The kinetoplastida endocytic apparatus. Part I: a dynamic system for nutrition and evasion of host defences

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The endocytic system of kinetoplastid parasites is a highly polarized membrane network focused on the flagellar pocket localized at one end of the cell. When first characterized, the endosomal network was envisioned as a simple system for uptake of extracellular material by fluid-phase or receptor-mediated mechanisms. Subsequently, it has become clear that the kinetoplastid endosomal system has an active and vital role in avoiding the host immune system and virulence, as well as providing the basic functions to fulfil cellular nutritional requirements. In two reviews, recent advances in the definition and comprehension of kinetoplastida endocytosis are discussed and, in *Trypanosoma brucei* in particular as the more developed experimental system. In Part 1, the endocytic system is considered in context of the surface molecules and their potential roles in virulence.

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The life cycle of most trypanosomatids that are pathogenic to humans and to livestock includes at least one developmental stage in the insect and vertebrate host; thus, the parasite is exposed to multiple environments. Transfer between hosts results in differentiation of the parasite to a form suited to the new environment. One central feature of this differentiation is extensive restructuring of external cell surface proteins and glycoconjugates. The switch in cell surface composition not only requires coordinate changes to gene expression, but also depends on the protein trafficking system. Recent work has identified several components of the kinetoplastid endocytic and exocytic pathways (Fig. 1). These investigations have revealed distinct mechanisms of control, both quantitative and qualitative, for different cell surface proteins reflecting the diverse needs of the cell at specific stages of the life cycle. A clear example of such a developmental difference is the rate of endocytosis in *Trypanosoma brucei,* which is ~10-fold higher in the mammalian bloodstream form than in the insect procyclic form, and which might be explained by proposing a dual role for endocytosis in bloodstream forms. In both stages, the acquisition of nutrients is essential and is reflected in a basal rate on endocytosis but, in addition, there is a high rate of plasma membrane turnover (i.e. internalization and re-emergence) in bloodstream forms, possibly linked to the removal of surface-bound antibody.

The *T. brucei* cell is polarized, and endocytosis and exocytosis occur through membrane turnover at the flagellar pocket, an invagination of the plasma membrane where the flagellum enters the cell body (Fig. 2). The flagellar pocket membrane is highly specialized, and forms a discrete domain of the cell surface that is at least partly isolated from the bulk plasma membrane. As was described in early ultrastructural analysis of trypanosomatids, the flagellar pocket has apparent similarity in morphology to the cytostome (the feeding organelle, capable of engulfing large particles from the medium) of other protozoa such as *Paramecium.* The flagellar pocket is distinguished structurally and functionally from the cytostome of *Paramecium* because phagocytosis has never been reported in trypanosomatids. However, the flagellar pocket is the site of all endocytosis in *T. brucei*, which is characterized by the budding of vesicles and flask-shaped structures of ~20–40 nm diameter (Fig. 2), and so, in this regard, function remains conserved. The high degree of polarization of plasma membrane endocytic activity is reflected in the localization of underlying endocytic membrane networks. While the entire cytoplasmic volume of *T. brucei* is pervaded by membrane-bound compartments, such as the endoplasmic reticulum (ER), those associated with late macromolecular secretion and endocytosis are concentrated within the region of the cell posterior to the nucleus, which occupies ~30–40% of the total cell volume, and especially in the region between the nucleus and the flagellar pocket [1,2]. Within this area, there is a complex network of interconnected tubules and vesicles, a stacked Golgi complex and other membranous structures in close proximity to the flagellar pocket [3].

