

Protein transport and flagellum assembly dynamics revealed by analysis of the paralysed trypanosome mutant *snl-1*

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SUMMARY

The paraflagellar rod (PFR) of *Trypanosoma brucei* is a large, complex, intraflagellar structure that represents an excellent system in which to study flagellum assembly. Molecular ablation of one of its major constituents, the PFRA protein, in the *snl-1* mutant causes considerable alteration of the PFR structure, leading to cell paralysis. Mutant trypanosomes sedimented to the bottom of the flask rather than staying in suspension but divided at a rate close to that of wild-type cells. This phenotype was complemented by transformation of *snl-1* with a plasmid overexpressing an epitope-tagged copy of the PFRA gene. In the *snl-1* mutant, other PFR proteins such as the second major constituent, PFRC, accumulated at the distal tip of the growing flagellum, forming a large dilation or 'blob'. This was not assembled as filaments and was removed by detergent-extraction. Axonemal growth and structure was unmodified in the *snl-1* mutant and the blob was present

only at the tip of the new flagellum. Strikingly, the blob of unassembled material was shifted towards the base of the flagellum after cell division and was not detectable when the daughter cell started to produce a new flagellum in the next cell cycle. The dynamics of blob formation and regression are likely indicators of anterograde and retrograde transport systems operating in the flagellum. In this respect, the accumulation of unassembled PFR precursors in the flagellum shows interesting similarities with axonemal mutants in other systems, illustrating transport of components of a flagellar structure during both flagellum assembly and maintenance. Observation of PFR components indicate that these are likely to be regulated and modulated throughout the cell cycle.

Key words: Cytoskeleton, Flagellum, Intraflagellar transport, Paraflagellar rod, Trypanosome

INTRODUCTION

The assembly of cilia and flagella is a complex process that has intrigued cell biologists for several decades. These large structures, usually but not always motile, are composed of several hundred proteins (at least 250 in *Chlamydomonas*; Piperno et al., 1977). Synthesis of these proteins must take place in the cytoplasm as there are no ribosomes in the flagellum matrix. However, the assembly site of most precursors is localised at the distal tip of the elongating flagellum (Rosenbaum et al., 1969; Witman, 1975; Sherwin and Gull, 1989a; Johnson and Rosenbaum, 1992; Piperno et al., 1996), implying the existence of a transport mechanism (Rosenbaum et al., 1999). This anterograde intraflagellar transport has been observed in *Chlamydomonas* where particles or 'rafts' are moved underneath the flagellar membrane (Kozminski et al., 1993). These rafts presumably contain motor proteins and flagellar precursors (Cole et al., 1998). This anterograde transport is powered by at least one motor protein, the kinesin-like molecule FLA10. The activity of this kinesin is essential for flagella or cilia assembly in several organisms (Shakir et al., 1993; Kozminski et al., 1995; Tabish et al., 1995; Piperno et al., 1996; Morris and Scholey,

1997; Nonaka et al., 1998; Marszaleck et al., 1999). However, retrograde transport of particles has also been seen (Kozminski et al., 1993). The dynein light chain 8 (LC8) is essential for this process. Loss of its activity leads to production of short, abnormal and immotile flagella, characterised by the dramatic accumulation of rafts between the outer doublets of the axoneme and the flagellar membrane (Pazour et al., 1998). Mutations in the dynein heavy chain 1b produced similar although more pronounced alterations of flagellum assembly (Pazour et al., 1999; Porter et al., 1999). Therefore, both anterograde and retrograde intraflagellar transport are required for flagellum assembly.

Flagella and cilia are found throughout the eukaryotic world, from protists to mammals, where they are present in sperm, in cells from the epithelium of the respiratory tract or of the oviducts, and in sensory cilia such as in the rod and cone cells of the eye or the olfactory receptor neurons. In such metazoan systems, the application of modern molecular genetic approaches is hindered by the fact that all such cells are differentiated and do not replicate in culture. The flagellum of *Trypanosoma brucei* represents an interesting system to study the assembly of this type of organelle. Trypanosomes are kinetoplastid parasites that possess a

single flagellum, emerging from the flagellar pocket at the posterior end of the cell. The flagellum is attached throughout most of its length to a region of the cell body termed the flagellum attachment zone (Kohl and Gull, 1998). The basal body of the flagellum is tightly connected to the kinetoplast, the condensed DNA of the single mitochondrion (Robinson and Gull, 1991). During progression through its cell cycle, the trypanosome duplicates its basal body complex and produces a new flagellum from the one segregated to the posterior end of the cell (Sherwin and Gull, 1989b). The mature or old flagellum remains present, but in a more anterior position. This allows direct comparison of growing and non-growing flagella in the same cell.

The trypanosome flagellum contains an extra-axonemal structure, the paraflagellar rod or PFR, which is present inside the flagellum (Vickerman, 1962). This lattice-like structure of about the same diameter as the axoneme is composed of filaments elegantly arranged at defined angles (Vickerman and Preston, 1976; Farina et al., 1986; Sherwin and Gull, 1989b). The PFR is composed of two major proteins of M_r 69,000 (PFRA) and M_r 73,000 (PFRC) in *T. brucei* (Russell et al., 1983) which show immunological cross-reactivity (Gallo and Schrevel, 1985; Schlaeppli et al., 1989; Birkett et al., 1992; Kohl et al., 1999). Each are encoded by tandem arrays of identical genes (Schlaeppli et al., 1989; Defflorin et al., 1994) and homologues have been found in *Trypanosoma cruzi* (Beard et al., 1992; Fouts et al., 1998), in *Leishmania mexicana* (Moore et al., 1996) and in *Euglena gracilis* (Ngo and Bouck, 1998). A few low-abundance PFR proteins have also been identified (reviewed by Bastin et al., 1996a) including the antigen recognised by the monoclonal antibody ROD-1 (Woods et al., 1989; Woodward et al., 1994) and possibly some calcium-binding proteins called calflagins (Wu et al., 1992, 1994).

