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Review

Intracellular Membrane Transport Systems in *Trypanosoma brucei*

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Trypanosomes belong to the order kinetoplastida, an early diverging group of organisms in the eukaryotic lineage. The principal reasons for interest in these organisms are twofold; they provide a superb distant triangulation point from which to assess global features of eukaryotic biology and, more importantly, they are representative of a number of pathogenic parasitic protozoa with a huge public health impact – *Trypanosoma brucei*, *T. cruzi* and *Leishmania* spp. Recent advances in the study of intracellular transport in *T. brucei* have been considerable, and a fuller picture of the complexity, function and role that the endomembrane system plays in trypanosomes is finally emerging.

Key words: clathrin, Rab, small G protein, trypanosoma, vesicle trafficking

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Trypanosoma brucei is the causative agent of African sleeping sickness and a range of livestock diseases endemic in 36 countries and afflicting most of tropical Africa (http://www.who.int/tdr/diseases/tryp/diseaseinfo.htm). *T. brucei* is transmitted by dipterans of the genus Glossina, more commonly called tsetse. Both the mammal and tsetse harbour proliferative forms of the trypanosome. The 'bloodstream form' lives in the mammalian bloodstream, lymphatic system and tissue spaces and the 'insect' or 'procyclic form' lives in the tsetse. These forms have adapted to exploit radically different environments (Figure 1). *T. brucei* is exclusively extracellular, unlike most other protozoan parasites, for example *Plasmodium*, *Leishmania* and *T. cruzi*, the American trypanosome. Both the mammalian and the procyclic forms of *T. brucei* are amenable to culture in the laboratory and can be transfected, facilitating genetic manipulation.

The maintenance of a chronic infection by an extracellular pathogen potentially creates unusual selective pressures, in particular the need to evade the adaptive immune response. For the mammalian stage this is primarily achieved by antigenic variation (1). In insects, which have a less sophisticated immune system, the parasite appears to achieve protection by a more passive mechanism involving surface expression of highly stable proteins, procyclins, which prevent direct attack of the plasma membrane (2).

Several general features of the trypanosome intracellular transport system are of specific interest. Firstly, the exocytic and endocytic systems are highly polarised and contained within the posterior region of the cell; the polarized organelles include the Golgi complex, endosomes and lysosomal apparatus but not the endoplasmic reticulum (ER), which is distributed throughout the cytoplasm (Figure 2). Secondly, all membrane exchange with the plasma membrane is restricted to the flagellar pocket – an an invagination that surrounds the flagellum as it enters the cell to engage with the basal body. A number of molecules have restricted surface localisation to the flagellar pocket, indicating a specialised membrane composition (3, 4). A third unusual property is the dominance of the cell surface by glycosyl phosphatidylinositol (GPI)-anchored molecules, which is a common feature amongst the kinetoplastida (5). In the bloodstream form, the plasma membrane contains $\sim 1 \times 10^7$ copies of the variant surface glycoprotein (VSG) – this molecule is responsible for antigenic variation mediated by sequential expression of one VSG gene from a reservoir of several hundred (see (1) for review of this topic). The insect stage, which does not exhibit antigenic variation, displays 5×10^6 copies of procyclin, a small acidic and highly glycosylated GPI-anchored protein – it is likely that this molecule has multiple roles in the survival of the parasite within the tsetse fly (6). Fourthly, the bloodstream form has an extremely active endocytic system, which is at least an order of magnitude more active than in the procyclic form. This differential endocytosis is unlikely to directly reflect nutrient requirements as both stages complete their cell cycle within a similar period. Several authors have argued that the up-regulation endocytic activity in the bloodstream form is a component of the immune evasion mechanism (7, 8).

The completion of the genome sequence for *T. brucei* (http://www.genedb.org/genedb/tryp/index.jsp) and several other protozoa has facilitated a more complete overview of the potential that resides within trypanosomes for protein and lipid processing and transport, and importantly,