Concentration of an internal membrane system between the nucleus and flagellar pocket could reflect a functional requirement; in particular, the short distances between compartments that arise from this arrangement result in extremely rapid transport through the pathways. This idea is consistent with the observation that the components of the secretory pathway are not only concentrated in the posterior part of the cells, but are also organized in a polarized
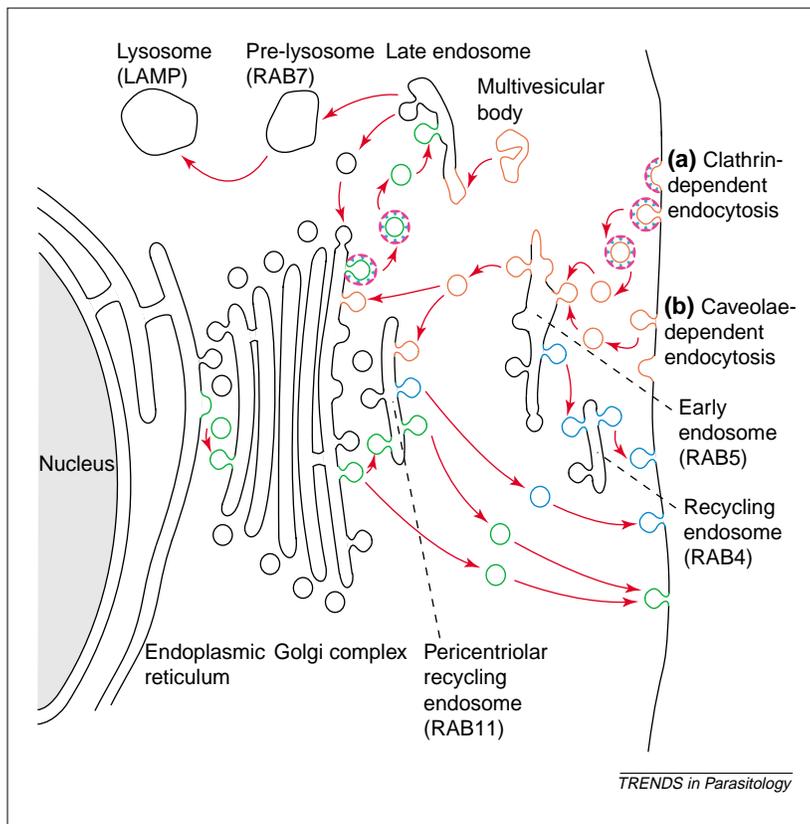
Fig. 1. The eukaryotic endosomal system. At least two modes of endocytosis (orange) from the plasma membrane are represented: (a) clathrin-dependent (pink); and (b) caveolae. Each serves to concentrate selected molecules within membrane microdomains, from where plasma membrane transport vesicles are formed (orange). Both pathways merge within the endocytic system, probably equivalent to the early endosome, responsible for separating ligands from receptors and for targeting molecules to one of at least four subsequent routes. These are: (1) delivery to lysosomes, which occurs via several intermediate stages; (2) rapid recycling; (3) recycling via the *trans*-Golgi network; and (4) direct recycling to the pericentriolar-recycling endosome. Homologues of each of these pathways have been detected in trypanosomatids. Key: blue, recycling pathways; green, synthetic (exocytic) routes; orange, endocytic pathway intermediates.

manner with the nucleus, ER exit sites, the Golgi apparatus and flagellar pocket all lying along an axis. Alternatively, the distribution of the secretory organelles might be dictated by the organization of the cytoskeleton, and is more a result of the positioning of the flagellum basal body, the microtubule-organizing centre. Early and late compartments can be discriminated by temperature shifts to block import of fluid-phase markers, such as horseradish peroxidase [2]. The early compartments, termed collecting tubules, are structures within the cytoplasm that contain material immediately following import. The later compartments are lysosomal and they receive imported material from the earlier compartments.

The mechanism of the quantitative difference in rate of endocytosis between bloodstream and procyclic forms has been investigated by determining the density and morphology of endocytic structures associated with the flagellar pocket. Clathrin-coated vesicles containing fluid-phase markers were detected in the bloodstream but not in the procyclic forms [5,6]. Recent molecular data confirms that this is broadly correct [7], although clathrin is present at a reduced level in procyclic forms. Interestingly, in the bloodstream non-dividing stumpy stage, the flagellar pocket becomes enlarged, which might suggest an increased requirement for membrane turnover. This is also accompanied by increased cellular levels of the lysosomal protein p67/CB-1 [8], suggesting an expansion of the lysosomal compartment. These non-proliferative cells might have a heightened requirement for endocytosis in the context of immune evasion.

Surface proteins, endocytosis and immune evasion
The *T. brucei* bloodstream form can be readily killed by antibody and complement. During an infection, the killing is mediated by increasing concentrations of antibodies as the immune response is mounted. Although antigenic variation is the primary mechanism for maintaining chronic infection, there is also evidence of a role for surface-protein trafficking in the immediate survival of any one cell against an immune response of increasing antibody concentration. The major surface protein of bloodstream forms, the variant surface glycoprotein (VSG), rapidly cycles through the flagellar pocket, between the cell surface and membrane-bound structures within the cytoplasm. This process has been reported to be constitutive because surface-labelling experiments demonstrate that recycling occurs in the absence of apparent stimulation by crosslinking or differentiation [9]. Recycling of VSG is distinct from shedding into the medium, which occurs slowly, because VSG has a half-life equivalent to 4–6 cell generations. Shedding is presumed to be a result of proteolysis [9], most likely a cysteine protease [10]. VSG polypeptide itself appears to be stimulatory for macrophages [11] and is highly immunogenic, and hence prevention of VSG release by endosome-associated proteases is potentially important for parasite survival.