We now report a detailed characterisation of flagellum assembly dynamics in the *snl-1* mutant of *T. brucei*. PFRA protein expression is ablated in this mutant which cannot assemble a normal PFR and shows a paralysed phenotype (Bastin et al., 1998). We show here that the defect can be complemented by expression of an epitope tagged version of PFRA. Careful examination shows that other PFR proteins such as the second major constituent, PFRC, accumulate at the distal tip of the growing flagellum, forming a large dilation or 'blob'. The old flagellum does not possess a blob. Strikingly, the blob of unassembled material is shifted towards the base of the flagellum after cell division and is not detectable when the daughter cell produces a new flagellum in the next generation. The dynamics of blob formation and regression are likely indicators of anterograde and retrograde transport systems operating in the eukaryotic flagellum.

MATERIALS AND METHODS

Trypanosomes and transfection

Strain 427 of procyclic *T. brucei brucei* was used throughout this study. Trypanosomes were grown at 27°C in semi-defined medium 79 containing 10% foetal calf serum. The *snl-1* mutant has been described (Bastin et al., 1998). For transfection (ten Asbroek et al., 1990; Lee and Van der Ploeg, 1990; Eid and Sollner-Webb, 1991), *snl-1* cells were electroporated in the presence of 20 µg of linearised DNA (Beverley and Clayton, 1993), either pPFRATAG430 or

pyTUBTAG430, and transformants were selected, screened and subcloned as described (P. Bastin et al., unpublished).

DNA constructs

Plasmid p α T6451 (Bastin et al., 1998) has been described. The Ty epitope tag was inserted at the 3' end of the trypanosome γ -tubulin gene by PCR amplification using the γ -tubulin gene (Scott et al., 1997) as template, with 5'GCAAGCTTATGCCACGGGAAATC-3' as forward primer (*Hind*III site underlined, start codon in bold) and 5'-GCAGATCTATGCATTTCAGTCAAGTGGATCCTGGTTAGTATGGA CCTCAAAGTTTCGTATATAATCGG-3' as reverse primer (*Bgl*III site underlined, in-frame stop codon in bold, epitope tag sequence in italics). The PCR product was digested with *Hind*III and *Bgl*III and ligated in matching sites of pHD430 (A. Ploubidou, P. Bastin and K. Gull, unpublished). DNA was purified from cesium chloride gradients or Qiagen resin.

Antibodies, immunofluorescence and immunoblotting

Monoclonal antibodies L8C4 (recognising exclusively PFRA) and L13D6 (recognising PFRA and PFRC; Kohl et al., 1999), ROD-1 (recognising a doublet of minor PFR proteins; Woods et al., 1989), BS7 (recognising proteins present at the distal end of the flagellum; Woodward et al., 1995), and BB2 (recognising the Ty epitope-tag; Bastin et al., 1996b) were used as hybridoma supernatants. The antiserum recognising the calflagins (Wu et al., 1992, 1994) was a kind gift from Dr Larry Ruben (Methodist University, Dallas, USA). For immunofluorescence, trypanosomes were spread on poly-L-lysine coated slides, fixed in methanol and processed as described (Sherwin et al., 1987). For cytoskeleton extraction, trypanosomes were harvested by centrifugation and washed once in PEM (100 mM Pipes, pH 6.9, 2 mM EGTA and 1 mM MgSO₄). Cells were resuspended in PEM containing 1% Nonidet P-40. After 2 minutes, the mixture was spun down and the pellet containing the cytoskeleton was resuspended, spread on poly-L-lysine coated slides and settled for 15-30 minutes in a humid atmosphere. Samples were fixed in methanol and processed for immunofluorescence. Slides were examined with a Zeiss Axioskop or a Leica DMRXA microscope. Images were captured using a cooled slow scan CCD camera and processed in Adobe Photoshop. For immunoblotting, 1×10⁷ trypanosomes were washed twice in PBS, resuspended in Laemmli buffer in the presence of proteases inhibitors and boiled before loading. Proteins were transferred to nitrocellulose membranes and stained with India ink before processing. The following dilutions of primary antibodies were used: L13D6, 1:20; L8C4, 1:20; BB2, 1:50; anti-calflagins, 1:20 and ROD-1 undiluted. Final detection was carried out using an ECL kit according to the manufacturer's instructions (Amersham).

Motility analysis

Trypanosomes were grown at ~5×10⁶ cells/ml in normal culture medium and visualised at 20 times magnification. Images were captured every second for a total of 25 seconds using a CCD camera and a trace of individual cells was monitored. For sedimentation assays, 5×10⁶ trypanosomes were resuspended in 1 ml of medium and incubated at 27°C. Optical density (600 nm) was measured every 2 hours and compared to controls in which cells were resuspended.

RESULTS

snl-1 cells are paralysed

Wild-type trypanosomes swim actively in any direction in the culture medium, with their anterior, thin end leading. We monitored the comparison of wild-type and mutant motility by video microscopy and traces of typical cell movements are illustrated in Fig. 1. Wild-type trypanosomes were seen to swim at speeds up to 20 µm per second (Fig. 1A) whilst *snl-1*