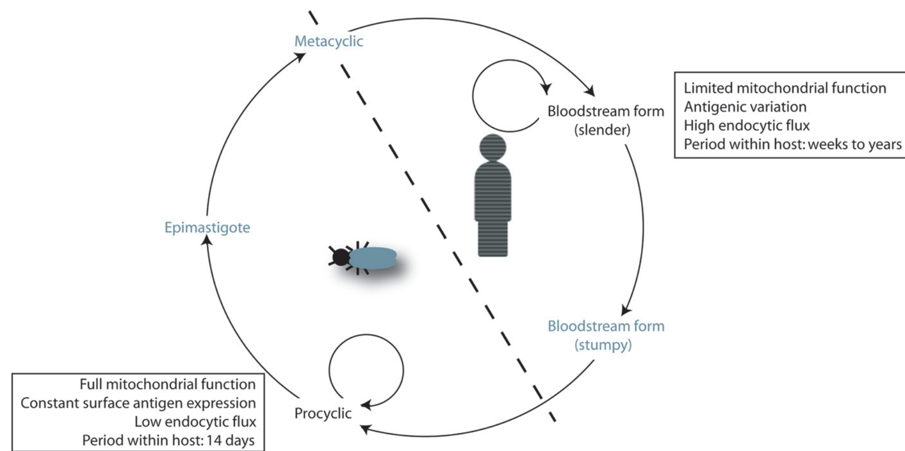


Figure 1: A simplified trypanosome life cycle with emphasis on developmental alterations accompanying the two major proliferative stages. The bloodstream form exists primarily within the mammalian circulatory system and tissue spaces, invading the central nervous system in late stage infections. In the insect form a complex maturation accompanies the development of infective forms within the tsetse; the proliferative procyclic form is located within the midgut. Several additional forms of the parasite have been described, but very little is known concerning their trafficking ability and they are omitted for simplicity. Experimentally accessible stages are shown in black, together with a number of characteristic features of the stage (box). Other life stages are shown in grey. Hosts are shown iconized; the tsetse portion of the life cycle is on the left and that in humans and other mammals on the right.

a comparison with other parasites and hosts. In a synergistic manner the development of very robust tools for RNA interference (9) has surmounted a number of technical obstacles to working with trypanosomes. In this review we will consider some recent advances in studies of the trypanosome membrane trafficking system. In particular, we will emphasise progress towards answering two major questions. First, what does the configuration of the membrane trafficking apparatus in a highly divergent eukaryote tell us about the essential core functionality required by all eukaryotic cells? Second, how has the trypanosome transport system evolved to facilitate the completion of the life cycle in multiple host environments?

The Pathways from Genomics

There are several productive ways in which to initiate molecular level studies of the trafficking system of a poorly defined eukaryote. The first step of any strategy must be to define the compartments by the use of molecular markers. These markers must satisfy a number of criteria; specific in location, be integral to the transport process, be experimentally amenable, have a comprehensive involvement in trafficking pathways and be readily identifiable from primary structure. The Rab family of proteins meets all these criteria. Rab proteins are central control elements for many specific steps in vesicle transport, and moreover exhibit a high degree of specificity in their subcellular localisation.

In silico analysis of the completed *T. brucei* genome indicates that there are 16 members in the *T. brucei* Rab family and five ARF proteins. Ten trypanosome Rab proteins have been experimentally characterised. Two show evidence of a divergent location (Rab23 and Rab18) when compared with their metazoan orthologues (10,11), confirming that, for the most part, sequence orthology is a reliable predictor of function for this gene family. There is clear evidence for a nuclear role for Rab23 and potentially Rab28 (10); this is at variance with recent data for Rab23 in metazoans, which is endosomal (12).

A comparison of the number of Rabs in trypanosomes, yeast and mammals makes it possible to estimate the level of complexity of the processes mediated by them. Trypanosomes are by far the most divergent eukaryote to be characterised in such detail, and these findings indicate a very ancient origin for the basic overall form of the endocytic and exocytic system. A conserved core of Rab proteins is also found in the apicomplexan *Plasmodium* (13). *Saccharomyces cerevisiae* has 11 Rab proteins, whereas trypanosomes have 16, indicating the potential for a more complex and highly regulated vesicular transport system. The major exocytic (Rab1, 2 and 6), endocytic (Rab5, 7), recycling (Rab4, 11) and *trans*-Golgi-associated (Rab11,14) Rab proteins are represented in the trypanosome genome, and the majority have been shown to possess analogous functions (14–20). Trypanosomes possess two Rab1 homologues and one Rab2 homologue, which are involved in early exocytic transport of proteins (21), making them substantially more complex than yeast in this regard. This is likely indicative of a loss of function by the fungal system. Several Rab functions appear absent. For example, there is no evidence for a Rab3 homologue functioning in regulated secretion, and biochemical studies agree. In contrast, several trypanosome Rabs (X1,X2 and X3) have unique functions. Two of these, RabX1 and X2, are associated with the

