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Review

Intracellular Trafficking in the Trypanosomatids

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Trypanosomes are members of the kinetoplastida, a group of divergent protozoan parasites responsible for considerable morbidity and mortality worldwide. These organisms have highly complex life cycles requiring modification of their cell surface together with engagement of immune evasion systems to effect survival; both processes intimately involve the membrane trafficking system. The completion of three trypanosomatid and several additional protist genomes in the last few years is providing an exciting opportunity to evaluate, at the molecular level, the evolution and diversity of membrane trafficking across deep evolutionary time as well as to analyse in unprecedented detail the membrane trafficking systems of trypanosomes.

Key words: endocytosis, immune evasion, *Leishmania*, trypanosomes, vesicle transport

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Trypanosomatids represent important pathogens that are transmitted by insect vectors, with the majority of the human population potentially exposed to one or more species. The three model organisms that have been most extensively studied are *Trypanosoma brucei*, the causative agent of African sleeping sickness, *Trypanosoma cruzi*, responsible for Chaga's disease, and *Leishmania major*, which causes Leishmaniasis. *Trypanosoma brucei* persists extracellularly in the bloodstream of the infected mammalian host, whereas *T. cruzi* invades a wide range of cells, preferring smooth and cardiac muscle cells, while *Leishmania* principally infects macrophages (Figure 1A). Excellent reviews on the current states of management, treatment and disease burden are available; these aspects of trypanosomatid biology will not be considered further here.

Trypanosomatids are highly divergent protozoa and provide an excellent opportunity for exploration of evolutionary biology and examination of specialised mechanisms present in pathogenic protists and divergent systems. Membrane trafficking plays a crucial role in life cycle progression, infectivity and immune evasion of these pathogens. A recent review in this organ focusing on transport in the African trypanosome (1) provides a primer for much of the relevant background. Hence, the present article focuses on novel data from the last couple of years with a special focus on data gleaned from the recently completed genomes.

Trypanosomatid cells adopt a wide range of morphologies during the life cycle, but all feature a subpellicular microtubule array that serves to maintain an elongated spindle shape. The cell also features a flagellum, which emerges from the parasite body through a structure termed the flagellar pocket – the pocket is a small invagination whose membrane is maintained as a distinct domain. In some trypanosomatids, including *T. brucei*, the flagellum is attached to the cell body, while in others, such as *Leishmania*, the flagellum is free. The majority of the expected endomembrane machinery has been identified in these organisms, including the endoplasmic reticulum (ER), a stacked Golgi complex, and several endosomal compartments (see 1 for more details). Trafficking in trypanosomatids is highly polarised, with all plasma membrane transport directed through the flagellar pocket (2,3). Further, as molecular markers have become available it is now clear that the exocytic and endocytic compartments are highly polarised, and, with the exception of the ER that is distributed throughout the cell, endomembrane compartments are restricted to the region of the cell between the nucleus and the kinetoplast (4,5) (Figure 2). In *T. brucei*, immune evasion is a dominating facet of cell biology, likely comprising both antigenic variation and efficient mechanisms for endocytosis of immune effectors. By comparison, *T. cruzi* and *L. major* do not exhibit antigenic variation or have especially active endocytosis, but do present a role for secretion of factors from the cell in disease progression, a feature not seen in *T. brucei*.

All three organisms have surfaces dominated by glycosylphosphatidylinositol (GPI)-anchored antigens (Figure 1B, Table 1). The surface of *T. brucei* bloodstream stage is dominated by the variant surface glycoprotein (VSG); sequential expression of antigenically distinct variants is the principal mechanism of antigenic variation (6). In the insect form (procyclics), VSG is replaced by procyclin (7). In *T. cruzi*, the major surface antigens are a family of extensively *O*-glycosylated mucins and a GPI-based lipid, lipophosphopeptidoglycan (LPPG). *Leishmania* presents a surface dominated by lipophosphoglycan (LPG), a surface