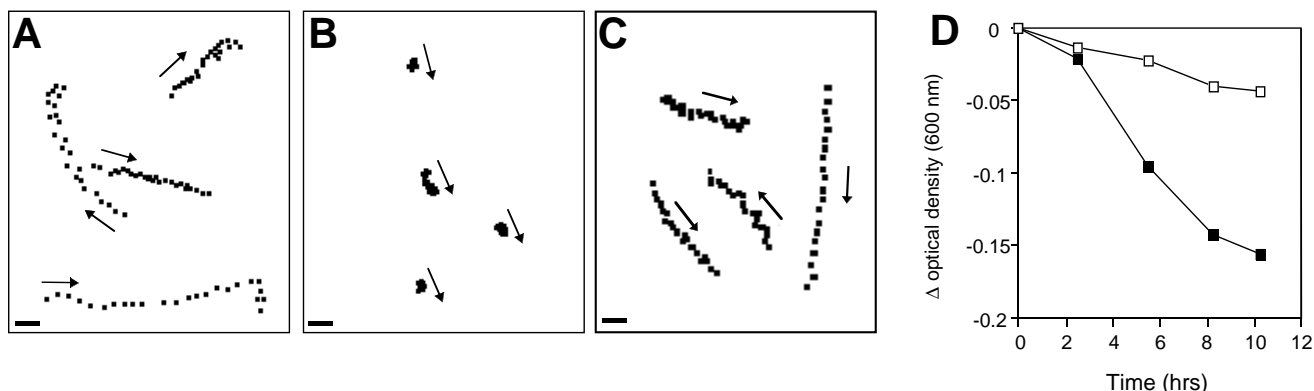


Fig. 1. Mutant *snl-1* trypanosomes are paralysed. Images of live wild-type trypanosomes (A), *snl-1* mutant (B) cells and *snl-1* transformed with pPFRATAG430 (C) were captured every second and the anterior end of four representative cells was labelled with a black dot. The arrows indicate the direction of movement. Wild-type cells were very motile and quickly crossed the field in any direction whereas mutants moved hardly at all and merely drifted with the flow of the medium. Bar, 20 μm . (D) For sedimentation assay, cells were grown in cuvettes and the optical density was measured every 2 hours. After 10 hours, wild-type trypanosomes were still swimming in the medium and only a small proportion of cells was found at the bottom (open squares). In contrast, *snl-1* mutants sedimented to the bottom of the cuvette (closed squares).

cells did not swim at all and simply drifted with the slow flow of the medium (Fig. 1B). Video analysis showed that their flagellum was still moving a little but at a much reduced frequency. The paralysis was so extreme that trypanosomes sedimented at the bottom of the flask rather than staying in suspension. This was illustrated in a sedimentation assay that showed the obvious difference between wild-type and *snl-1* trypanosomes (Fig. 1D). With the exception of these motility defects, *snl-1* cells appeared healthy and their DNA content showed the classic G₁-S-G₂ profile by FACS analysis (data not shown). However, their growth rate was consistently slightly slower than wild-type populations.

***snl-1* cells are PFRA depleted and can be complemented by overexpression of epitope-tagged PFRA**

The *snl-1* mutant was obtained in an experiment aimed at expressing PFRA antisense RNA and molecular analysis revealed that the phenotype was produced by insertion of an antisense version of the PFRA within one allelic PFRA multigene complex (Bastin et al., 1998). The mechanism of ablation of gene expression appears likely to be related to the RNA interference phenomenon described in *C. elegans* (Fire et al., 1998; Ngo et al., 1998; P. Bastin and K. Gull, unpublished). PFRA expression was evaluated by immunoblotting analysis using the monoclonal antibody L8C4, that recognises PFRA without cross-reactivity with PFRC (Kohl et al., 1999). The M_r 69,000 band was barely detectable in the *snl-1* sample, whereas a strong signal was present in wild-type (Fig. 2A). The amount of PFRA left in the *snl-1* mutant is about 1% of that of wild-type trypanosomes. This was estimated by immunoblot comparison of *snl-1* samples with successive dilutions of wild-type samples (data not shown). Immunofluorescence confirmed the almost complete disappearance of PFRA (data not shown). To confirm that these modifications were due to PFRA depletion, the *snl-1* mutant was transfected with the plasmid pPFRATAG430 (P. Bastin et al., unpublished) containing the full length PFRA gene under the control of the trypanosome

procyclin promoter (Sherman et al., 1991). This gene was epitope-tagged (Bastin et al., 1996b) to discriminate its products from those of the endogenous PFRA genes. As control, the same vector was used but with an epitope-tagged γ -tubulin gene instead of the PFRA gene. When the pPFRATAG430 plasmid was transfected in *snl-1*, the transformants expressed the expected epitope tagged PFRA protein identified by western blotting with the anti-tag antibody BB2 (Fig. 2B). This was confirmed by using the PFRA specific L8C4 monoclonal antibody, showing that the presence of the epitope-tagged PFRA (Fig. 2A, top band, larger because of the epitope tag) was accompanied by a small increase in the amount of the endogenous PFRA. These new transformants showed a total complementation of the paralysed phenotype with a return to a wild-type swimming pattern (Fig. 1C), also demonstrating that the epitope-tagged PFRA protein was functional. In *snl-1* transformed with the p γ TUBTAG430 plasmid, the epitope-tagged γ -tubulin (M_r ~50,000) was detected with the anti-tag antibody. PFRA ablation was not altered and the cells still exhibited the paralysed phenotype (Fig. 2). All the transformants were then analysed by double immunofluorescence with the anti-tag antibody BB2 and with ROD-1, a monoclonal antibody recognising a minor PFR protein, localised to the outer distal zone of the PFR and hence a good marker of a fully assembled PFR (Woods et al., 1989; Woodward et al., 1994). ROD-1 stained the flagellum brightly in wild-type trypanosomes but not in the *snl-1* mutant which of course lacks the full PFR structure (Fig. 2C). A strong immunofluorescence signal was detected in the flagellum of most complemented trypanosomes using the BB2 anti-tag antibody (Fig. 2C). Such trypanosomes also exhibited a bright signal with ROD-1, that colocalised with that obtained for BB2. Finally, in the γ -tubulin controls, the signal obtained with the BB2 anti-tag antibody was located mostly over the cell body (Scott et al., 1997) and the ROD-1 signal remained as weak as in the original *snl-1* and did not colocalise with that obtained with the anti-tag antibody. These data confirm that the paralysis phenotype was due to the ablation of PFRA protein expression.

