Host–parasite interactions and trypanosome morphogenesis: a flagellar pocketful of goodies

Keith Gull

Trypanosomes are characterised by the possession of a single flagellum and a subpellicular microtubule cytoskeleton. The flagellum is more than an organelle for motility; its position and polarity along with the sub-pellicular cytoskeleton enables the morphogenesis of a distinct flagellar pocket and the flagellar basal body is responsible for positioning and segregating the kinetoplast – the mitochondrial genome. Recent work has highlighted the molecules and morphogenesis of these cytoskeletal/flagellum structures and how dynamic events, occurring in the flagellar pocket and kinetoplast, are critical for host–parasite interactions.

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Current Opinion in Microbiology 2003, **6**:365–370

This review comes from a themed issue on
Host–microbe interactions: parasites
Edited by David Sibley

1369-5274/$ – see front matter

DOI 10.1016/S1369-5274(03)00092-4

Abbreviations

CAP	cytoskeletal associated protein
FAP	flagellum associated proteins
FP	flagellar pocket
GPI	glycosylphosphatidylinositol
kDNA	kinetoplast DNA
MAP	microtubule associated protein
MTOC	microtubule-organising centre
PFR	paraflagellar rod
PM	plasma membrane
SRA	serum resistance associated
TAC	tripartite attachment complex
TfR	transferrin receptor
VSG	variant surface glycoprotein

Introduction

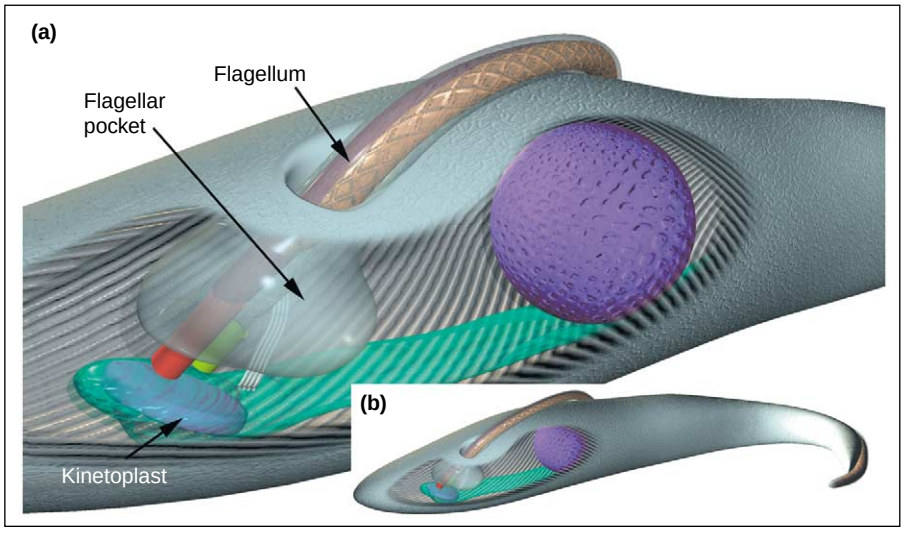
It is axiomatic that as the flagellum emerges from a eukaryotic cell it defines a new membrane surface distinct from the more general plasma membrane (PM). However, in trypanosomes something else occurs. A combination of the emergence of the flagellum and the lack of subpellicular microtubules in that domain, together with a cytoskeletally defined neck region, produces an internal 'ballooning' of a discrete type of PM. This structure is known as the flagellar pocket (FP). The main architectural features of the pocket have been defined in a set of computer drawn images derived from electron microscope images (see [1••] and Figure 1). Flagellum morphogenesis defines three PM domains: the surface membrane, the FP membrane and the flagellar membrane. All vesicular traffic, both into and out of the cell, passes through the FP and it defines the dynamic portal to host or vector environment. The molecular components of the important secretory and endocytic systems associated with the FP are being revealed through the impact of the genome projects and the efforts of individual laboratories [2–5].

Cytoskeletal filaments of unknown identity are important for defining the FP neck. Importantly, we have no idea why microtubules are so firmly attached to the inner face of the surface PM yet are excluded from that of the FP. Discrete protein targeting phenomena and differential lipid constituents (lipid rafts) are likely to play a role in this membrane/cytoskeletal partitioning. In *Trypanosoma brucei* however, the flagellum pocket has started to reveal its bag of molecular 'goodies'.