Upon experimental exposure to high concentrations of VSG antibody, the cell-surface VSG is rapidly capped and endocytosed via the flagellar pocket. In an early investigation, addition of anti-VSG Fab or $F(ab)_2$ fragments resulted in rapid movement of VSG–antibody complexes from the cell body or flagellum towards the flagellar pocket [12]. Subsequent to reaching the flagellar pocket, antibody fragments were internalized into structures between the flagellar pocket and nucleus, and VSG-bound antibody was degraded internally. Crosslinking with bivalent antibodies was not required for endocytosis in this study. In fact, internalized VSG is returned to the cell surface intact, but bound immunoglobulin (Ig) is cleaved by a L-1-chloro-3-(4-tosyl-amido)-7-amino-2-heptanone (TLCK)-sensitive (presumably serine) protease, and the Ig fragments are secreted [13].Several additional proteases, including a homologue of the *Leishmania* cell surface metalloproteinase gp63, have been implicated as being present in the flagellar pocket and associated recycling compartments, which might indicate an ability to degrade proteins
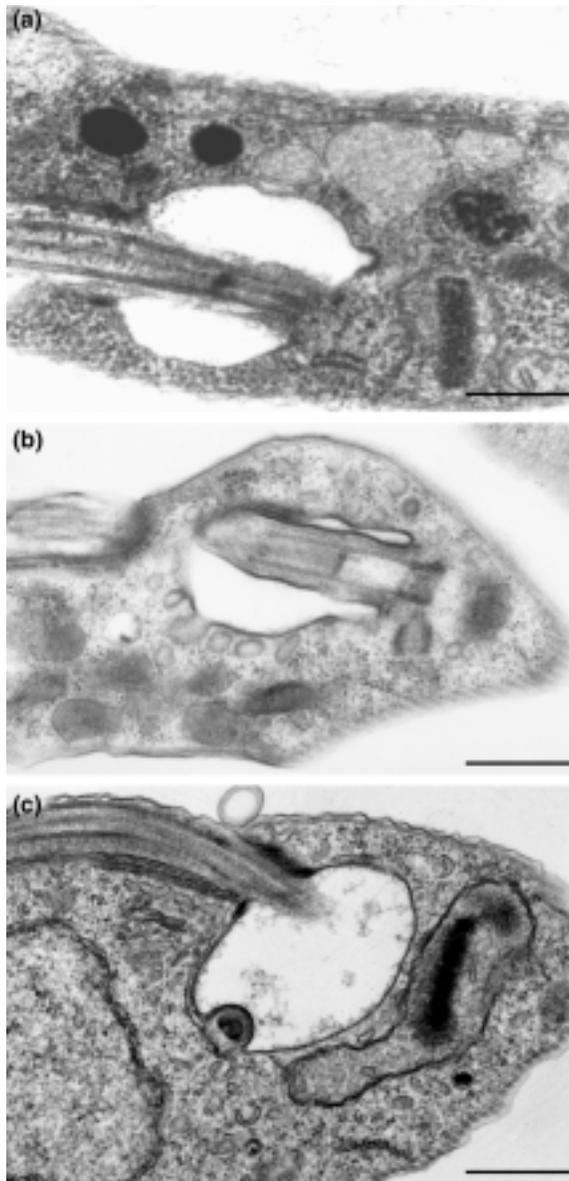
Fig. 2. Ultrastructure gallery of transport structures associated with the flagellar pocket of procyclic and bloodstream stages of *Trypanosoma brucei*. (a) Single clathrin-like coated vesicle budding from/or to the posterior wall of the flagellar pocket from a procyclic cell. (b) Bloodstream-form cell with multiple flask-like structures engaged with the flagellar pocket membrane. It is not clear if these structures are endocytic or exocytic, but they are clearly devoid of clathrin or other prominent coat structures associated with the membrane. (c) Multilamellar membrane structure associated with the anterior wall of the flagellar pocket of a procyclic trypanosome. A lack of molecular markers continues to confound assignments of these various membrane figures to specific roles in membrane transport processes. Scale bar in (a–c) = 500 nm.