Other flagellar proteins in *snl-1* mutants

Given the absence of a fully formed PFR, we examined what happened to other flagellar proteins in the *snl-1* mutant. First, we used the monoclonal antibody L13D6 to look for the other major PFR protein, PFRC. This antibody recognises both PFRA and PFRC but given the absence of PFRA in *snl-1*, here it can be used as a marker for PFRC. An immunoblot of total proteins showed a slight reduction in the amount of PFRC in *snl-1* (Fig. 3A). Much more dramatic was the change of its localisation: only a weak signal was detected along the length of the flagellum, with in some cells, a bright signal at the distal

tip. The amount of the antigen recognised by ROD-1, a doublet of M_r 180-200,000, was considerably reduced in *snl-1* mutants (Fig. 3B). Moreover, its localisation was severely affected: it was mostly found in the cytosol and, in only some cells, in the flagellum where it produced a punctuate staining with also some labelling at the distal tip. The calflagins are calcium binding proteins localised in the flagellum (Wu et al., 1992, 1994). The signal produced by an antiserum recognising three of these proteins on immunoblots was identical in wild-type and in *snl-1* mutant (Fig. 3C). However, immunofluorescence showed the presence of an extra signal at the distal tip of the

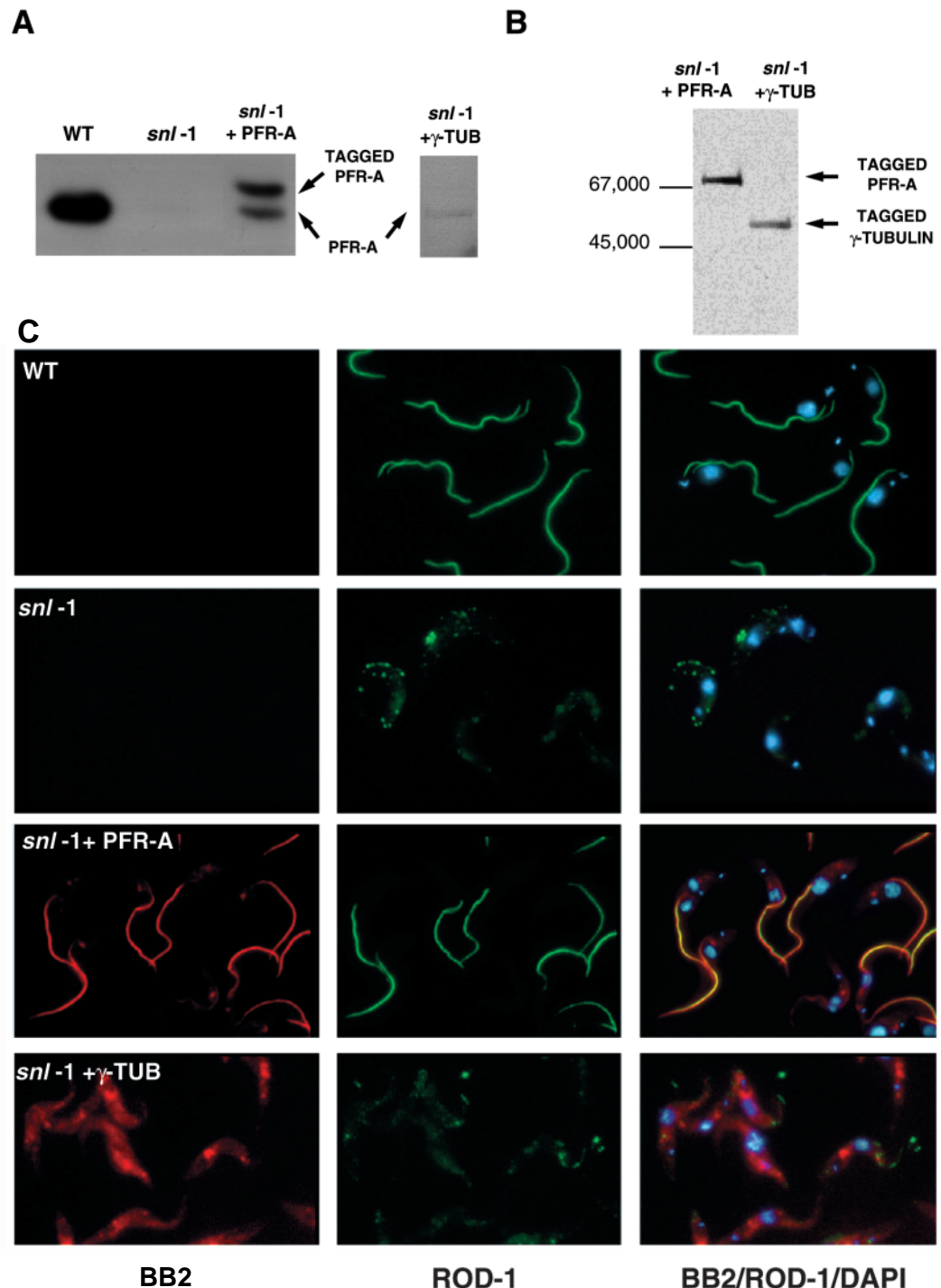


Fig. 2. *snl-1* trypanosomes are missing the PFRA protein but can be complemented.