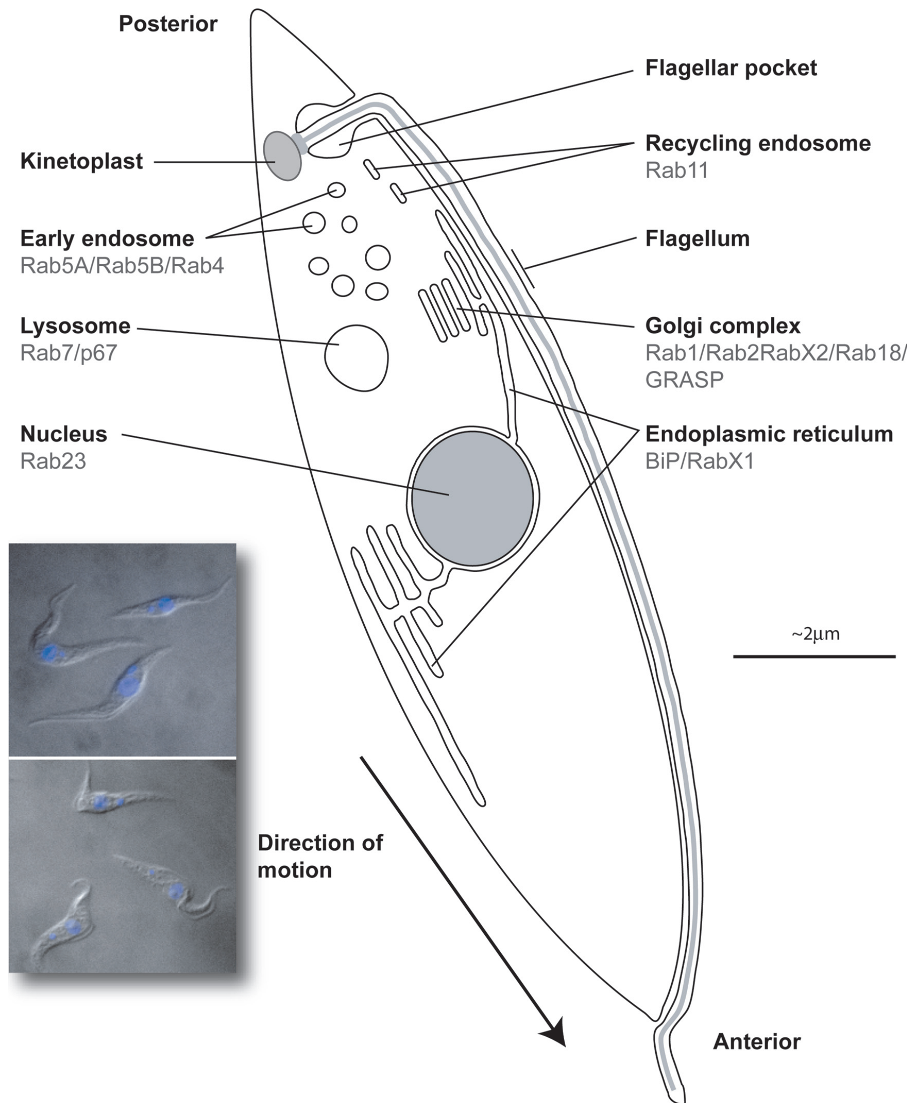


Figure 2: Schematic of a trypanosome cell highlighting the positions of various intracellular organelles and their associated marker proteins. *Trypanosoma brucei* is a spindle-shaped cell approximately 15 μm $\times$ 4 μm. A single flagellum emerges from the posterior of the cell through the flagellar pocket, but remains associated with the plasma membrane. The majority of the membrane transport pathway is confined to the posterior of the cell. The inset shows phase contrast images of procyclic form parasites, stained with DAPI to visualise the DNA (blue); note the presence of the nucleus plus a small extranuclear spot, which is the mitochondrial DNA and is called the kinetoplast. In this life stage the distance between the kinetoplast and nucleus is less than in the bloodstream stage.

ER and Golgi complex, respectively (15,22), and may control a process that is specific to the kinetoplastida. The initial characterisation of the trypanosome Rab protein repertoire is nearing completion; the way is now open for a more detailed analysis of the integration of these small GTPases in the control of trafficking.

