Intracellular Transport Systems in Trypanosomes: Function, Evolution, and Virulence

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Abstract

The surface of pathogenic trypanosomatids represents the host–parasite interface, and characterization of the surface molecules and their functions has yielded major insights into mechanisms of disease progression, immune modulation and immune evasion. Additionally, trypanosomatids place a particularly heavy emphasis onto use of the GPI anchor as a means for membrane attachment of proteins, glycoconjugates and glycolipids. Synthesis, degradation and maintenance of the surface are a function of the endomembrane system. Recent advances have begun to unravel the complexity of the trypanosome trafficking system, how it is regulated and the mechanisms that underpin protein sorting. Availability of complete genome data now allows molecular level comparisons between the TriTryps and their hosts, identifying unique aspects to trafficking pathways in trypansomes, as well as providing potential for more rational experimental manipulation of transport systems. These aspects together with our current state of knowledge of the trypanosome trafficking system are discussed.

Introduction and overview

The characterization of the organelles and pathways followed by cargo proteins during exocytosis or endocytosis is an important area in eukaryotic cell biology (Palade, 1975; Helenius *et al.*, 1983), especially in mammalian and yeast systems where major advances have been made in the analysis of transport steps at the molecular level. Amongst the biggest challenges remaining are determining how the different molecular assemblies and compartments are integrated to control the various transport steps. The principles coordinating the itinerary of lipids and proteins and the precise organization of each compartment are still poorly understood in any system (van Vliet *et al.*, 2003).

Both endocytosis and exocytosis are of significant interest in trypanosomatids as membrane trafficking is responsible for construction of the host—parasite interface, responses to the host immune system and expression of components required for cell invasion and adhesion (Figs. 9.1 and 9.2). Common to all trypanosomatids is the polarization of the majority of the membrane transport system—including the Golgi complex and endocytic system—to the posterior region of the cell, with all membrane exchange with the plasma membrane restricted to the flagellar pocket. The very distinct life cycles, surface molecules and mechanisms for immune evasion exploited by *Leishmania*, *Trypanosoma cruzi*