before, or without, the involvement of a lysosomal compartment [14]. The ability to degrade surface-bound Ig represents a mechanism by which the trypanosome can survive during a period of rising concentrations of VSG antibodies, and thus increase the likelihood of transmission, but the identity of this activity remains unresolved. This requirement is unique to bloodstream forms and is a plausible explanation for the basis behind evolution of a highly active endocytosis and/or recycling system in haemoflagellate trypanosomes. The presence of a proteolytic activity in the early endosomal system and, possibly, also the recycling arm suggests that trypanosomes might have substantial abilities for degrading extracellular material [10,13].

Because VSG is a glycosylphosphatidylinositol (GPI)-anchored molecule, it does not have a cytoplasmic domain necessary to bind the adaptin and/or clathrin-dependent endocytic pathway. It is possible that VSG endocytosis uses either a co-receptor with a cytoplasmic domain, a raft-mediated mechanism, or that endocytosis is nonselective but, at present, this is unknown. Internalization of surface proteins of bloodstream form *T. brucei* which do have cytoplasmic domains, such as the non-variant ISG_{65} and ISG_{75}, has not been well studied. It has been suggested that oligomannose glycans present on some VSG variants could act as binding sites for soluble ligands, and tumour necrosis factor (TNF)-α in particular [15]. TNF-α has oligomannose-specific lectin activity [16], and the data implicate VSG as an important factor in endocytosis and subsequent trypanocidal activity of TNF-α [17]. Little is known about endocytosis or recycling of procyclins, the major surface proteins of procyclics. Recent data on modulation of procyclin isoform expression on the cell surface by genetic manipulation of the endocytic pathway indicate that these proteins do recycle, albeit at a low rate (B.S. Hall and M.C. Field, unpublished).

Leishmania studies of endocytosis have focused on the amastigote, which resides and replicates within a parasitophorous vacuole (PV) inside the host macrophage that resembles a late endosomal and/or lysosomal compartment. Any molecule taken up by the amastigote first has to enter the PV, but once there, macromolecules can be endocytosed by the amastigote itself via the flagellar pocket [18,19]. The PV has similarities to specialized compartments of antigen-presenting cells, where complexes between the peptide and major histocompatibility complex (MHC) class II are formed before their exposure at the cell surface. Significantly, in *Leishmania*-infected cells, amastigotes endocytose and degrade MHC class II molecules and the cofactor β-2 microglobulin [20,21], presumably allowing the parasite to block or hinder presentation of *Leishmania*-derived antigenic peptides. Lysosomal hydrolytic activity is highly upregulated in *Leishmania mexicana* amastigotes compared with that in insect-stage promastigotes, which correlates with the ability to internalize and degrade MHC and other host molecules, and the appearance of multivesicular megasomes within the amastigote [22].

Interest in the *Leishmania* promastigote endocytic processes has been less intense. However, recently, a morphologically unusual tubular lysosomal compartment has been described in several *Leishmania* spp. [23–25]. This compartment stretches from the flagellar pocket towards the posterior end of

the cell, and its multivesicular composition is reminiscent of the megasomes of *L. mexicana* amastigotes. The significance and function of this structure remains unknown.

Surface receptors

Parasitic trypanosomatid species share many nutritional requirements, but the sources and receptors used to fulfil these needs differ between species and developmental stage. For the essential nutrient iron, a variety of mechanisms of acquisition have evolved to suit the environment. In *T. brucei* bloodstream form, in *T. cruzi* epimastigotes, and in both the promastigote and amastigote forms of *Leishmania* spp., iron is taken up via specific transferrin receptors, but the mechanisms are unique to the individual parasite. The pathway of iron uptake is well understood in metazoans; transferrin binds a cell-surface receptor, is endocytosed to early endosomes, and a reduction in pH within the endosomes leads to release of bound iron from transferrin. The apo-transferrin is then recycled, intact, back to the surface via recycling endosomes and is released [26]. In *T. brucei*, transferrin is taken up initially into a *T. brucei* Rab5 (TbRAB5A)-positive compartment [27], the transferrin is degraded and the empty receptor is recycled [28]. Fragments of endocytosed transferrin can be recovered in the medium, suggesting that uncoupling of receptor and ligand, and return of the transferrin fragments to the cell surface takes place without the involvement of the lysosomal compartment [28]. This process involves a compartment associated with *T. brucei* Rab11 (TbRAB11), similar to the recycling system in mammalian cells [29].