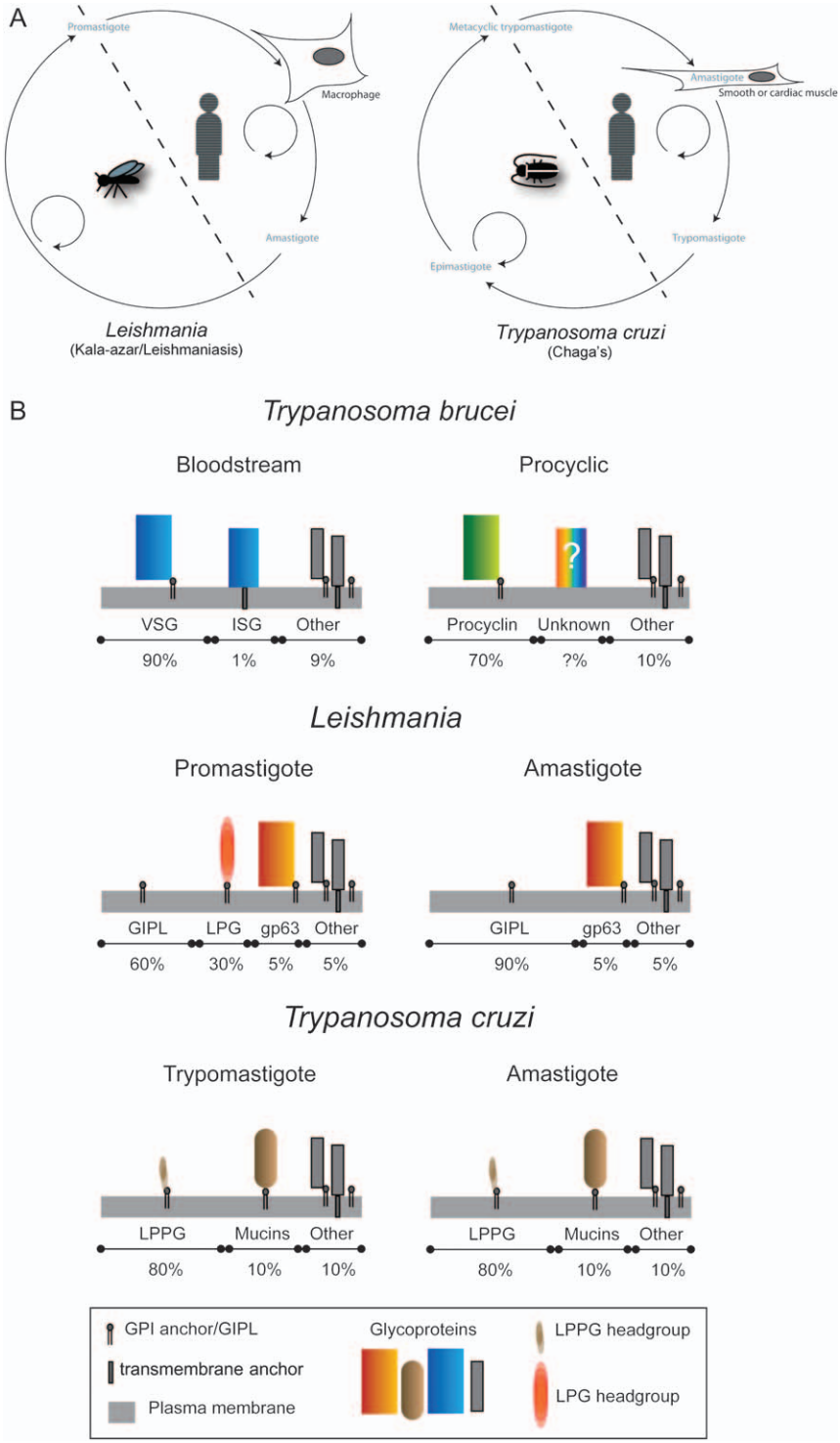

Figure 1: Legend on next page.

protease (gp63) and a family of GPI lipids, glucoinositol phospholipid (GIPLs) (8). *Leishmania* and *T. cruzi* also differ from *T. brucei* in having an established role for secretion of factors from the cell in disease progression – the African trypanosome does not secrete detectable levels of macromolecules. These distinct surfaces imply a differential burden on the exocytic system, for example in expression of glycosyltransferases and chaperones. For degradation, turn over and maintenance of the surface, the endocytic system is the major mechanism (9).

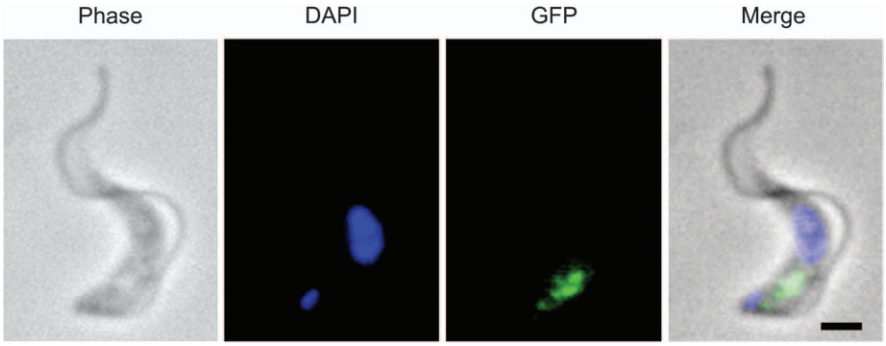

Figure 2: Polarised location of major trafficking pathways in *T. brucei*. The images show a bloodstream form trypanosome that has been genomically tagged at the clathrin light chain locus with GFP at the C-terminus to create a CLC-GFP-fusion protein. CLC is present on the flagellar pocket membrane, the Golgi complex and also endosomal compartments. Shown is a cell stained with anti-GFP (green) and counterstained with DAPI to visualise the nucleus and kinetoplast. Note that the stain is concentrated in the region between the nucleus and kinetoplast, the latter subtending the flagellar pocket. Scale bar 1 μm.