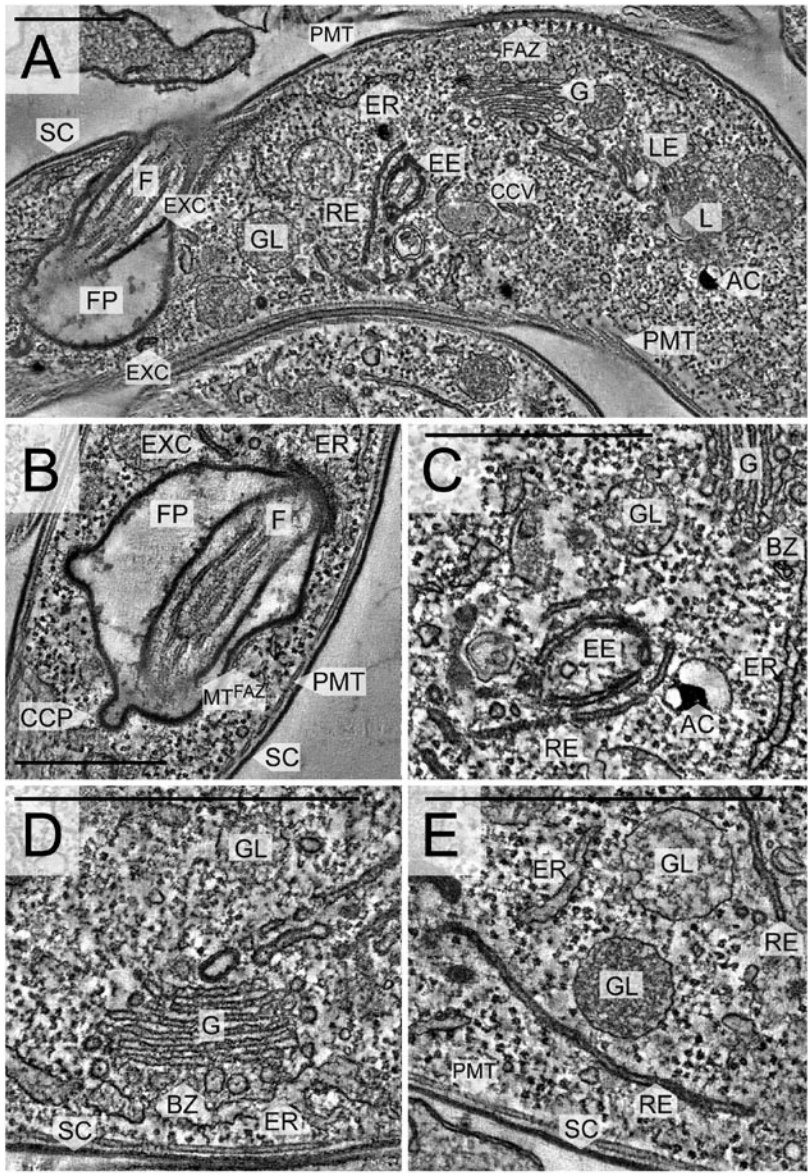


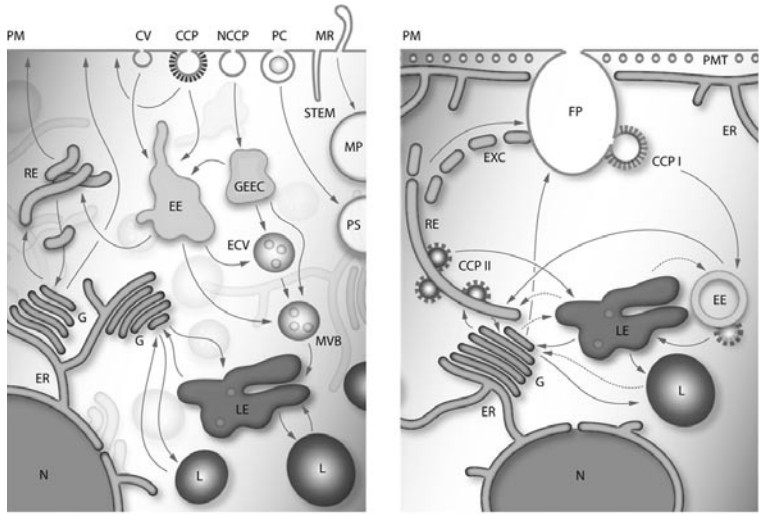
Figure 9.1 Ultrastructure of the posterior part of bloodstream stage *Trypanosoma brucei*. The images show representative electron micrographs of non-dividing trypanosomes. (A) Overview of the intracellular membrane system between the flagellar pocket and the nuclear area. (B) Enlarged view of the flagellar pocket with budding clathrin-coated pits. (C) Early endosomes and recycling endosome can locate in close proximity to the single Golgi. (D) Detailed view of the Golgi apparatus and the ER exit site. (E) Recycling endosomes are flat and extended cisternae. For details see text. AC, acidocalcisome; BZ, budding zone; CCP, clathrin-coated pit; EE, early endosome; ER, endoplasmic reticulum; EXC, exocytic carrier; F, flagellum; FAZ, flagellar attachment zone; FP, flagellar pocket; G, Golgi apparatus; GL, glycosome; L, lysosome; LE, late endosome; MT-FAZ; microtubules of the flagellar attachment zone; PMT, pellicular microtubules; RE, recycling endosome; SC, surface coat; size bars: 1 µm (images: M. Morphew, J. R. McIntosh and M. Engstler).

and *Trypanosoma brucei* are likely to be reflected in specific adaptations within the secretory system. A very strong emphasis on synthesis and surface expression of macromolecules linked to the membrane via a glycosylphosphatidylinositol (GPI) membrane anchor in all of these organisms may require specializations within the exocytic pathway. Antigenic variation in African trypanosomes mediated by the variant surface glycoprotein and production of highly complex glycoconjugates related to the lipophosphoglycan (LPG) in *Leishmania*, are all linked to virulence mechanisms in the mammalian and insect hosts (Ilg 2000a,b; Spath *et al.*, 2003). In the African trypanosome a massive increase in endocytic capacity and proteolytic activity within the recycling system in mammalian stages may reflect activation of a further immune evasion system.

Genome data are providing insights into the likely presence or absence of proteins and pathways involved in trafficking. Following emergence of RNA interference, completion and annotation of the genome together with significant technical advances, trypanosome molecular cell biology has now become an accessible and attractive model system, with a valuable contribution to offer to specialized and evolutionarily divergent biological problems as well as in exploring protozoan parasite virulence systems. The highly organized trypanosome cell architecture and programmed changes to structure and function during development provide unique opportunities to study regulation of secretion, endocytosis, recycling and the destruction of membranes and proteins.