In *T. cruzi* epimastigotes, which have both a flagellar pocket and a cytostome, transferrin is initially taken up into the cytostome and then is transported via vesicular tubular structures to the reservosome at the posterior end of the cell [30,31]. The reservosome is most likely the equivalent of the lysosome [32,33]. Interestingly, the reservosome is associated with *T. cruzi* Rab11 (TcRAB11), suggesting the possibility that the transferrin receptor could recycle to the surface from here [34]. The trypomastigote form of *T. cruzi* does not appear to bind or internalize transferrin [30]; it is a non-proliferative stage and the metacyclic forms rely instead on the nutrients stored in the reservosome, whereas the bloodstream trypomastigotes have a relatively short extracellular life and are non-proliferative, and presumably do not require *de novo* iron uptake.

Integration of the *Leishmania*-containing PV into the host cell endosomal system allows the parasite to subvert normal endosomal processes. Transferrin uptake by the infected macrophage occurs normally, but the endosomes containing the transferrin receptor and transferrin then fuse with the PV, and *Leishmania* amastigotes can bind and endocytose the released transferrin, which is trafficked to lysosome-like compartments within the parasite [19]. By contrast, *T. cruzi* amastigotes express a specific receptor for transferrin and cannot grow in a transferrin-free environment [35]. However, this finding applies to extracellular amastigotes, and the importance of transferrin for *in vivo* intracellular amastigotes, which lie free in the cytoplasm and not inside a vacuole, is unclear because cytosolic iron is sequestered by ferritin.

Transferrin receptors also differ in location between different species and stages. Transferrin binding is limited to the flagellar pocket in the *T. brucei* bloodstream form and *Leishmania* procyclic form, but is found in the cytostome of *T. cruzi* epimastigotes [30,31]. In *Leishmania*-infected macrophages, the parasites have transferrin-binding sites distributed over the surface of the intracellular amastigote [19]. These distinct sites of receptor expression could reflect the environments in which the parasite has to survive. When *T. brucei* is in the bloodstream, restriction of expression to the flagellar pocket limits the access of effector cells of the immune system to the comparably invariant receptor [36]. However, *Leishmania* amastigotes, within a PV inside a host cell, do not come into contact with effector cells of the host immune system, and the receptor is present over the entire surface of the cell.

The transferrin receptors expressed in the various parasites are specific to the individual species. The *T. brucei* receptor is a heterodimer containing two closely related polypeptides, ESAG6 and ESAG7, and is attached to the external face of the plasma membrane through a GPI-anchor at the C-terminus of ESAG6. There are multiple copies of genes encoding both ESAG6 and ESAG7, although only one of each is expressed at any one time. Within each gene family, there is minor sequence variation, which has probably evolved to produce a family of receptors with different affinities for transferrin from a range of mammalian host species. A transferrin receptor repertoire has been suggested as an adaptation to facilitate survival in a wide range of hosts [28,37]. A similar ESAG6- and/or ESAG7-based receptor has recently been identified in the closely related parasite *Trypanosoma evansi* [38] The leishmanial transferrin receptor is only partially characterized, but appears to be a 70-kDa monomeric integral membrane protein, distinct from either the mammalian or the *T. brucei* receptors [39]. The *T. cruzi* receptor remains to be identified.

Host-serum-derived low-density lipoprotein (LDL) serves as an important source of sterols for haemoflagellates [40]. Even some lifecycle stages restricted to the insect vector, such as the procyclic form of *T. brucei*, show an absolute requirement for exogenous LDL for growth [41]. Potentially, sufficient LDL is present in a tsetse bloodmeal to provide lipoprotein *in vivo*, but it is also possible that related insect proteins, such as lipophorins, might be used, especially by the proliferating epimastigotes in the salivary glands. Specific LDL-binding proteins

appear to be expressed in *Leishmania* spp., *T. brucei* and *T. cruzi*, but the molecular nature of the LDL receptors is unclear [30,41–43].