Genomics and Trafficking

Completion of the genomes of *T. brucei*, *T. cruzi* and *L. major* allows *in silico* comparisons of the endomembrane systems between these parasites and other organisms (10), and together with experimental advances in *T. brucei* and *Leishmania* we now have an improved map of transport pathways within kinetoplastids. In *T. brucei*, current models suggest a simple, exclusively clathrin-dependent/dynamin-independent endocytic and recycling system with three major arms; lysosomal targeting and two recycling pathways mediated by Rab4 and Rab11 (Figure 3) (1,11,12). This system contrasts to higher eukaryotes where at least five endocytic modes are recognised (13), and potentially places additional burdens on the parasite with limited scope for selective uptake as all sorting must be achieved at internal sites and not at the initiation of endocytosis. A recent report suggests that dynamin may be involved in endocytosis in the *T. brucei* procyclic form and suggests distinct mechanisms in the two life stages (14). Experimental evidence for exclusive reliance on clathrin has been extended through genome mining as caveolin and flotillin, proteins mediating clathrin-independent endocytosis, are restricted to higher eukaryotes (15).

Trypanosome genomes encode approximately 20 Rab proteins. The core Rab repertoire associated with basic endocytosis and exocytosis is fully conserved, but additional Rab proteins are also present, including Rab 23/28 (all three trypanosomatids), Rab14 (*T. cruzi* and *Leishmania*) and Rab32 (*T. cruzi*). A group of three trypanosomatid-unique Rabs (X1, X2 and X3) are present in all three systems; their functions have not been fully elucidated (10). Interestingly, in *T. brucei* a total of 14 TBC domain-containing open-reading frames are present in the genome, several of which interact with specific Rab proteins (A. O'Reilly, Carme Gabernet-Castello and Mark C. Field, unpublished data); as the Tre-2/Bub2p/Cdc16p (TBC) domain is exclusively associated with Rab GTPase activating proteins (GAP), these data suggest that there is promiscuity within the Rab–RabGAP interaction network

Figure 1: Simplified life cycles and surface architectures of parasitic trypanosomatids. Panel A, left: *Leishmania* parasites are transmitted by sandflies between mammalian hosts. While there is a strong relationship between the sandfly and *Leishmania* species, for the mammalian host the parasite is widely infectious. Flagellated metacyclic promastigote forms are injected into the mammalian host, which rapidly infect macrophages. There the parasite differentiates to the non-flagellated amastigote and replicates. Amastigotes are able to infect additional macrophages following lysis, and the life cycle is completed when a sandfly takes a blood meal that contains infected macrophages. Right-hand panel: *T. cruzi* is transmitted by reduviid beetles; the insects inject bloodstream form trypomastigotes that invade a wide variety of cells, with preference for smooth muscle tissues. Within these cells, the parasite differentiates to an amastigote. Lysis releases trypomastigotes to the bloodstream and it is these forms that are taken up by the reduviid bug again for completion of the life cycle. A period of amplification as an epimastigote within the insect vector serves to generate large numbers of infectious trypomastigotes. Small circles represent rapidly replicating stages. See Field and Carrington (1) for a similar representation of the *T. brucei* life cycle. Panel B: Extensive differences both between trypanosome species and life stages dominate the output of the trafficking systems. Cartoon versions of the surfaces of major life stages of the three principle trypanosomatid parasites. These surfaces are dominated by GPI-anchored antigens, which vary from small glycolipids (GIPLs), through glycoconjugates (LPG) to true glycoproteins (VSG, gp63, mucins). Note that while the surfaces of the two principle stages of *T. cruzi* appear identical, the structure of the mucins is altered. The approximate proportion of total surface antigen (excluding phospholipid) is also indicated. These cartoons are highly idealised. Unknown refers to a recently described structure on the surface of insect stage trypanosomes.

Table 1: Some commonly studied secretory pathway and surface antigens of kinetoplastida parasites

Name (MW)[a]	Location	Function	Topology/ anchor	Targeting signal	Copy number protein/gene
Trypanosoma brucei					
p67 (150)	Lysosome	Presumed to be LAMP like; extensive *N*-glycosylation and progressive proteolysis	Type I	Dileucine related	NK/2
CRAM (200)	PM – FP only	Possible lipoprotein receptor; highly repetitive sequence	Type I	Cytoplasmic endocytic targeting signal	NK/1
ISG65 (65)	PM/endosome	Unknown; possible receptor; partially endosomal; rapidly turned over	Type I	Lysine/Ub[b]	10^5/~6[c]
ISG75 (75)	PM/endosome	Unknown; possible receptor; partially endosomal; rapidly turned over	Type I	Lysine/Ub[b]	10^5/~6[c]
VSG (60)	PM	Immune evasion; highly stable	GPI	Probably none	10^7/1[d]
ESAG6/7 (65)	PM – FP only	Transferrin receptor; unrelated to metazoan receptor	GPI	Not known	10^3/NK/1[e]
Procyclin (30)	PM	Surface coat; unknown role; highly repetitive polypeptide; extensive GPI glycan	GPI	Not known	10^7/6[c]
Leishmania major					
LPG (15)	PM	Surface coat; sandfly interaction; possible role in mammalian infection	GPI	Probably none	10^6/NA[f]
gp63 (65)	PM	Cell invasion; defence	GPI/type I	Not known	10^5/Variable[c]
GIPL (1.5)	PM	Surface coat; implicated in virulence	GPI	None	10^7/NA
Trypanosoma cruzi					
LPPG	PM	Surface coat; virulence	GPI	None	10^7/NA
Mucin	PM	Surface coat; virulence; display of sialic acid	GPI	None	10^7/100s