Trypanosome endomembrane systems

Trypanosomes reveal a unique cellular architecture. In contrast to most eukaryotes, the organism contains one Golgi (in the bloodstream stage) and a single lysosome, both located within the posterior part of the cell, together with essentially the entire endosomal system (Field and Carrington, 2004; Overath and Engstler, 2004). The flagellar pocket (FP), a flask-shaped invagination of the plasma membrane, continuous with the pellicular and flagellar membrane, surrounding the emerging flagellum and representing ~5% of the total cellular membrane area is the only site for communication between endomembranes and the cell surface by exocytosis and endocytosis (Overath *et al.*, 1997; Grünfelder *et al.*, 2002; Gull, 2003); the lack of pellicular microtubules in the pocket area may explain this exclusivity. A hemidesmosome-like zone encloses the pocket neck making it a secluded, yet extracellular, compartment that contains an electron-dense carbohydrate-rich matrix. Molecules entering the flagellar pocket have to pass through the hemidesmosomal zone and/or the pocket matrix. There are no conclusive data on the exclusion limit or rates of diffusion in either direction, little information on factors controlling access to the pocket, or on the physical properties or biochemical constituents of the lumen of the pocket (but see Atrih *et al.*, 2005). While the density of the variant surface glycoprotein (VSG) coat in the flagellar pocket equals that of the pellicular plasma membrane, other proteins are clearly enriched in the pocket membrane. This is especially true for receptors, which in the flagellar pocket are protected from attack by the host's immune system. The GPI-anchored transferrin receptor (TfR) shuttles between endosomes and the flagellar pocket, but is virtually absent from the pellicular cell surface. Membrane turnover at the flagellar pocket is exceptionally high. After internalization cell surface proteins (e.g. VSG, TfR, ISGs) are



diluted and re-concentrated in endosomal membranes to a very similar extend (about 10-fold) (Engstler *et al.*, 2004; Engstler *et al.*, unpublished). The functional role of the flagellar pocket machinery in secretion and internalization of molecules remains to be analysed but the possession of a pocket structure may influence integration between intracellular membrane systems and the cell surface, with significant consequences for sorting and targeting of cargo molecules (Overath and Engstler, 2004).

Endoplasmic reticulum and Golgi apparatus

The ER consists of a system of tubes and cisternae (sac-like structures) (Voeltz *et al.*, 2002). Portions of the ER membrane are continuous with the outer membrane of the nuclear envelope, and the luminal space of the ER is continuous with the nuclear intramembrane space (Mattaj, 2004). While in most eukaryotes the ER is distributed throughout the cytoplasm, in *T. brucei* the ER is more distinct. A sheet-like system of interconnected, cisternal membranes attaches to a specific subset of four subpellicular microtubules that mark the flagellar attachment zone (FAZ) (Ersfeld and Gull, 2001). The ER stretches beneath the FAZ from the anterior pole of the cell to the FP. A distinct, tubular part of the ER passes by the FP and reaches to the posterior of the cell. The elongated, polarized structure of the trypanosome ER could be reflective of a functional compartmentalization of this organelle. When compared to other cells, the ER of *T. brucei* bloodstream stage cells, at ~25% of total internal membrane, represents less of the total cellular membrane. This area doubles early in the cell cycle (M. Samwer and M. Engstler, in preparation).

In the posterior half of the cell, the ER features an exit site intimately connected to the location of the Golgi and is characterized by an extensive network of tubular structures and abundant variable sized vesicles. The bloodstream stage Golgi typically consists of 5–8 discrete stacked cisternae (Grab *et al.*, 1987). Abundant budding of predominantly clathrin-coated vesicles occurs from the rims of all Golgi-cisternae. The size of those vesicles is smaller than that of plasma membrane-derived or endosomal clathrin-coated vesicles (Engstler, unpublished). In the procyclic stage, the