Characterization of the affinity of LDL for its binding sites revealed high- and low-affinity lipoprotein binding sites in *T. brucei*, which are, most likely, two forms of a single receptor [27,44]. Both bloodstream and procyclic forms possess a similar number of LDL-binding sites, mostly in the flagellar pocket and flagellum. However, LDL uptake levels are vastly different: endocytosis of lipoprotein is barely detectable in procyclics, but is at least ten times more rapid in bloodstream forms [27,44]. Procyclic forms, which can synthesize their own sterols, are probably less dependent on continuous uptake of host LDL [41]. LDL is internalized initially into a TbRAB5A-containing early endocytic compartment, similar to transferrin but, by contrast, is ultimately degraded in the lysosome [27,44]. It is assumed that the receptor is recycled to the surface. In bloodstream forms, surface-bound LDL is degraded within 20 min in a process that is dependent on temperature, and is sensitive to acidotropic agents [45]. In addition to LDL, *T. brucei* appears to use high-density lipoprotein (HDL) as a source of lipid. Specific binding sites are present on the parasite surface, and endocytosis of HDL has been detected. Endocytosis of LDL by other kinetoplastids is less well studied, but the pattern of uptake seems to be similar to that described for transferrin. LDL binds to *T. cruzi* epimastigotes and amastigotes, but not to trypomastigotes, and gold-labelled lipoprotein is bound to the flagellar pocket and, in epimastigotes, the cytostome at 4°C [30].

Endocytosis and the evasion of lytic factors

Trypanosoma brucei brucei does not infect humans because it is killed by trypanosome lytic factor (TLF), whereas *T. b. rhodesiense* is resistant to TLF. TLF is a component of normal human serum, and can be separated into two parts, TLF-1 and TLF-2, both of which are in the HDL fraction [46]. TLF-1 contains apolipoprotein AI, apolipoprotein AII, paraoxonase and haptoglobin-related protein (Hpr), whereas TLF-2 contains apolipoprotein AI, IgM and Hpr. The killing of trypanosomes by TLF-1 is inhibited by haptoglobin, whereas TLF-2 is not [46]. The component of TLF-1 that is probably responsible for killing is Hpr because HDL containing the other three components has no effect on viability [47]. Haptoglobin itself is a serum protein that binds haemoglobin released as a result of tissue damage. Hpr is also a serum protein, present at <1% of the concentration of haptoglobin. A functional *Hpr* gene is observed only in primates resistant to *T. b. brucei* [48,49] and it has no known function other than its putative role in protection against *T. b. brucei*

infection. The action of TLF-1 is dependent on endocytosis; after binding to a cell-surface receptor, TLF-1 is internalized and trafficked to the lysosome. By a mechanism not fully characterized, TLF-1 causes release of lysosome contents into the cytoplasm and cell death [50]. The receptor for TLF-1 has not been identified at the molecular level. However, there are both high- and low-affinity binding sites, and the low-affinity sites, at least, overlap with HDL-binding activity. The binding to the high-affinity sites is dependent on the presence of Hpr in the HDL [51]. The mode of action of TLF-2 is less well understood.

TLF resistance is dependent on interference with the endocytic pathway. Screens to find messenger RNAs (mRNAs) expressed in lines resistant to human serum, but not expressed in sensitive lines, identified a serum-resistance-associated (*SRA*) gene [52,53]. Ectopic expression of the *SRA* gene is necessary and sufficient to confer resistance to human serum in a normally sensitive strain [54]. The mode of action of the SRA protein in protecting the normally sensitive trypanosome appears to involve a reduction in the endocytosis of TLF-1, as opposed to a reduction in binding. The mechanism of the process will be fascinating for two reasons: (1) resistance operates through a specific reduction in endocytosis of TLF-1 because uptake of essential nutrients, such as transferrin, presumably continues at a normal rate; and (2) the *SRA* gene must function as a dominant, negative factor blocking the uptake of TLF-1, and hence SRA must interact with the components directly involved in the endocytosis process in trypanosomes. How SRA expression protects against TLF-2 is not known.

Conclusions

Our views of the endocytic system of trypanosomes have advanced considerably in the past decade. In particular, the importance of this system in evasion of host defense mechanisms, particularly resistance to TLF and avoidance of antibody recognition, are vital to propagation of *T. brucei* within the mammalian host. The view of endocytosis as an active virulence mechanism among the kinetoplastida is extended by the potential importance of the system in compromising immunological surveillance in *Leishmania*. A deeper understanding of the endocytic processes at the molecular level is promising following the advances in the genome analysis of trypanosomatids. Recent insights into the kinetoplastid endocytic machinery will be the focus of our second article on the endocytic apparatus of African trypanosomes and kinetoplastida.

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