Microtubule- or cytoskeletal-associated proteins and the cytoskeleton

Electron microscopy reveals a distinct set of regularly spaced projections that link the microtubules to each other and to the inner face of the PM [6,7]. We have some information as to the molecular identities of microtubule- or cytoskeletal-associated proteins (MAPs or CAPs), however recent progress has been slow. Two CAPs have been defined in a study by the Baltz laboratory [8]. Both CAP15 and CAP17 are low molecular weight proteins (15 kDa and 17 kDa respectively) and share some sequence motifs. CAP15 is expressed in both procyclic (Tsetse form) and bloodstream form parasites whereas CAP17 is expressed only in procyclics. Biochemical and cytological studies revealed that both CAPs locate to subpellicular microtubules but not those of the flagellum or mitotic spindle. The association with the subpellicular array is relatively uniform except at the posterior end of the trypanosome where the plus ends of the microtubules terminate [9]. Over-expression leads to a pronounced increase in the doubling time with the concomitant production of aberrant trypanosomes that exhibit difficulty in coordinating cytokinesis. The suggestion is that this might be due to microtubule hyperstabilization. This idea has the more interesting implication that normally some intrinsic regulation modulates (and limits?) the stoichiometry of interaction with particular subpellicular microtubules. Understanding control of cytoskeletal modulations at this level will no doubt

Figure 1



A computer generated cartoon based on electron microscopy of the *T. brucei* procyclic form parasite. **(a)** shows an enlarged section of **(b)** and illustrates the relationship between the flagellum and the flagellar pocket morphogenesis. Basal bodies and probasal bodies are shown in red and yellow respectively, the nucleus is the purple ball and the mitochondrion is shown in green. (Figure reproduced with permission from [1••].)

start to become possible as the catalogue of interacting proteins lengthens.

Rather confusingly, another MAP termed p15 has been partially characterised from *T. brucei*. This was first identified and termed p15 over 10 years ago and shown to bind to the subpellicular microtubules. New evidence [10] shows that the p15 gene encodes a polypeptide containing a set of short repeats of positively charged and nonpolar amino acids. There is evidence for a p15 gene-family and although some biochemical evidence has been presented for an interaction of p15 with membranes and microtubules, we await a more definitive functional analysis of p15 in the trypanosome cytoskeleton.

Flagellum and associated proteins

Just as these MAPs and CAPs characterise the subpellicular microtubules, flagellum associated proteins (FAPs) show an exclusive location to this cellular compartment. Kinetoplastids and Euglenoids share many features, particularly the presence of a paraflagellar rod (PFR) alongside their flagellar axonemes. In *Leishmania*, *T. brucei* and *T. cruzi* the PFR is composed of two major proteins PFR A (PAR1) and PFR C (PAR2). The sequences of PFR A and PFR C genes are similar in Euglenoids and Trypanosomes, indicating a gene duplication event before the branching of a progenitor line into Euglenoids and Kinetoplastids [11].

The presence of the major PFR proteins and PFR structure is critical for motility in *T. brucei* and *Leishmania* [6,9], however, there are many other proteins present in the PFR or interacting with it. One class appears to be the adenylyl cyclases and recent evidence has revealed a calcium stimulated adenylyl cyclase that may interact with the PFR [12]. Trypanosome genomes encode many adenylyl cyclases and at least some of these signalling proteins appear to be in the flagellum. This emphasises my view of the importance of the flagellum as an organelle of signalling/recognition in addition to motility and attachment [6].

The PFR proteins of *T. cruzi* can raise a protective immunity against this parasite. Recently this work [13] was extended using knockout mice to show that although the protective immunity requires MHC class I-restricted T-cell function it appears that the critical role played by CD8(+) T cells might be the secretion of type 1 cytokines rather than lysis of infected cells.

It is unclear as to whether certain other proteins are CAPs or FAPs [14,15••,16••] but when subjected to RNAi knockdown some produce interesting phenotypes illustrated by trypanin [16••], which appears to be required for progressive cell motility.