Figure 9.2 (opposite) Comparison of secretory and endosomal traffic in bloodstream stage *Trypanosoma brucei* and in other eukaryotes. The left panel shows a schematic overview of membrane pathways discussed in the mammalian or yeast systems. There are several portals of entry into the cell. Clathrin-mediated internalization is the best-known endocytic process (Robinson, 1987; Kirchhausen *et al.*, 2005). It is widely accepted that transmembrane proteins, especially, receptors and their ligands, are concentrated in clathrin-coated pits (CCP) due to the interaction of sequence motifs in their cytoplasmic domains with constituents of the clathrin-coat (Sorkin, 2004). After loss of the clathrin coat, the internalized vesicles fuse with one another and with existing early endosomes (EE) (Woodman, 2000; Lemmon, 2001; Maxfield and McGraw, 2004). Non-clathrin processes often lack specific markers and are believed to be less concentrative (Kirkham and Parton, 2005). Caveolae (CV) are small invaginations (50–60nm) at the cell surface that are commonly defined by the presence of caveolin. Caveolae can form vesicles, however, this seems to be a rather sporadic event that requires stimulating signals (Anderson, 1998; Nabi and Le, 2003; Nichols, 2003; Parton and Richards, 2003). Phagocytosis (PC) is defined as the uptake of large particles by an actin dependent process. This process and likewise the STEM pathway (surface-connected tubules entering macrophages) occur habitually in specialized cells such as in macrophages (Jutras and Desjardins, 2005; Stuart and Ezekowitz, 2005; Touret *et al.*, 2005). Macropinocytosis comprises membrane ruffling (MR) and can result in the formation of large endocytic compartments (MP) when the "ruffle" fuses back with the plasma membrane (Kirkham and Parton, 2005). From non-clathrin-coated pits (NCCP), GPI-anchored proteins are trafficked to RAB5-negative early endosomes (GEEC; GPI-anchored-protein-enriched early endosomal compartment). From EE and GEEC, membrane and proteins are trafficked via multivesicular bodies (MVBs) and late endosomes (LE) to the lysosomes (L). Recycling to the plasma membrane occurs either directly from early endosomes or via recycling endosomes (RE). To which degree secretory cargo is routed from the Golgi (G) directly or via recycling endosomes to the plasma membrane (PM) is still a matter of debate. In trypanosomes (right panel), all membrane and surface proteins are internalized at the flagellar pocket (FP) in clathrin-coated pits of distinct size (CCP I). These vesicles fuse to circular early endosomes, from which a second class of clathrin-coated pits (CCP II) buds that carries fluid phase cargo via late endosomes to the single lysosome. The bulk of membrane and embedded proteins are sorted in the flat, sheet-like recycling endosome (RE). From this compartment CCPs II abundantly bud carrying cargo and endocytosed ligands via late endosomes to the lysosome. The recycling endosome gives rise to disk-shaped exocytic carriers (EXC), which transport membrane and concentrated surface proteins back to the flagellar pocket. As in the mammalian system, it is as yet unclear to which degree traffic from the Golgi to the plasma membrane occurs via endosomes or directly. CCP I, clathrin-coated pit class I; CCP II, clathrin-coated pit class II; CCP, clathrin-coated pit; CV, calveolae; ECV, endosomal carrier vesicle; EE, early endosome; ER, endoplasmic reticulum; EXC, exocytic carrier; FP, flagellar pocket; G, Golgi apparatus; GEEC, GPI-anchored-protein-enriched early endosomal compartment; L, lysosome; LE, late endosome; MP, macropinosome; MR, membrane ruffling; MVB, multivesicular body; N, nucleus; NCCP, non-clathrin-coated pit; PC, phagocytosis; PM, plasma membrane; PMT, pellicular microtubules; PS, phagosome; RE, recycling endosome; STEM, surface-connected tubules entering macrophages

Golgi apparatus is almost double in size and contains significantly more cisterna. The Golgi is one of the earliest organelles to replicate during mitosis, and recent evidence suggests this process may require PI3P (Field *et al.*, 2000, M.C.F. and Belinda Hall, unpublished data). Recent elegant work has shown that Golgi duplication in *T. brucei* is intimately connected to the formation of a new ER export site and involves transfer of material from the old Golgi stack (He *et al.*, 2004).