More recently, a second strategy for charting endocytic pathways has become available through the genome project by examining the coat proteins encoded by the trypanosome genome. The 'greater' adaptin family is well represented; COP-I and three adaptin complexes (AP-1, 3 and 4) are encoded by the genome, whilst experimental evidence confirms the presence of COP-I (47), AP-1 (26), AP-3 and AP-4 (C.L. Allen and M.C. Field, unpublished data). The absence of AP-2 is a secondary loss as this complex is found in the related kinetoplastida *Leishmania* and *T. cruzi* (see below). Interestingly, neither the GGA family nor the stonins are present in trypanosomatids; these appear to be comparatively late additions to the adaptin repertoire, and their emergence probably post-dates the separation of the Trypanosoma from the higher eukaryotes (23). By contrast, the adaptins and COP-I are ancient gene families. The above data suggest that the extreme complexity that appears to be present at the *trans*-Golgi in mammals may be absent from trypanosomes, only the core adaptin-mediated systems being present. Subunits of the COP-II coat are also encoded by the genome, but at present no experimental evidence for their function is available. Therefore, *T. brucei* possesses a comparatively minimal adaptin/coatomer set comprising COPs I and II and AP-1, 3 and 4.

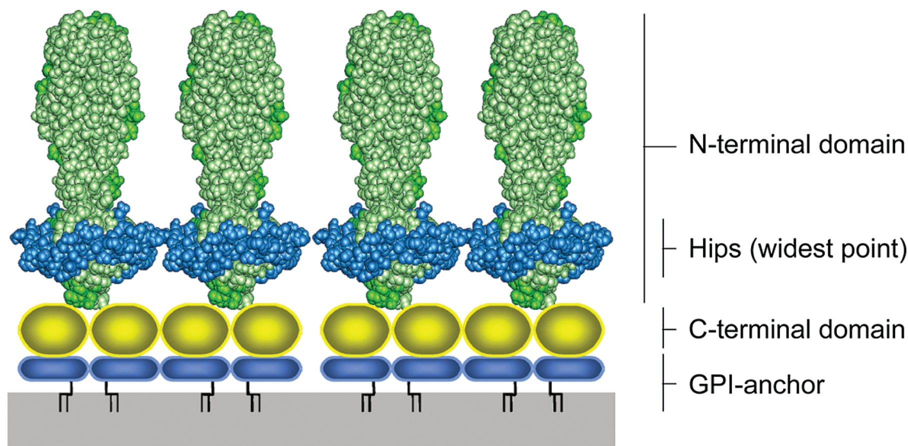
Recent data indicate that in *T. brucei* the large GTPase dynamin is not required for the fission of vesicles in the secretory or endocytic pathway. Only two near identical dynamin homologues are encoded by the genome, and functional analysis indicates that the proteins are involved in mitochondrial biogenesis and not endocytosis (24). Several evolutionarily widely represented factors are absent.

For example, there appears to be no caveolin gene in trypanosomes, and expression of mammalian caveolin results in mistargetting of the transgene (G.W. Morgan and M.C. Field, unpublished), suggesting that the machinery for caveolin targeting is either absent from trypanosomes or highly divergent.

What emerges from these studies is that in trypanosomes, a conserved core functionality is maintained. The secondary loss of some factors (e.g. AP-2) suggests specialisation rather than simplicity, whilst the absence of some higher eukaryote factors (GGAs and stonins) provides graphic evidence for lineage-specific development and elaboration of the endomembrane system. The absence of a dynamin activity involved in endocytosis clearly begs the question of how endocytic vesicle fission is achieved in this system. Further, the absence of an AP-2 may indicate a fundamentally different solution to endocytosis.

The Exocytic System

T. brucei has one very clear feature that distinguishes it from the vast majority of eukaryotes; the cell surface is dominated by GPI-anchored proteins as opposed to transmembrane domain (TMD) proteins. The homogeneity of the *T. brucei* cell surface is extreme as the plasma membrane is covered by $\sim 5 \times 10^6$ homodimers of a single GPI-anchored protein, VSG. The surface is saturated with a monolayer of VSG, precluding immune recognition of less abundant invariant surface determinants (Figure 3). The VSG represents $\sim$10% of total cell protein and $\sim$90% of total cell surface protein. A similar situation occurs in procyclic cells that express 5×10^6 copies of procyclin on their surface. One consequence of the abundance of these GPI-anchored molecules and their dominance of the trypanosome surface is that the bulk protein cargo within the exocytic system is essentially a single polypeptide species – the consequences of this somewhat flat secretory landscape for ER chaperone function, for example, have not yet been explored. A second consequence arises from the consideration of growth rates and expression levels of VSG and procyclin. As both bloodstream and procyclic stages proliferate at approximately equal rates in culture, are of comparable sizes and hence surface areas, and also synthesize similar levels of GPI-anchored proteins, the overall synthesis and export rates for surface proteins must remain relatively constant. Finally, the abundance of GPI-anchored proteins has led many authors to speculate that the trypanosome