[a]Approximate molecular weight (MW) of mature protein, in kiloDaltons.
[b]Protein can become ubiquitylated on cytoplasmic lysine residues. For ISG75 this is assumed based on ISG65 data.
[c]Variable number between subspecies and strains.
[d]One gene only is active at any one time; there are over 1000 transcriptionally silent genes in the genome.
[e]The receptor is a heterodimer; one gene per subunit is active at any time, but there are many silent copies.
[f]NA, not applicable. Molecule is a glucoconjugate and hence the product of multiple genes.
[g]It is not known how many genes are expressed at one time.

similar to *Sacharomyces cerevisiae* with 11 Rabs and six TBC GAPs. Despite levels of conservation of the core machinery that governs transport in these systems, downstream effectors of Rab GTPases such as tethering factors have proven difficult to identify due to their diversity (16). This likely reflects both adaptive pressures placed upon these organisms to meet the demands of specialised host environments as well as a deep divergence due to early separation from model systems.

The SNARE family has only been experimentally investigated in *Leishmania*. Twenty-seven SNARE candidates have been identified, although sequence conservation is poor (17). The study did not identify SNAP-25 or brevins of the R family and hence there may be additional *Leishmania* SNAREs. Analysis of the *T. brucei* genome identifies 20 candidate SNAREs, also likely an undersampling. These sequences represent SNARE families implicated in transport to early and late endosomes, intra- and post-Golgi transport and ER to Golgi trafficking. In common with *Leishmania* there are several SNAREs that cannot be assigned to a higher eukaryote SNARE family and may indicate the presence of novel transport steps. The size of the trypanosomatid SNARE families compares well with *S. cerevisiae* (24 SNAREs), indicating that trypanosomes possess a relatively complex trafficking system, also implied by their repertoire of Rab proteins.

By contrast the repertoire of vesicle coat proteins in trypanosomes appears limited. The two principal coatomer systems are fully represented, including two copies of genes for Sec13p and Sec23p, suggesting potential heterogenetity in ER-derived transport vesicles. There is good evidence that only the tetrameric adaptor protein (AP) complexes have wide evolutionary distributions and the GGA and stonins are only found in the Opistokhonta (15). Further, both *T. brucei* and *Leishmania* lack one AP complex, AP2 or AP3, respectively. The absence of AP2

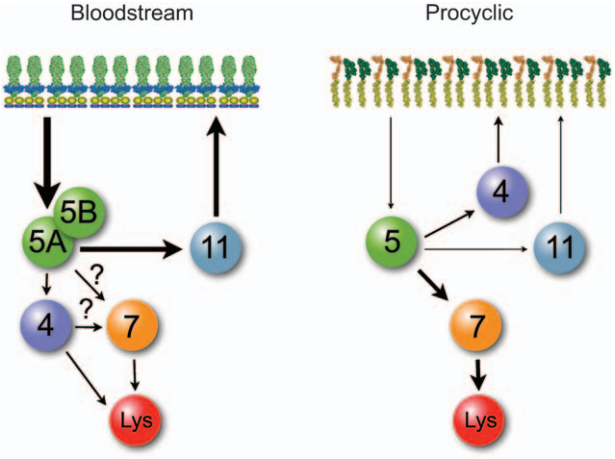

Figure 3: Developmental alterations to trafficking pathways in *T. brucei*. The image shows the endocytic and recycling systems of bloodstream (left) and procyclic (right) form trypanosomes in highly schematic fashion. Endocytosis in both stages originates at the flagellar pocket region of the plasma membrane and rapidly delivers cargo to the early endosome (green circles). Sorting takes place from this, or a closely related, compartment to either deliver material for a lysosomal (red circle) destination or entry into a recycling (grey or blue circle) pathway. In bloodstream forms, recycling is the predominant route and lysosomal delivery is secondary, at least in terms of flux. In procyclics, the dominant route is lysosomal delivery. Most dramatically, the Rab11-mediated recycling system is under direct developmental control due to increased expression of Rab11 in the bloodstream stage, while the early endosome population becomes more complex in the bloodstream stages with at least two distinct subpopulations present compared to one in the procyclic form. In addition, the role that the Rab4 recycling system plays is substantially more important in the procyclic form, with a predominant role in lysosomal delivery in the bloodstream stage. An increase in endocytosis *per se* in the bloodstream stage is underpinned, at least in part, by upregulation of clathrin. Arrow thickness corresponds to flux through a particular route, and numbers on circles denote the Rab family GTPase associated with a specific compartment. Interrogation signs indicate that a route has not been established, but is predicted.