The endosomal system

Classifications of endosomal compartments tend to highlight particular characteristics such as morphology, function and composition. Intracompartmental pH,

lipid composition and cytoskeletal organization have been suggested as principal determinants of the orderly flow of material through endosomes but the distinction between compartments can be lost at the molecular level (Gruenberg and Maxfield, 1995; Vorisek, 2000; Sachse *et al.*, 2002). Many proteins that "mark" a particular organelle can shuttle between their typical locations and other sites within the cell (Bishop, 2003), and appointing a single protein as marker for an entire organelle oversimplifies.

In view of the highly dynamic nature of endosomal subcompartments we will use the classical terms of "early", "late" and "recycling endosome". All three compartments have been identified in trypanosomes by the presence of accepted molecular markers. The characteristic morphology and distinct intracellular localization of endocytic subcompartments in *T. brucei* provides unique opportunities for studies on the interface between different endosome types. In fact, trypanosomes may challenge some generally acknowledged features of endosome structure–function relationships.

Early endosome

Like other cells, trypanosomes deliver internalized material to early endosomes (EE). In most cell types, early endosomes are peripherally located. They appear as tubular–vesicular structures with a luminal pH of ~5.5–6.0. In contrast, the *T. brucei* early endosomes are predominantly located close to the nucleus, where they are juxtaposed to late endosomes (Field *et al.*, 1998; Grünfelder *et al.*, 2003; Engstler *et al.*, 2004). Structurally, trypanosome EE appear as rather large circular cisternae and are morphologically distinct from the more irregularly shaped late endosomes (Engstler *et al.*, 2004). Interestingly, the individual cell contains not more than four EE (Engstler, unpublished). The small GTPase Rab5, and early endosome antigen 1 (EEA1) have been implicated in the fusion of coated-pit-derived primary endocytic vesicles with EE (McBride. 1999; Woodman. 2000; Simpson and Jones. 2005); this machinery is likely conserved in *T. brucei* (Pal *et al.*, 2002; and see below).

The EE acts as primary branch point; cargo can be directed for recycling or to late endosomes. As a consequence of lower EE luminal pH many ligands are uncoupled from their receptors represents the first step in sorting (Kurten. 2003). In trypanosomes uncoupling occurs predominantly in the recycling endosome, while fluid phase cargo can be segregated in both compartments (M. Engstler, unpublished).

To maintain the endosomal membrane balance is an essential task of all eukaryotic cells (Steinman *et al.*, 1983). In mammalian cells membrane is removed from EE by detachment of narrow diameter tubules favouring sorting of recycled membrane from soluble molecules. Hence internalized membrane proteins would be routed from the EE with the bulk membrane by default (Rodriguez-Boulan *et al.*, 2005; Verma and Hong, 2005). Thus, sorting in early endosomes is based to a large extend on the geometry of the compartment (Maxfield and McGraw, 2004). In trypanosomes the characteristic pinching off of tubules from EE has not been observed. Do these cells then lack the geometric sorting system that appears to function in sorting from early endosomes in other eukaryotes? Recent studies suggest that *T. brucei* in fact employs rather sophisticated geometric sorting, however, mainly at a later stage during the endosomal itinerary (Engstler *et al.*, 2004; Overath and Engstler. 2004; Engstler, unpublished).

Recycling endosomes

Recycling endosomes (RE) continuously exchange membrane with late endocytic compartments, the plasma membrane, and the *trans*-Golgi-network (TGN) (Mallard, 1998). In the mammalian system two main routes have been described that direct material back to the cell surface from EE (Mayor *et al.*, 1993; Palfrey and Artalejo, 1998). Recycling molecules can either be delivered directly back to the plasma membrane or be trafficked to RE (Mayor *et al.*, 1993; Hao and Maxfield, 2000). The molecular differences regulating sorting of components back to the plasma membrane via the direct or indirect recycling route are not known. In fact, it has proven technically difficult to analyse the rapid recycling of membrane proteins, and thus, it is not known what fraction returns to the surface using the direct route. In mammalian cells the RE is primarily a group of tubules (diameter 50–70 nm) that are associated with microtubules. REs can be distributed widely throughout the cytoplasm or be mostly clustered around the microtubule-organizing centre (Sonnichsen *et al.*, 2000; Lin *et al.*, 2002).