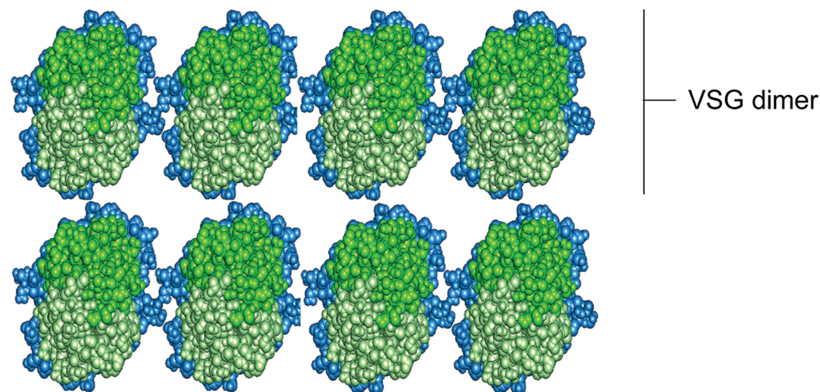


Figure 3: Model of the surface of the bloodstream form trypanosome. Both an elevation and a plan view are shown. The surface is essentially a monolayer of VSG molecules, which exist primarily as stable homodimers and are shown as space-filling models based on the X-ray crystal structures of the N-terminal region and schematically for the remainder of the molecule for which the three-dimensional structure is not available (45). The density of the VSG is an accurate representation of the *in vivo* situation (see 46 for a detailed discussion of this topic). The plasma membrane lipid bilayer is represented by a grey rectangle. In the plan view, the two monomers, shown in different shades of green, are more apparent. Significantly, despite substantial primary structural variation, all VSGs appear to share a common tertiary structure (45).

secretory system has novel features. So far there is little evidence to suggest a major divergence between trafficking mechanisms within the exocytic arm of the trypanosome and higher eukaryotes, but this part of the trypanosome endomembrane system has received little direct attention.

The abundance of the VSG has enabled the kinetics of exocytosis to be determined. VSG is synthesised as a 56 kDa precursor and rapidly modified to a 58 kDa form within the ER ($t_{1/2} < 2$ min) as a result of N-glycosylation and GPI-addition (25). The 58 kDa form is elaborated to a 59 kDa mature polypeptide with a $t_{1/2}$ of ~ 13 min by maturation of both the N- and GPI-glycans within the Golgi complex, followed by rapid delivery to the surface ($t_{1/2} \sim 14$ min). VSG also rapidly dimerises within the ER. Sorting of VSG at ER exit sites is highly efficient: the concentration of VSG at the cell surface is 50-fold that in the ER, indicating a powerful concentration mechanism within the exocytic pathway (26).

Extensive slasher mutations, predicted to affect the secondary structure of VSG, result in decreased expression and little or no surface transport. This is consistent with the operation of an efficient ER quality control (QC) system (27). Mutation of N-glycan addition sites indicates that N-glycosylation is required for stable expression (21). VSG mutants that are defective for GPI addition and retain their C-terminal signal sequence are also unstable and fail to be efficiently exported from the ER (28). Removal of the C-terminal GPI-signal from VSG results in slowed exit from the ER and possible delivery to the lysosome (29). Lysosomal involvement in degradation of poorly exported VSG mutants was also suggested (27). It is unclear how the VSG mutants progress from the ER to the lysosome, but these studies may suggest the presence of a second QC system later within the exocytic pathway. A similar post-ER QC system is present within higher eukaryotic cells. This area is deserving of more direct investigation as the analysis of a system that is presumably optimised for the synthesis and export of a single GPI-anchored species has the potential to provide novel insights into GPI-specific trafficking mechanisms.