from *T. brucei* is possibly due to saturating levels of VSG at the cell surface, eliminating the endocytic concentration step, but the reason for loss of *Leishmania* AP3 is not clear. Adaptin complexes interact with many additional protein factors through highly conserved motifs. While many of these factors are fully conserved in the kinetoplastida, including epsin N-terminal homology domain proteins (but not epsin itself), AAK1, RME-8 and synaptojanin (18), whole domain sets and their interacting motifs seem to be missing in these genomes (15). Interestingly, the epsin-R configuration, which lacks the canonical ubiquitin interaction motif (UIM), is probably the ancestral form as the UIM was recruited only in the Opistokhonta lineage (15).

Exocytosis has received substantially less attention than endocytosis in trypanosome trafficking studies, and consequently our understanding is somewhat limited. The basic *N*-glycosylation machinery, GPI-addition system and components of the Sec61 translocon are all present, in agreement with the presence of canonical N-terminal ER-targeting and C-terminal GPI anchor signals in cell surface protein sequences and the extensive biochemical analysis of post-translational modification of trypanosomatid surface antigens. In terms of protein folding, few factors have been analysed in detail, but a wide range of chaperones are present in the genome, including multiple PDIs, HSP family proteins, EDEM homologues etcetera (18). Given the high level of GPI-anchored proteins produced by the trypanosomes, analysis of these factors should be of significant value.

Molecular Aspects of Trafficking in Trypanosomatids

Endocytosis in T. brucei

In terms of developmental regulation of trafficking, most is again known functionally about *T. brucei*. The principal changes accompanying differentiation are alterations to the endocytotic rate, with a massive increase in bloodstream stages and remodelling of the surface coat. Immune evasion by *T. brucei* is an absolute requirement for survival and is known to occur principally by antigenic variation (19). However, the mammalian stage parasite can not only internalise surface-bound anti-VSG antibodies with considerable rapidity and efficiency (20) but also subsequently degrade the antibody as well (21,22). The significant level of developmental regulation of this process is compelling circumstantial evidence for involvement in stage-specific survival.

At the molecular level, most significant is upregulation of clathrin and Rab11 in the bloodstream form (3,23). A more complex morphological arrangement of the early endosomal system is evident from studies of Rab5 where the two isoforms have distinct functions (22,24). These represent the only known endocytic trafficking factors that display stage-specific regulation but are consistent with increased activity together with changes in the manner in which the different pathways and compartments are interconnected. p67, a major lysosomal protein, is also subject to developmentally regulated post-translational modification (25), while upregulation of bloodstream form lysosomal activity has been known for some time (18).

RNAi against Rab4 and Rab11 has demonstrated significant developmental alterations to the manner in which molecules are sorted. In bloodstream forms, sorting is very rapid (26) with separation of VSG and fluid-phase cargo occurring early and VSG recycled while soluble material is delivered to the lysosome. Rab11 is implicated in recycling and endocytosis; RNAi results in an enlarged flagellar pocket and decreased receptor-mediated endocytosis, with no apparent function in fluid-phase endocytosis (27). In the procyclic form, internalised surface protein along

with fluid-phase material is delivered to the lysosome (27) and fluid-phase endocytosis is strongly affected by Rab11 RNAi in procyclics (27). Rab4 also has a role in fluid-phase endocytosis in *T. brucei*. In the bloodstream form, Rab4 is involved primarily in delivery of fluid-phase endocytic cargo and endogenous proteins to the lysosome (28,29).

The lysosomal protein p67 has also provided insight into developmental aspects of endocytic transport (25). Firstly, glycosylation of this protein is more elaborate in the bloodstream stage, but the extensive proteolytic processing that occurs during, or after, lysosomal delivery is essentially equivalent. However, the protein is turned over more rapidly in the bloodstream stage. Significantly, lysosomal targeting of p67 constructs lacking the transmembrane domain is retained, but in the procyclic a significant amount of material can also be shed from the cell.

Recent data indicate remodelling of the adaptor expression profiles between life stages in *T. brucei*. Specifically, we have observed that most subunits of the AP3 and AP4 complexes are upregulated at the messenger RNA (mRNA) level in procyclics (our unpublished data) while γ-adaptin from AP3 has been shown to be overexpressed by procyclics at the protein level (unpublished data). This observation is consistent with the paradigm that the procyclic is devoted to degradative pathways, and indicates multiple levels of regulation encompassing both downregulation of recycling and upregulation of adaptin-mediated lysosomal pathways.