(A) Immunoblots of total protein samples (10 μ g per lane) of wild-type trypanosomes (WT), of *snl-1* mutant or of *snl-1* transformed with pPFRATAG430 or with p γ TUBTAG430 probed with the antibody L8C4, specific for PFRA. A single band of the expected M_r 69,000 is present in wild-type cells that is barely detectable in *snl-1* mutant or in *snl-1* transformed with p γ TUBTAG430. Both tagged and endogenous PFRA can be detected in the complemented cell line (*snl-1* transformed with pPFRATAG430). (B) Immunoblot of total protein samples (10 μ g per lane) of *snl-1* trypanosomes transformed with pPFRATAG430 or with p γ TUBTAG430 probed with the anti-tag antibody BB2. Positions of molecular weight markers are indicated on the left. (C) Immunofluorescence of wild-type trypanosomes, of *snl-1* mutant, of *snl-1* transformed with pPFRATAG430 or with p γ TUBTAG430 double-labelled with the anti-tag antibody BB2 (red, left panel) and with the anti-PFR antibody ROD-1 (green, central panel). These last images were merged to each other and to the 4,6-diamidino-2-phenylindole (DAPI) staining (blue, right panel), showing colocalisation, when present, in yellow.

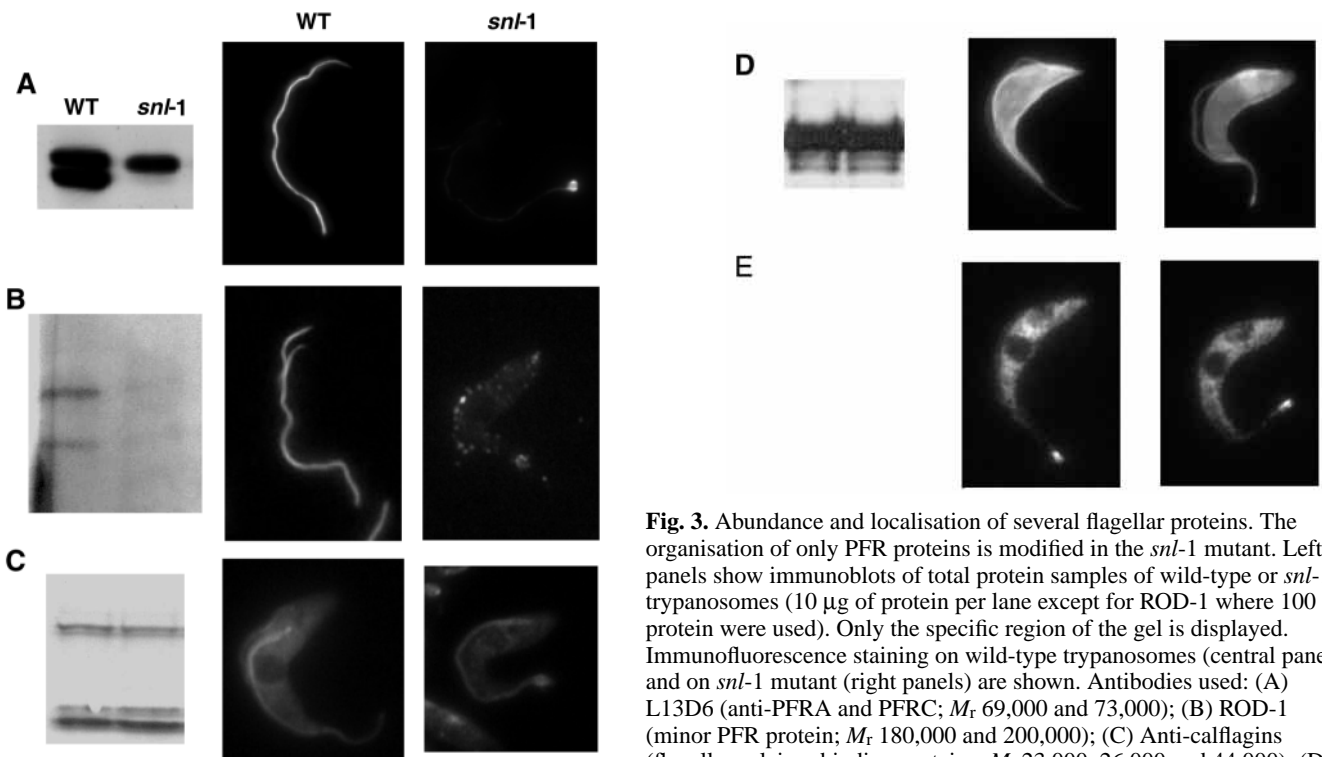


Fig. 3. Abundance and localisation of several flagellar proteins. The organisation of only PFR proteins is modified in the *snl-1* mutant. Left panels show immunoblots of total protein samples of wild-type or *snl-1* trypanosomes (10 μ g of protein per lane except for ROD-1 where 100 μ g protein were used). Only the specific region of the gel is displayed. Immunofluorescence staining on wild-type trypanosomes (central panels) and on *snl-1* mutant (right panels) are shown. Antibodies used: (A) L13D6 (anti-PFRA and PFRC; M_r 69,000 and 73,000); (B) ROD-1 (minor PFR protein; M_r 180,000 and 200,000); (C) Anti-calflagins (flagellar calcium binding proteins; M_r 23,000, 26,000 and 44,000); (D) TAT-1 (anti-tubulin); (E) BS7 (anti-flagellum tip; this does not blot).

snl-1 flagellum, absent from wild-type cells. The amount and localisation of non-PFR proteins such as tubulin (Fig. 3D), procyclin and flagellum attachment zone antigens (not shown) was not modified at all. Finally, given the obvious modification of the distal tip of the *snl-1* flagellum, we also checked the signal produced by BS7, a monoclonal antibody recognising a

structure normally present at this site (Woodward et al., 1995). This antibody does not blot but the immunofluorescence looked absolutely identical in both *snl-1* and wild-type trypanosomes (Fig. 3E). Therefore, only proteins normally localised to the PFR showed modification in localisation/abundance in *snl-1* mutants.