Tubulin modifications

Cytoskeletal dynamics are undoubtedly modulated by MAPs, CAPs and FAPs. In addition, tubulin itself is likely to be an active player in this phenomenon. Tubulin is amongst, if not the most, modified of polypeptides. Modifications include acetylation, detyrosination and tyrosination, polygycylation and polyglutamylation [7]. Trypanosomes have an interesting position in evolution in that they exhibit all modifications except polyglycylation [17], which has been shown to be functionally important in ciliates [18,19]. There is also some recent *in vitro*

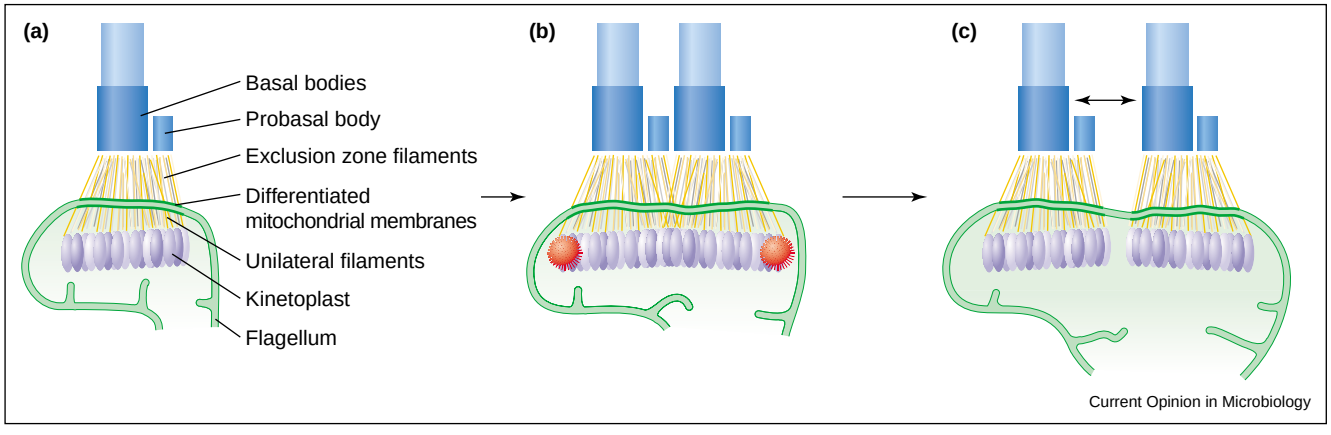
evidence for tubulin phosphorylation [20]. Enzymes (tubulin tyrosine ligase and the HDAC6 deacetylase) responsible for two of these modifications have been identified in other systems, but others have eluded molecular characterisation. An intriguing recent report on polyglutamylase centred on a protein extract from *Crithidia* which had polyglutamylation activity and whose major polypeptide turned out to be a novel member (CfNek) of the NIMA family of protein kinases that act as cell-cycle regulators [21•]. Unfortunately, recombinant expression did not produce an active enzyme preparation and thus it could not be ruled out that the actual glutamylase is associated with the CfNek in the extract and regulated through phosphorylation. However, this is a very intriguing finding and is now amenable to further study in this and other trypanosomes.

Flagellar basal bodies and the kinetoplast

The role of the basal body as the microtubule-organising centre (MTOC) for the flagellar axonemal microtubules has been clarified by work in *T. brucei* using RNAi. Gamma tubulin is a critical feature of MTOCs, acting to nucleate microtubules. We have shown that gamma tubulin functions to nucleate the central pair microtubules of the eukaryotic flagellum but that the axonemal doublets appear to be nucleated directly on the existing basal body triplet microtubules [22••]. RNAi ablation of gamma tubulin expression is ultimately lethal due to effects on the spindle and cytoplasmic microtubule nucleation. However, early phenotypes show that the new flagellum produced under such conditions is immotile and lacks the two central pair microtubules. This discovery provides insight as to how gamma-tubulin activity might be orchestrated across the cell cycle when different MTOCs become activated at different times in the trypanosome.