In summary, there is increased recycling function in the bloodstream form that returns VSG to the cell surface compared to the equivalent pathway in the procyclic stage. This supports a potential role for endocytosis in immune evasion, although an additional function may be present as maintenance of VSG surface density is required for cell-cycle progression in bloodstream forms (30), while depletion of surface GPI-anchored proteins is tolerated in procyclic stage (31). The procyclic appears devoted to delivery of material to the lysosome, presumably for efficient degradation, although the capacity of this system may be more easily saturated than in bloodstream forms – the role that this pathway has in progression through the insect vector is not known.

Exocytosis in T. brucei

There is little evidence for major stage-specific changes within the exocytic system, although there are highly significant changes to the cell surface during differentiation (32). The exocytic system is devoted to production of GPI-anchored molecules, which account for >80% of protein biosynthetic output from the ER but there are major differences between life stages (Figure 1B). VSG is globular, predominantly α-helical and *N*-glycosylated (33,34), presumably requiring a sophisticated chaperone/folding system. *N*-glycan processing capacity is extensive as some glycoproteins bear high molecular weight lactosamine repeat structures (35). Procyclins are mainly unstructured as 50% of the mature polypeptide forms a proline-repeat helix, the N-terminal domain is small, and only one of the isoforms is *N*-glycosylated. Hence, chaperone requirements are probably rather less than for bloodstream stage, while glycosyltransferase activity is also decreased due to the absence of procyclic *N*-glycan maturation beyond oligomannose forms. A highly elaborate processing of the procyclin GPI anchor is observed, which contains structures with broad similarity to lactosamine *N*-glycans; it is not known if the glycosyltransferases responsible for GPI glycan elaboration are unique or common with the *N*-glycan pathway.

Trypanosome BiP interacts directly with VSG and is essential (36–38). Two PDI homologues, PDI-1 and PDI-2, have been described; paradoxically they have been localised to the lysosome, which is unexpected particularly as PDI-2 has a functional ER retention tetrapeptide (39). Recent data indicate that ER glucosidase II is non-essential (40), but is required for correct maturation of VSG *N*-glycans. This study also provided evidence for a distinct glycosylation pathway in *T. brucei*, involving transfer of a $Man_5GlcNAc_2$ glycan to the nascent polypeptide, rather than $Man_9GlcNAc_2$. The absence of glucosylated lipid-linked *N*-glycan donors in trypanosomes has been known for some time, and recent data using RNA interference approaches suggest that calreticulin (there is no calnexin orthologue) is non-essential (T. Sergeyenko and Mark C. Field, unpublished data). A moderate increase in BiP expression (41), increased morphological complexity in the Golgi complex (4) and upregulation of Rab18, a Golgi GTPase (42), have all been described in bloodstream forms. By contrast, expression of Rab1 and Rab2, key regulators of ER and Golgi transport processes, is constitutive (43).

Leishmania

Considerably less is known about the molecular basis for developmental regulation in *Leishmania*. For *Leishmania*, reorganisation of the endosomal system accompanies differentiation, and surface remodelling is also extensive, as the predominant LPG antigen is down-regulated in mammalian stages. The morphology of the lysosome in the insect stage is unusual, comprising a multivesicular tubule (44,45). The presence of additional membranous structures within this organelle may indicate a fusion between the true lysosome and the multivesicular body, consistent with a comparatively high lumenal pH and correspondingly low lytic capacity. The MVB matures into a more conventional lysosome in the metacyclic promastigote, the form that invades the mammalian host, and there is good evidence for an effective endocytic and lytic system in amastigotes. In infected macrophages amastigotes endocytose and degrade major histocompatibility complex class II molecules, presumably to interrupt antigen presentation (46). Augmented proteolytic activity in the amastigote probably correlates with further alterations

to lysosomal morphology and appearance of multivesicular megasomes (47). Interestingly, there is no change in Rab7 expression through the life cycle, suggesting that modulation of lysosomal activity resides elsewhere (48). Further, both clathrin and AP1 are constitutively expressed, indicating that early endocytosis is unlikely to exhibit any great degree of developmental regulation (49). Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) analysis of the *Leishmania* Qa SNAREs indicates potential developmental regulation of these factors, and by inference the pathways they mediate (17) but assignment of specific *Leishmania* SNAREs to specific steps has not been achieved.

An active endocytic sorting system and autophagy appear required for differentiation from the promastigote to the infective metacyclic (50). This has been shown through increased expression of the autophagy marker Atg8 during differentiation, deficient differentiation in Atg4 mutants and defective differentiation and endosome sorting in overexpressors of a mutant form of Vps4. A major consequence of compromised differentiation is reduced infectivity and survival of the parasites in macrophages. Autophagy is also implicated as required for differentiation from the metacyclic to the amastigote within macrophages, again through tracking the autophagy marker Atg8 (51). Further, two cysteine peptidases (CP-A and CP-B) are important for autophagy in *L. major*, as either inhibition or gene deletion prevents autophagosome degradation and thus proper differentiation of the cells and reduced virulence. A coherent analysis of the intracellular trafficking pathways in *Leishmania* is still required, and the very many biological differences between *Leishmania* species add an additional level of complexity that awaits resolution.