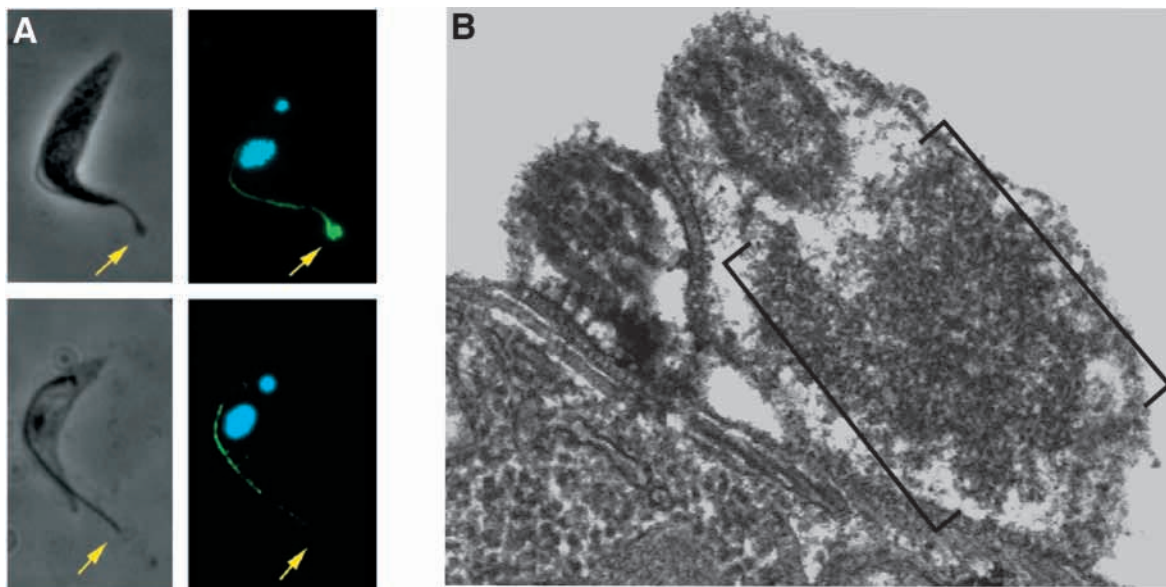


Fig. 4. The dilatation of the distal tip of the flagellum of *snl-1* corresponds to non-assembled material. (A) Immunofluorescence with the monoclonal antibody L13D6 (anti-PFRA and PFRC, shown in green) on *snl-1* whole cells (top) or cytoskeletons (bottom) stained with DAPI (blue). In the *snl-1* mutant, in the absence of PFRA, this antibody indicates the localisation of PFRC. The yellow arrow indicates the distal tip of the flagellum. (B) Section through the distal tip of a *snl-1* flagellum. The membrane and the axoneme are visible, and the blob, a mass of unassembled material is comprised between the two brackets.

Unassembled PFR precursors accumulate in a blob at the distal tip of the flagellum

The localisation of PFRC in the *snl-1* mutant was investigated in more detail. When a strong immunofluorescence signal with the L13D6 antibody (detecting mainly the PFRC protein in the *snl-1* mutant) was observed at the distal end of the flagellum, it always colocalised with a blob at the distal tip of the flagellum seen by phase contrast microscopy (Fig. 4A, top panel). Phase-contrast examination of detergent extracted cytoskeletons from *snl-1* showed that they never possessed the blob, and immunofluorescence with L13D6 never produced a bright signal at the distal end of the flagellum in such preparations (Fig. 4A, bottom panel). However, the faint signal along the length of the flagellum remained present and its intensity was not affected. Thin section electron microscopy of the *snl-1* mutant revealed that the blob consisted of a mass of unassembled material, with no PFR filaments structure visible (Fig. 4B).

Interestingly, the blob of unassembled material was not present in every cell: its occurrence showed cell cycle regulation. Observation of biflagellated cells (i.e. in the later stages of the cell cycle) revealed that only the new flagellum possessed a blob whereas the mature flagellum never presented such a structure. Careful examination of trypanosomes during progression through the cell cycle showed that a small amount of PFRC was detectable at the end of a short new flagellum, and this amount became larger as the flagellum elongated (Fig. 5A). Immunofluorescence with the anti-PFRC antibody often produced two bright bars separated by a dark zone, the latter

representing the axoneme, as determined by double immunofluorescence with anti-PFR and anti-tubulin (data not shown). The blob of PFRC material appeared therefore to collect around the tip of the new, growing axoneme. Cytokinesis of such trypanosomes at the end of the cell cycle should then produce a daughter cell, possessing the new flagellum with a blob, and a sibling possessing the old flagellum without a blob. One might envisage therefore a 50:50 ratio of 'blob/no blob' unflagellated cells with one kinetoplast and one nucleus. However, only 34.6% of G₁ cells ($n=1008$) possessed a blob illustrating its loss before initiation of growth of the new flagellum. A few cells (about 1%) were also seen where the entirety of the blob was not present at the distal tip but further towards the proximal end of the flagellum, at the region where the flagellum was attached to the cell body (Fig. 5B). The retrograde movement of the blob, from the tip of the flagellum to the region where the flagellum comes in close contact with the cell body, was directly observed on video