In mammalian cells, GPI-anchored proteins are sorted into detergent-resistant membranes (DRMs), or rafts, during transit through the medial-Golgi complex, whilst in yeast, incorporation into rafts occurs earlier (30). DRMs have been described in *T. brucei*, although the function that these assemblies has in exocytosis, if any, remains unclear (31). During export from the ER in higher eukaryotes, both GPI and TMD proteins are initially recruited into COP-II-coated transit vesicles. There is a significant degree of specificity in this process and for yeast some evidence suggests that GPI and TMD proteins are packaged into distinct populations of vesicles (32). In yeast the segregation of GPI and TMD proteins depends on Ypt1p, the tethering factor Uso1p, several members of the SNARE family (33), Lag1p and Dgt1p (34). Orthologues of all of these factors are present in trypanosomes. In mammals there are two Rab proteins associated with ER to Golgi transport, Rab1 and Rab2. Trypanosomes also contain Rab1 and Rab2 homologues; these are the most conserved Rab proteins in the trypanosome genome, suggestive of a strong selective pressure on this particular aspect of the secretory pathway. Trypanosome Rab1 can complement ypt1 and both TbRAB1 and TbRAB2 are correctly targetted to the ER/Golgi in mammalian cells (21). Further, RNAi indicates that both of these Rab proteins are required for export of VSG. This study represents the only detailed mechanistic analysis of this step in the exocytic pathway, and no evidence for selective transport was obtained. Perhaps with an exocytic system already full of GPI-anchored proteins, there is no need for sorting away from a minor TMD population.

There is no suggestion that the exocytic rate and pathway is altered between the two proliferative stages. In contrast, there are multiple lines of evidence for developmental regulation of endocytosis. One morphological difference, however, is that the Golgi apparatus is rather more elaborate in the bloodstream form than in the procyclic form. This may reflect a change in glycan processing; bloodstream form cells synthesise complex-class N-glycans, whereas procyclic forms do not maturate their N-linked carbohydrates beyond the oligomannose stage (35,36).

Endocytosis and Recycling

The observation that endocytosis in trypanosomes is developmentally regulated is a comparatively old one, and the very high rate of endocytic transport in the bloodstream form has been known for decades. Direct measurements of endocytosis in the procyclic form are all consistent with low activity (18,20). Evidence for a role for endocytosis in the turnover of bloodstream form surface ligands can also be found in the early literature (37). In particular, the potential for participation in clearance of immunoglobulins that recognise surface VSG was noted (7).

More recent quantitative evidence for an active uptake of IgG–VSG immune complexes, the degradation of the IgG and return of VSG intact to the surface was described by Voorheis and co-workers (38). In this work it was demonstrated that IgG was rapidly degraded within 30 min, but the location of the IgG within the cell was not defined. Subsequently, anti-VSG IgG was found in compartments that co-stained for TbRAB5A and TbRAB11, highly suggestive of an active recycling pathway (14). Further, following fluorescently tagged IgG cycled through living trypanosomes that were expressing mutant isoforms of various Rab proteins, it was confirmed that TbRAB5A and TbRAB11 are indeed involved in the recycling of immunoglobulins and transferrin. Interestingly, transferrin was recycled rather faster than immunoglobulin in this assay

(18). Further, this study confirmed earlier observations of a highly active proteolytic system within the endocytic pathway of *T. brucei*; IgG and transferrin were degraded, with ~70% of both proteins reduced to short peptides containing at most four amino acids. Most significantly, this material was efficiently secreted, indicating that substantial degradative capacity is located within part of the active recycling system, as opposed to a terminal endocytic compartment (18). When considered together, these observations suggest that the recycling system has the potential to act as a system for the removal of surface immune complexes, and therefore may be an adjunct mechanism for immune evasion. Degradation of transferrin has no obvious function, and may be the result of essentially nonspecific degradative capacity within the recycling system. However, some degree of specificity most likely exists, as VSG, which is highly sensitive to proteases *in vitro*, is returned to the surface essentially intact (38, 39). It is not clear at present whether differential degradation is the result of distinct intracellular itineraries for VSG and IgG, or reflects the evolution of specific proteases. Certainly, a recent elegant study using fluorescent-labelling of surface VSG and following a cohort through the recycling system suggested that VSG is recycled much faster than IgG (39). This may indicate a short recycling pathway for VSG and a longer path for IgG, or alternatively that membrane is recycled more rapidly than soluble luminal contents. Clearly, there is a lot still to determine about this important pathway, including the similarities and differences in the VSG and IgG itinerary, the location where the immune complex components are presumably separated, and also the identity of the proteases within the recycling system.

How is the mainly GPI-anchored surface internalised? As described above, trypanosomes do not encode a caveolin homologue or an AP-2 complex. Also, the two near identical dynamin-like proteins do not appear to participate in endocytic events (24). There are clathrin-coated pits at the flagellar pocket but also apparently noncoated invaginations (8), suggesting the possibility that there is more than one mechanism for endocytosis from the trypanosome surface.