Evidence for lack of transcriptional control

In kinetoplastids, regulation of gene expression is mostly post-transcriptional through mRNA stability (52–54). Recent data from our laboratory indicate absence of transcriptional flexibility in trypanosome cells dealing with artificially elevated gene expression. *Trypanosoma brucei* overexpressing Rab5A or Rab5B displays increased endocytosis (22) and a concomitant increase in clathrin (our unpublished data). However, transcriptome analysis with microarray and qRT-PCR shows no significant variation in steady-state mRNA levels for clathrin or for any other of 700 genes with a possible role in endocytosis, suggesting that trypanosomes may have rather limited capacity to respond to altered circumstances via control of gene expression (our unpublished data).

GPI-Dependent Trafficking

GPI addition is essential for ER exit and surface transport of most GPI-anchored proteins (55), but in trypanosomes an additional feature of GPI addition has been uncovered. Comparison between secretion kinetics of the same ectodomain bearing either a GPI or no membrane attachment (soluble) suggests that the GPI acts to increase the rate of secretion in *T. brucei* (36,37,56). The number of GPI anchors per protein complex may also influence the ultimate fate of membrane proteins. Specifically, VSG, a dimer, has two GPI anchors and is efficiently excluded from lysosomal degradation and recycled. By contrast, the trypanosome transferrin receptor has one GPI anchor and is quickly degraded during multiple rounds of recycling (57). VSG lacking a GPI addition signal is extremely rapidly delivered to the lysosome and degraded (56). How the presence of the GPI anchor affects trafficking and ultimate fate is not clear, but may suggest a role for membrane microdomains in recognition of trypanosome membrane proteins.

Immune Evasion

The abundance of VSG in *T. brucei* immediately suggests a model whereby the VSG monolayer acts as a barrier against antibody recognition of invariant surface determinants, but this paradigm neglects both the dynamic nature of biological membranes and the presence of comparatively high abundance invariant surface glycoproteins of equal or greater molecular mass than VSG (58). Anti-VSG immunoglobulin bound to the surface of living parasites in *in vitro* cultures is rapidly internalised, degraded and recycled (20–22), and this mechanism was recently found to extend to invariant surface glycoproteins (59). Internalisation of immune complexes may afford trypanosomes additional protection from antibody-dependent destruction beyond antigenic variation.

Detailed examinations of the expression of endocytic system markers, clathrin and Rab11, in different life stages of *T. brucei*, reveals rapid down-regulation of endocytic activity within 24 h of entering the insect host which remains low throughout subsequent development within the midgut and proventriculus, indicating that endocytosis is probably not involved in combating insect defence mechanisms or in remodelling the parasite surface. Increased endocytosis is observed in metacyclic forms in the insect salivary glands where trypanosomes preadapt to the mammalian host by re-expression of VSG and alteration of metabolic systems, consistent with a specific role in survival in the mammalian host (Natesan, Peacock, Matthews, Gibson and Field, submitted, 60,61).

Spectacular advances in understanding the host range of African trypanosomes have been made in recent years, and clearly this process involves the endocytic system (62). In humans and some other higher primates, a trypanosome lytic factor (TLF) has been described, which is absent from cattle and most other mammals. This material is taken into the cell and is delivered to the lysosome where it

appears to produce pores in the lysosome membrane, leading to swelling of the cells and ultimately rupture (63, Pays). The precise identity of the lytic factor in TLF remained controversial for some time, but clearly requires Apo LI, which is probably the major lytic factor (64,65). However, human infective subspecies of *T. brucei* express the serum resistance-associated gene, or SRA (66). Recent work demonstrates that SRA is present within the endosomal system of *T. brucei*, binds to TLF and in some manner inactivates it preventing lysosomal rupture (62). The co-evolution of SRA and TLF, together with the role of trafficking in resistance to innate immunity, is an intriguing example of the utilisation of the endocytic pathway for new functions.

Golgi replication

The Golgi apparatus is a highly dynamic organelle that forms the core of the eukaryotic secretory system. Golgi replication occurs in a coordinated manner with the cell cycle, but the complexity of the structure has made it difficult to fully explain the mode of inheritance. Two principal models for Golgi replication are *de novo* synthesis and template-directed synthesis. In the former, the new Golgi complex is created independently of the old, in the latter it appears as an extension of the old Golgi, followed by partitioning. It appears that different organisms have evolved distinct modes of Golgi replication. In *T. brucei*, the structure of the Golgi apparatus is simpler than in many systems, being composed of a single stack of cisternae, making it an appealing model for study of Golgi biogenesis. The Golgi reassembly-stacking protein (GRASP) repertoire is simpler as there is only a single paralogue for GRASP55/65 in the genome. In addition, the trypanosome Golgi complex undergoes duplication at a very early point in the cell division process, followed by relocation in a manner that is coupled to migration of the kinetoplast (4).