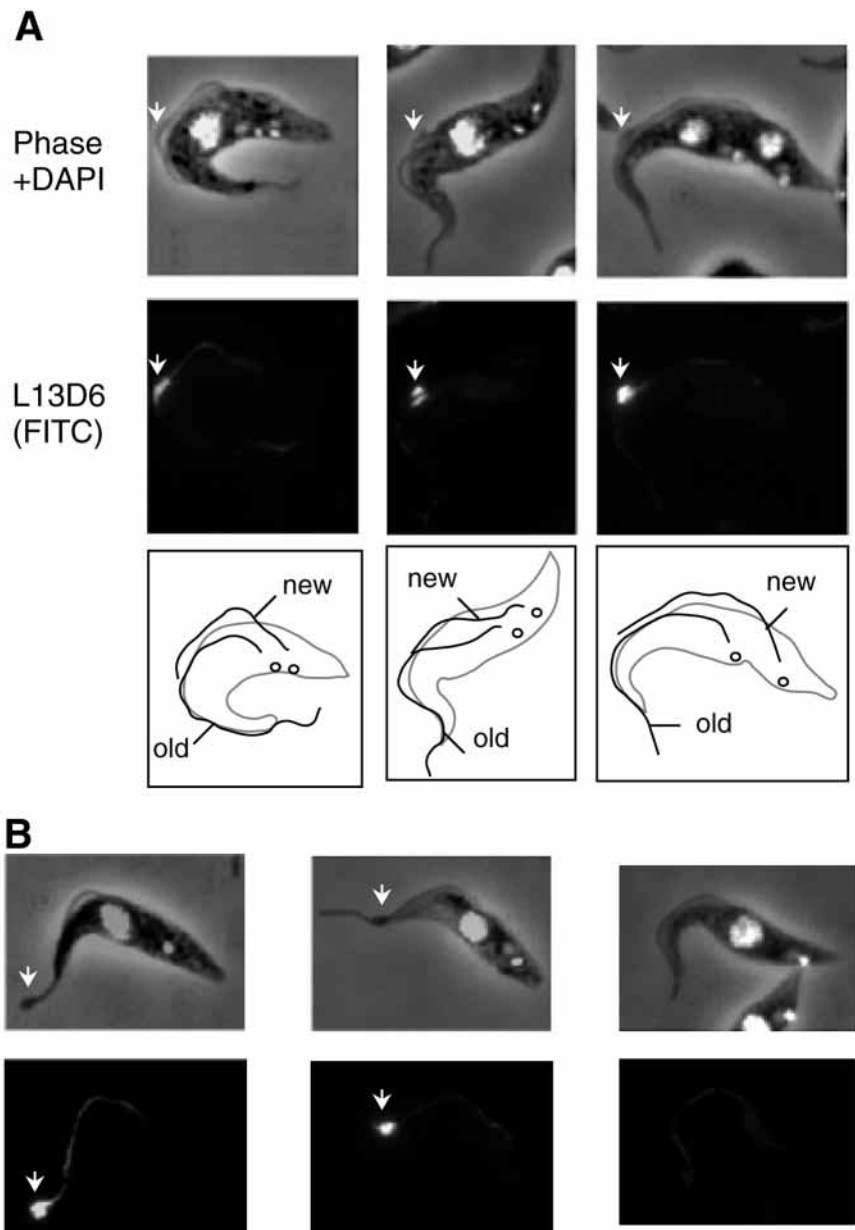


Fig. 5. The blob shows cell cycle regulation. (A) Images of *snl-1* trypanosomes stained with the monoclonal antibody L13D6 showing progression through the cell cycle. In the *snl-1* mutant, in the absence of PFRA, this antibody indicates the localisation of PFRC. The bottom panels show cartoons of the above cells indicating the new and old flagella and the separated kinetoplasts (circles). After duplication and segregation of the basal body/kinetoplast complex, the new short flagellum can be seen at the posterior end of the cell (right-hand side of the picture). As the duplicated basal body/kinetoplast complex migrate apart, the nucleus undergoes mitosis and the new flagellum keeps on elongating (Sherwin and Gull, 1989; Robinson and Gull, 1991). The blob (indicated by a white arrow) becomes larger and stains more brightly with the monoclonal antibody L13D6, revealing progressive accumulation of PFRC at the tip of the flagellum. (B) The three types of unflagellated G₁ *snl-1* cells: where the blob is present at the distal tip of the flagellum (left panels), where it is found further towards the anterior end (central panels) and where it is absent (right panels). Top panels show phase contrast image superimposed to DAPI staining (white) and bottom panels show L13D6 immunofluorescence staining.

microscopy of live *snl-1* trypanosomes. Unfortunately, the proximity of the cell body prevented a clear view of further movements.

DISCUSSION

An intact PFR is required for flagellum and cell motility

In the absence of PFRA, a normal PFR could not be assembled and consequently flagellar and cellular motility were severely reduced. The *snl-1* mutants divided normally, albeit slightly more slowly than wild-type populations. Their most obvious feature was a dramatic paralysis, with cells that failed to swim and which sedimented at the bottom of the flask instead of staying in suspension. A double knock-out of the *PFR2* gene, the *PFRA* homologue in *L. mexicana* also led to serious motility defects marked by a pronounced reduction in both amplitude and frequency of flagellar beating pattern (Santrich et al., 1997). The kinetoplastid parasites require therefore an intact PFR for normal flagellum and cell motility. This raises the question of how this structure actually contributes to motility but also provides an excellent opportunity to study the role of motility during their life cycle (Bastin and Gull, 1999).

Intraflagellar anterograde movement of PFR precursors

In the absence of PFRA, the other major protein, PFRC could not form a normal PFR although portions were still incorporated in a rudimentary PFR, a structure resembling the proximal domain of the PFR (Bastin and Gull, 1999). This rudimentary proximal region was still attached to the axoneme at the correct location and connected to the FAZ (Bastin et al., 1998). However, most of the PFRC pool was not assembled and accumulated at the distal end of the growing flagellum, colocalising with a blob visible by phase contrast. The size of the blob increased as the flagellum elongated. The minor PFR antigen recognised by the antibody ROD-1 and some of the calflagins were also found in this blob. Proteins present in the axoneme such as tubulin, or the antigen recognised by BS7, showed their usual location and were never present in the blob. Our interpretation of this *snl-1* phenotype is that PFRC and probably other PFR precursors are not able to assemble a PFR structure in the absence of PFRA. The major site of addition of PFR precursors is at the distal tip of the growing flagellum (P. Bastin et al., unpublished). Given this distal assembly site, one can envisage the existence of a mechanism for anterograde movement of the precursor material along the flagellum to the tip. We interpret the blob of PFRC and associated unassembled PFR precursors as the physical manifestation of this anterograde transport system. It produces the progressive accumulation of unassembled PFR material at the distal tip in the *snl-1* mutant. The molecular identity of the motor proteins is not known in trypanosomes but in *Chlamydomonas* the kinesin-like molecule FLA10 is responsible for anterograde transport of complexes in the flagella (Kozminski et al., 1995). Homologues of this motor protein appear to be critical for assembly of cilia and flagella in several organisms such as the nematode *C. elegans*, the sea urchin and the mouse (Shakir et al., 1993; Tabish et al., 1995; Morris and Scholey, 1997; Nonaka et al., 1998; Marszalek et al., 1999). This anterograde