A resolution for these issues was recently provided by elegant ultrastructural work using high pressure freezing to preserve membrane structure (40). This work definitively demonstrated the presence of VSG in clathrin-coated pits located at the flagellar pocket, based on immunogold co-localisation. Significantly, the density of VSG within the clathrin-coated pits is similar to that on the bulk plasma membrane, indicating the absence of any significant concentration during endocytosis. However, given the extreme density of VSG on the surface, further concentration is difficult to envisage, and this probably explains the secondary loss of the AP-2 complex from this organism. Further, following a cohort of surface-biotinylated VSG as it progressed through the recycling system, a second clathrin-dependent transport step was identified. In this case, internal complex elongated membrane tubules were rapidly filled with VSG, which was concentrated within the central regions of these structures. Clathrin buds could be observed on the periphery, but these were devoid of VSG (40). The tubules probably correspond to TbRAB5A early endosomes (20, 39) and are actively sorting VSG. As the trypanosome expresses an AP-1 complex (C.L. Allen and M.C. Field, unpublished data), it is possible that the clathrin buds on these endosomes are actively engaged in the sorting of non-GPI-anchored proteins from VSG. Hence in this system the concentration mechanism for internalised molecules has been relocated from the plasma membrane to the early endosome. At later times, VSG is found in disc-shaped structures at high density; these structures are also positive for TbRAB11 and rapidly fuse with the flagellar pocket (26). Clearly, these TbRAB11 recycling endosomes are the origin of the nonclathrin-coated membrane profiles that are associated with the flagellar pocket.

The absence of a caveolin homologue and the presence of the major surface protein in clathrin-coated vesicles suggest that this may be the major mechanism by which proteins enter the trypanosome endocytic system. However, there are numerous examples of proteins that are able to use alternative mechanisms in the absence of clathrin. The possibility that clathrin represented the sole endocytic route was tested by RNA interference (41). In contrast to yeast and metazoan systems, clathrin ablation is lethal. Remarkably, cells with suppressed clathrin expression contained a grossly enlarged flagellar pocket that presumably arose from an imbalance between exocytic mechanisms that are still active and endocytosis that is compromised. Essentially no endocytic activity could be detected in these cells, whilst export of VSG to the cell surface was normal. These data suggest that all endocytosis is clathrin-mediated. Interestingly, in a recent study of actin function in trypansomes, a very similar phenotype was observed for actin RNAi compared to the clathrin RNAi, including loss of uptake of specific markers and enlargement of the flagellar pocket (42). Therefore in trypanosomes, endocytosis is actin- and clathrin-mediated but dynamin- and AP-2-independent.

By contrast to the single mechanism underpinning endocytosis, at least two endocytic routes have been identified in the bloodstream form trypanosome. Specifically, the two Rab5 isoforms, TbRAB5A and TbRAB5B, localise to distinct endosome populations that contain different cargo molecules (VSG, transferrin receptor and ISG_{65} in the case of TbRAB5A, and ISG_{100} in the case of TbRAB5B) (20,48). Both of these routes are essential, as RNAi of either Rab5 isoform is lethal and results in an enlarged flagellar pocket (43). Further, RNAi of TbRAB5A or TbRAB5B decreases the cellular levels of clathrin; hence both pathways are indeed clathrin-dependent. Interestingly, the situation in the procyclic stage is different: TbRAB5A and TbRAB5B are co-localised

and both TbRAB11 and clathrin expression are down-regulated. These observations indicate both a simplification of the endosomal population and presumably changes to gene expression that result in decreased activity.

Recycling must represent the major pathway for addition of membrane to the flagellar pocket in the bloodstream form trypanosomes. Quantitation of the rate of endocytosis indicates that the trypanosome is able to internalise membrane equivalent to the surface area four times an hour (39). As the bloodstream cell divides once every 8 h, the contribution of biosynthetic exocytosis to membrane deposition at the surface must be $\sim$10% of the total. This also indicates that the vast majority of VSG inside the cell is in the process of being recycled, and is not newly synthesised VSG. Interestingly, the recycling and biosynthetic routes to the surface appear to be distinct, as RNAi ablation of TbRAB11 has no effect on delivery of VSG to the surface (B.S. Hall and M.C. Field, unpublished data). Analysis of TbRAB4 function, which in higher eukaryotes is involved in a fast recycling pathway, suggests little participation in recycling events but a major role in degradative trafficking to the lysosome (17). Thus, TbRAB11 mediates essentially all recycling in bloodstream form cells. The situation in procyclic forms is different; TbRAB11 expression is reduced and TbRAB4 may contribute significantly to recycling (B.S. Hall and M.C. Field, unpublished data).