An elegant live cell-imaging approach used a green fluorescent protein (GFP) fusion of trypanosome GRASP to follow replication of the organelle in detail. These data suggest that the new Golgi appears *de novo* in close association with the new ER export site and has an independent identity next to the old Golgi (67). On the other hand, photobleaching experiments using the Golgi-resident enzyme *N*-acetylglucosamine transferase B as a probe suggested that the old Golgi also played an important role in synthesis of the new copy by supplying at least some components to the new structure. These findings indicate that the mechanism of Golgi biogenesis in *T. brucei* could be explained by a combination of the two pre-existing models whereby *de novo* synthesis of the new Golgi would be dependent on traffic of components from the old Golgi and supply of material from the new ER export site. Video imaging following different Golgi markers showed that assembly of the new Golgi proceeds in an ordered fashion. Resident enzymes and certain components of the Golgi matrix are supplied first to the new Golgi, whereas those elements that are involved in traffic and sorting of cargo are provided at a later stage (68).

From an evolutionary point of view, *T. brucei* is also of interest as Golgi duplication occurs prior to the onset of mitosis, contrary to mammals, suggesting that the signals that orchestrate replication are different from those in higher eukaryotes. Recently, a *T. brucei* orthologue of phosphatidylinositol 3-kinase, TbVps34, was implicated in segregation of the Golgi apparatus during cell division, a requirement not observed so far in other systems (69). In a further study, TbCentrin2, one of four centrins in *T. brucei*, has been found to define a bilobed structure not previously reported in any other system. This structure is located near the Golgi apparatus and appears to dictate the position of assembly of the new Golgi and new ER export site (70).

Sorting signals for transmembrane proteins

Characterisation of transmembrane domain protein sorting signals is not well advanced in trypanosomes, but three examples suggest that the basic system is similar to higher eukaryotes. Evidence for tyrosine- and dileucine-based sorting signals comes from a *Leishmania* acid phosphatase, where the C-terminal cytosolic region appears to contain such sequences, including a C-terminal IIV motif for endocytosis (Weise et al., 2005). Additional evidence for the presence of dileucine signals comes from mutagenesis of the lysosomal protein, p67, in *T. brucei* – this type I transmembrane protein contains a classical dileucine signal embedded within a short cytoplasmic domain; mutation of these residues to AA compromises lysosomal targeting (Allen, Liao, Chung and Field, unpublished data). A lysine-based endocytosis signal has been identified within the conserved cytoplasmic region of ISG_{65} (59), and recent data indicate that these residues are acceptors for the covalent attachment of ubiquitin, implicating ubiquitination as an endocytic signal in trypanosomes (W. Chung and Mark C. Field, unpublished data). Overall, a remarkable degree of conservation of sorting signals and mechanisms between trypanosomes and higher eukaryotes is observed.

In an interesting study, Qiao et al. investigated the trafficking of CRAM a type I transmembrane highly repetitive protein that localises to the flagellar pocket, and which may be a lipoprotein receptor (72). Using an alanine insertion strategy for the cytoplasmic domain, these authors demonstrated effects on both ER exit and endocytosis; disruption mapped to distinct regions of the cytoplasmic tail for exocytosis and endocytosis. This approach is rather unusual in that the effect of insertion rather than substitution alters spacing rather than the identity of residues, but does confirm the presence of trafficking signals in the short cytoplasmic domain of CRAM. These authors also provided *in vitro* evidence for

interaction with TbAP1 via the m1 subunit, suggesting the involvement of adaptins.

An evolutionary aside

Endocytosis and the evolution of membrane-bound organelles represented a key evolutionary advance for the eukaryotes (73). The early divergence of trypanosomes from mammals, yeast and higher plants provides an excellent model for studying common factors among all eukaryotes (44,75). Earlier views of eukaryote evolution considered the trypanosomes as primitive forms close to the root (76); hence understanding the configuration of the trypanosome endomembrane system was expected to contribute to models of the origins of these systems. Recent work has indicated that contrary to earlier paradigms, the last common eukaryotic ancestor (LCEA) possessed a complex system, with a fully differentiated endocytic and exocytic system that included Rab subfamilies, SNARE and SM proteins, vesicle coatomers and tethering complexes (15,16,74,77). This sophisticated architecture is at odds with molecular phylogenies that can be constructed for many components of the vesicle trafficking system where the most obvious conclusion would be for expansion in complexity following sequential gene duplication events. The apparent contradiction can be resolved by recognising that a short period of rapid innovation followed by lineage expansion appears to have accompanied emergence of the LCEA, resulting in the complete loss of forms retaining transitional architectures (74). Therefore, examination of divergent systems, including trypanosomes, is unlikely to directly contribute to understanding evolutionary origins in the sense of being primitive systems. However, studies have demonstrated the range of strategies that eukaryotes exploit, and indicate that information from highly distal systems will be illuminating in a general context, as well as being essential for understanding specific taxa. With ~40% of the trypanosome gene complement lacking homology to sequences outside of the kinetoplastida (10), it is likely that considerable novel biology resides within trypanosomes.

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