The basal body is a polar structure and the above study provides insights into distal-end functions. What about the proximal end? Trypanosomes provide one of the very best opportunities to recognise proximal-end functions for centrioles/basal bodies as they act to segregate the genome of the mitochondrion — the kinetoplast. The *T. brucei* kinetoplast contains a mass of catenated minicircle and maxicircle DNA that is replicated in a periodic cell-cycle manner and then segregated before mitosis by the movement apart of the flagellar basal bodies [23]. This segregation suggests a 'hard-wired' linkage between the kinetoplast and the basal body proximal end [24]. Electron microscopy has now revealed that this connection indeed exists [25••] and can be recognised as a three-membered complex — the 'tripartite attachment complex' (TAC). The TAC is composed of a set of filaments linking the basal bodies to an area of differentiated mitochondrial membrane and a further set of filaments that link the inner face of this mitochondrial membrane zone to the kinetoplast (Figure 2). The architecture of the TAC provides the structural basis for kinetoplast positioning that defines each trypanosome type in their individual lifecycles. However, its presence and replication in the cell cycle also suggests a function in providing a structural and vectorial role during the replication of this complex mitochondrial genome. The TAC provides a mechanism whereby inhibition of replication of kinetoplast DNA can lead to basal body segregation and the formation of a dyskinetoplastic cell [25••,26•].

Figure 2



A schematic diagram of the tripartite attachment complex in Trypanosomes. Panel **(a)** illustrates the basal bodies, kinetoplast and the components of the TAC (exclusion zone filaments, differentiated mitochondrial membranes and unilateral filaments) in a trypanosome in G1 of the cell cycle. In this period there is a single flagellum, a basal body and a probasal body. Panel **(b)** shows the organization of the S-phase TAC. When the cell enters S phase discrete fibrous lobes appear at the poles of the kinetoplast, the probasal body matures into a basal body and subtends the new flagellum and two new probasal bodies are formed. Two nascent TAC complexes are discernable at this period of the cell cycle. Panel **(c)** shows the period where movement apart of the flagella basal bodies segregates the replicated kinetoplast DNA. Note that the position and orientation of the basal bodies have been idealised in this two-dimensional cartoon. (Figure reproduced with permission from [25••].)

The kinetoplast and dyskinetoplasty

Many studies have shown that dyskinetoplastic trypanosomes can survive as bloodstream forms, yet there is now good recent evidence that they cannot survive certain types of insults to kinetoplast DNA (kDNA) replication or gene expression [27••]. This intriguing conundrum and the concept of dyskinetoplasty has been reviewed in [26•].

Kinetoplast structure changes in S-phase and we now have some insight into the molecular events associated with periodic kDNA replication [28]. Four new polymerase proteins (TbPOLIA, IB, IC and ID), in addition to the pol β [29], have now been localised to the mitochondrion and two, TbPOLIB and TbPOLIC, are located close to the kDNA where replication occurs [30••]. Their knockdown by RNAi caused shrinkage of the kDNA network and accumulation of intermediates suggesting a direct role in replication. Trypanosomes therefore exhibit an expanded family of mitochondrial DNA polymerases in contrast to other eukaryotes as well as a mitochondrial RNA polymerase [31].

The Englund laboratory has also used knockdown of mitochondrial topoisomerase II expression to perturb the kinetoplast and assess the minimal network size compatible with viability [31]. These experiments revealed the potential of some mutant trypanosomes to survive network shrinkage by asymmetrical division of replicated kinetoplast networks at the expense of siblings inheriting small networks. Mitochondrial development during *T. brucei* lifecycle differentiations also suggests a requirement for kDNA in the bloodstream and a kinetoplast-dependent control point during differentiation to procyclic forms [32•].

Flagellar pocket: the parasite–host portal

The *T. brucei* bloodstream form surface is covered by a dense coat composed of a glycosylphosphatidylinositol-anchored (GPI) protein, the variant surface glycoprotein (VSG). The presence of the VSG coat and its switching in individual parasites allows evasion of the host's immune response [33–36]. GPI-anchored proteins are delivered to the cell surface via secretory events at the FP. A recent study [37••] of steady-state VSG distribution shows that around 90% is present on the cell surface, and of the 10% that is intracellular much is located in the endosomal compartment with only some associated with the ER, Golgi and lysosomes. Thus, the density of VSG in the PM including the membrane of the FP is some 50 times higher than the density in ER. Thus the high surface-density is achieved in two or three enrichment steps suggesting efficient sorting between several membrane compartments.