transport system has so far been studied mainly in relation to axonemal growth. The phenotype of the *T. brucei snl-1* mutant strongly suggests that such mechanisms are also operating to move extra axonemal material. Many cilia and flagella (particularly in sperm; Baccetti, 1986) possess such structures and insights into the molecular mechanisms of PFR transport are likely to be of significance in this wider context.

Intraflagellar retrograde movement of PFR precursors

A particular characteristic of the *snl-1* phenotype is that a blob is present only at the tip of the new flagellum. The cell divides into two siblings: one which inherits the old flagellum and one the new flagellum (Sherwin and Gull, 1989b). Obviously the new flagellum formed in a particular cell cycle becomes the old flagellum in all subsequent cell cycles. Thus, the blob of PFR material must be removed/resorbed at some point between cytokinesis and the moment of initiation of new PFR growth within the next cell cycle, a point determined as 0.52 of the unit cell cycle (Sherwin and Gull, 1989b; Woodward and Gull, 1990). Analysis of *snl-1* cells containing one kinetoplast and one nucleus (i.e. early in their cell cycle) show that 34.6% of them ($n=1008$) do possess a blob. This indicates that the blob is removed from the flagellum at some point early in the cell cycle, prior to the formation of the new flagellum. Our initial observations suggest that the blob is actually moved back down the flagellum as a unit structure, at least until the point where the flagellum meets the cell body. Whatever the precise kinetics, the fact that the removal of the blob occurs with such regularity suggests the presence of a specific retrograde transport system. Again, for the axoneme, work with *Chlamydomonas* has shown that a retrograde transport system does exist and involves at least one dynein light chain LC8, whose activity is required for correct flagellum assembly (Pazour et al., 1998). The sequence of LC8 is remarkably well conserved in a number of organisms including humans, *Drosophila* and *C. elegans* (King et al., 1996) but its importance for intraflagellar retrograde transport and flagellum assembly has only been demonstrated in the green algae (Pazour et al., 1998). More recently, it has been shown that the dynein heavy chain DHC1b is also involved in this process (Pazour et al., 1999; Porter et al., 1999). It may be that such dynein systems are also involved in the retrograde transport that must operate to remove the PFRC and other unassembled material from the flagellum of the *snl-1* mutant.

Cell cycle control of intraflagellar transport?

Our observation of the formation of the blob and its removal in the *T. brucei snl-1* mutant has significance in terms of cell cycle control of synthesis or intraflagellar transport of components. The progressive, mass accumulation of material at the distal tip during flagellum growth may indicate the dominance of the anterograde mechanism during the later cell cycle period (from around 0.5 of the unit cell cycle) when the new flagellum is being built. In such a model of cell cycle regulation of transport, the retrograde mechanism would be dominant after cytokinesis, during the early period of the next cell cycle (up to 0.5 of the cell cycle). One could envisage however, that the two mechanisms, anterograde and retrograde, operate with no modulation over the whole cell cycle. In which

case, the *snl-1* phenotype (blob at end of new flagellum before cytokinesis but not after) could result from a cell cycle regulation of synthesis of flagellar components. Lack of 'feed-in' of new precursors to the pipeline after cytokinesis would no doubt result in a shift in balance to removal of existing accumulated blob material.

***snl-1* reveals differences between old and new flagella**

A major feature of the *snl-1* phenotype is the appearance of the mass of unassembled PFR material only at the tip of the new flagellum. This emphasises the point that whilst a trypanosome cell possesses both a new and an old flagellum, there is a preferential regulation acting to direct new precursors to the new, forming flagellum. Whilst this may seem to be so self-evident as to be obvious, it is difficult to observe such a process without a mutant such as *snl-1*. Our observations now raise issues as to at which level this regulation between old and new flagellum operates. One could imagine selective precursor transfer only to the area of the new basal body. Alternatively, the new basal body complex could allow entry into its new flagellum whereas the old one acts to restrict entry. Other models such as free entry of precursors to both flagella but transport only operating in the new one, or other combinatorial models are clearly possible.

Import of PFR proteins in the flagellum

These results also show that PFRC can be imported and transported in the flagellum in the absence of significant amounts of PFRA, arguing against the necessity of pre-assembly of PFRA/PFRC complexes prior to import. On the contrary, the antigen recognised by ROD-1 was considerably affected by the absence of PFRA. First, its total amount was reduced and second, the remaining material was mostly found in the cytosol instead of the flagellum of *snl-1* cells. This suggests that the protein recognised by ROD-1 requires the presence of PFRA for efficient transport to the flagellum and might be an indication of pre-assembly of some PFR proteins. In *Chlamydomonas* and in *Tetrahymena*, dynein arms are assembled in the cytoplasm prior to transfer to the flagellum (Fok et al., 1994; Fowkes and Mitchell, 1998). Therefore, similar pre-assembly of different types of PFR precursors may also take place in *T. brucei*.

In conclusion, analysis of the *snl-1* mutant phenotype has allowed us to visualise and characterise complex events that must operate to orchestrate assembly of eukaryotic flagella within the cell cycle.

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