The length of the flagellum governs the cell body size: compare the cell body size of trypanosomes with normal flagellum (arrowheads), with a short flagellum (arrows) or without flagellum (stars). Reproduced from Kohl et al. (2003).





T. cruzi



L. major