The VSG journey to the FP is therefore a regulated pathway involving compartment changes and numerous modifications. The GPI-anchor is formed via a post-translational transamidation defined by a conserved 23 or 17 amino acid signal sequence. Cross and co-workers [38•] have interrogated the necessity of this evolutionary conservation. The omega amino acid, to which GPI is transferred, is Ser, Asp or Asn, the omega+2 amino acid is always Ser, and the omega+7 amino acid is almost always Lys. Mutational analysis revealed some surprises. Changing the most conserved amino acids and some more extensive changes had no detectable effect on the efficiency of GPI-anchoring or on VSG abundance. However, deleting stretches of sequence resulted in truncated proteins accumulating in the ER before lysosomal degradation. Given the acknowledged caveat of such experimental assessments of 'fitness' it appears that VSG GPI signal-sequence conservation is not necessary for efficient synthesis and GPI attachment, but appears essential for surface expression.

Location of VSG-like molecules in internal compartments has taken on a biological significance in the light of other recent findings. The critical feature of East African human trypanosomiasis, caused by *T. brucei rhodesiense* is the resistance of these parasites to lysis by normal human serum [39,40]. Resistance is conferred by a gene that encodes a truncated form of a VSG termed serum resistance associated protein (SRA) [41]. Further insight as to how SRA acts has now been provided [42••]. Although SRA is related to VSGs [43•] it turns out to be a lysosomal protein, and SRA interacts with the human-specific serum protein apolipoprotein L-I (apoL-I). It is this apoL-I host-factor that is suggested to be the effector lytic molecule and it appears to be trafficked via the FP through the endocytic pathway into the lysosome. SRA confers resistance to lysis by interaction with apoL-I in the lysosome [42••].

Some trypanosome proteins, such as the transferrin receptor (TfR), need to reach the FP and to remain there. FP residency allows them to interact with host proteins and participate in endocytic events but not to reveal themselves to the host immune system. The TfR is GPI-anchored and evidence has been presented [44••] to show that it is retained in the FP by a specific and saturable mechanism. Even modest overexpression of TfR in bloodstream-form trypanosomes resulted in escape from FP residency and accumulation on the surface. FP retention mechanisms for this type of receptor appear therefore to be easily saturable and control of the expression level is critical.

VSG and TfR are also highly glycosylated and these glycoproteins, as well as other FP glycoproteins, are known to contain galactose. *T. brucei* is unable to take up galactose and the Ferguson group [45••] has questioned the reliance of the parasite on UDPglucose 4′-epimerase for the conversion of UDP-Glc to UDP-Gal

and subsequent incorporation of galactose into glycoconjugates via UDP-Gal-dependent galactosyltransferases. Reverse genetic approaches showed that galactose metabolism in *T. brucei* does proceed via UDP-Glc-4′-epimerase and moreover is essential for parasite growth. Importantly, this provides a validation of galactose metabolism as a potential therapeutic target for African trypanosomiasis.

Conclusions

Modulation of the flagellum and other cytoskeletal elements of the trypanosome are responsible for the differing shapes and forms of trypanosomes, characteristic of their lifecycle and interactions with host and vector. A key feature is that the flagellum not only provides a mechanism for movement and attachment it also enables the morphogenesis of a lumenal region of the PM — the flagellar pocket. This pocket provides the portal through which most of the dynamic interactions with the host occur. These interactions facilitate resistance to innate and acquired immune responses as well as acquisition of growth factors from the host.

Acknowledgements

Work in my laboratory is funded by the Wellcome Trust and BBSRC.

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The transferrin receptor — a heterodimer attached to the surface membrane by a GPI anchor — is restricted to the flagellar pocket. These authors conclude that the receptor is retained in the flagellar pocket by a specific and saturable mechanism. The implication is that control of the expression level is critical in order for this restricted distribution to be maintained. Over-expression leads to escape from the pocket and 'exposure' on the whole surface.

45. •• Roper JR, Guther MLS, Milne KG, Ferguson MAJ: **Galactose metabolism is essential for the African sleeping sickness parasite *Trypanosoma brucei***. *Proc Natl Acad Sci USA* 2002, **99**:5884-5889.

Several critical molecules on the trypanosome surface are glycosylated. This study of the sugar requirements of the trypanosome demonstrates that galactose metabolism is essential for growth. Apart from the intrinsic interest, this approach validates the results that enzymes and transporters involved in galactose metabolism can act as potential therapeutic targets against African trypanosomiasis.