Endocytosis Signals in Transmembrane-Domain Proteins

GPI-anchored VSG is endocytosed from the flagellar pocket without being concentrated. In the absence of an AP-2 complex, how then are TMD proteins separated from the huge excess of VSG that is taken into the cell? A major TMD-containing surface protein family is the invariant surface glycoproteins (ISG), a group of type I membrane proteins with some structural similarities to VSG (44). ISGs comprise a number of families, and one family, ISG_{65}, has been analysed in some detail. The cytoplasmic C-termini of these proteins are quite short, but all contain a number of conserved lysine residues. ISG_{65} is found on the cell surface as well as in intracellular compartments, some of which co-stain with TbRAB5A, indicating an endosomal location. Recent data indicate that the lysine cluster in the C-terminus of ISG_{65} is required for endosomal localisation as mutations of the lysines to alanine results in increased surface expression and represents the first putative targetting signal to be identified in a trypanosome TMD protein (48). It is possible that the lysine residues are sites for ubiquitination, facilitating recognition of the modified protein by one more of the endocytic factors known to contain a ubiquitin-recognition motif. Such a mechanism may provide an alternative to adaptin-mediated transport.

Unanswered Questions and Issues for the Future

We are now in the position of knowing the major trafficking pathways occurring in trypanosomes and have identified most and characterised many of the orthologues of factors involved in endo- and exocytosis in mammals and yeast. Where next? Most probably, the key to understanding the role of the high level of endocytic activity in the bloodstream stages will come from an elucidation of regulatory mechanisms. The correlation between a high endocytic rate and a sophisticated adaptive immune system is compelling, especially when coupled with observations of the potent degradative capacity within the recycling system. It is possible that such a mechanism predates antigenic variation, and represents a primitive, but effective, strategy for prolonging persistence within a vertebrate host. However, the correlation between endocytic rate and the presence of an adaptive immune system does not equate to causality, and one authenticated exception would effectively scupper this model. Understanding the regulation of endocytosis will facilitate an overview of how the rate is increased so dramatically in bloodstream forms and hopefully will also identify activities that can be modulated, to allow a proper *in vivo* test of the importance of the system for persistence.

In a related area, it has only just become apparent that actin likely plays a very important role in endocytosis in trypanosomes (42). Whilst this is similar to the situation in higher eukaryotes, there are many potential differences in the manner in which actin functions in the trypanosome. The protein partners required to connect actin to, for example, Rab5, as recently reported for higher organisms, have not directly been identified so far in trypanosomes. Studies of both actin and trypanosome Rab-interacting proteins will be of interest here and, for the latter at least, will require direct experimental evidence as genome searches reveal limited homology for Rab-interacting proteins. Unique, or unusual, aspects of the trypanosome system have recently been described, and further such features can be anticipated. These include defining the functions of the protozoan-specific Rab proteins, such as X1, X2 and X3, and understanding the evolutionary significance of distinct functionality in sequence orthologues, as seen for mammalian Rab 23 (endosomal) and trypansome Rab23 (nuclear).

A most pressing question remains; can these findings be translated into therapy? The very clear essential nature of clathrin/Rab-mediated endocytosis, which we presume is due as much to cellular architecture as it is a true functional requirement by virtue of a restricted ability to add membrane to the bulk surface, provides an opportunity to turn the tables on the parasite and to exploit the flagellar pocket as a means to eradication. At present, none of the characterised factors we have available are suitable candidates for the development of therapies – highly conserved multigene families such as Rabs, clathrin or actin do not make

appealing targets. Deeper characterisation of the system may identify such factors, and is ongoing.

An Afterthought: No Apologies

Cobain was 'All Apologies', and many authors seem compelled to make similar attempts to justify their work on protozoan parasites. Protozoa are unglamorous to many, due to technical difficulties and a lack of immediacy to our scientific agendas. With a completed genome and excellent RNAi technology, trypanosomes are now much more accessible, but we must never forget why these organisms are so important. Trypanosomatids kill hundreds of thousands of people every year and are responsible for much of the poverty that is all too apparent in many regions of the globe. In a very tangible manner, parasitic protozoa exert a huge influence on the geopolitics of Africa. Our deep ignorance concerning the fundamental aspects of parasite cell biology, life cycles and interactions with mammalian hosts coupled with a poor understanding of their epidemiology stands in stark contrast to spectacular successes in postgenomics, integrative biology and other preoccupations of developed world scientists. For wishing to redress this imbalance, to provide an insight into the lifestyles of these mass killers, and perhaps also to provide a broader insight into the eukaryotic lineage, we respectfully make no apologies.

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