In trypanosomes, REs are the most prominent endosomal structure. Recent 3-D electron microscopic studies have shown that *T. brucei* bloodstream stage cells contain one giant, flat and fenestrated recycling endosome, which stretches from the flagellar pocket region throughout the posterior part of the cell (M. Murphy, C.G. Grünfelder, P. Overath, J.R. McIntosh and M. Engstler, in preparation). The endocytosed membrane along with fluid cargo is transported with biphasic kinetics via EEs to the RE (Engstler *et al.*, 2004). In this "recycling factory", ligands uncouple from their receptors and are sorted together with endocytosed fluid phase cargo into small clathrin coated vesicles (CCV class II), which abundantly bud from the rims of the cisternal recycling endosome. CCV class II have two destinations within the cell: late endosomes and the lysosome. The CCV class II are devoid of VSG, transferrin receptor and some transmembrane proteins (e.g. invariant surface glycoproteins (ISGs); M. Engstler, unpublished). Thus, by a negative mechanism, GPI-anchored proteins are passively (i.e. by default) concentrated in the recycling endosome of *T. brucei* (Grünfelder *et al.*, 2003). Interestingly, the recycling endosome that carries the concentrated VSG eventually gives rise to small, disk-shaped carriers that fuse with the flagellar pocket. These Rab11-positive exocytic carriers (EXCs) are profusely found within the posterior part of the cell (Grünfelder *et al.*, 2003). Since the RE is a long-lived compartment, transport from the recycling endosome demands the formation of vesicles or tubules as transport intermediates. Trypanosomes appear to avoid the formation of tubular transport intermediates, but rather employ clathrin-coated vesicles and disk-shaped carriers. It has been a matter of debate to whether the clathrin machinery is involved in sorting of recycling cargo or is just required for the physical formation of vesicles. To this end, the endocytic itinerary in trypanosomes appears to provide a more clear-cut answer, for protists at least.

Though from RE molecules can be sorted to several different intracellular destinations, the majority return to the cell surface. In mammalian cells recycling rates of many proteins and lipids are similar (Koval and Pagano, 1989; Maier *et al.*, 2002; Bishop, 2003), suggesting that protein-sorting signals are not important for export from recycling endosomes. Many trypanosomal proteins lack classical cytoplasmic sorting signals and recycling rates of transmembrane proteins are identical or

very similar to that of bulk membrane (M. Engstler, unpublished).

Late endosome

From early endosomes, proteins are also routed to late endosomes/lysosomes (Piper and Luzio, 2001; Gruenberg and Stenmark, 2004). Generally, proteins delivered to this pathway will be degraded and sorting to late endosomes is supposed to be particularly well controlled (Rouille *et al.*, 2000; Bonifacino and Traub, 2003; Raiborg *et al.*, 2003). The association between early and late endosomes is still debated. According to the endosome maturation model (Murphy 1991), EE are formed de novo, mainly by fusion of clathrin-coated vesicles (CCVs). The late endosome is thought to be refractory to fusion with plasma-membrane derived material and represents the remnant of the early endosomal compartment after exhaustion of recycling to the plasma membrane. The alternative model postulates that the EE is a stable compartment preserved by the balance of arriving and departing membrane material (Griffiths and Gruenberg 1991). Membrane destined for late endosomes leaves early endosomes in the form of endosomal carrier vesicles (ECVs). These spheroids are relatively large (400 nm) and contain internal vesicles. They fuse with late endosomes in a microtubule-dependent manner. Trypanosomes appear to lack ECV-related structures. Instead, membrane and cargo is delivered to late endosomes from both EE and RE in the form of clathrin-coated vesicles. It appears feasible that those CCVs class II are the only transport intermediates between endosomal compartments in trypanosomes. Furthermore, late endosomes in trypanosomes appear as morphologically clearly distinct units. The approximately six late endosomes that are observed in each cell are irregularly shaped and can be easily distinguished from the neighbouring circular early endosomes. The fact that even by electron tomography no "maturing" early endosomes have been found favours the view that late endosomes in *T. brucei* likely constitute a stable compartment. Thus, at least in trypanosomes the ongoing debate between the "maturation" and "stable compartment" models of endosome biogenesis can apparently be addressed. Furthermore, the clathrin-dependent mechanism of membrane delivery to late endosomes may not be unique to trypanosomes, since endosomal clathrin-coated pits have been described in mammalian cells.

A morphological landmark of the late endosomal pathway is a compartment with a characteristic accumulation of vesicles in its lumen. These structures are termed multivesicular bodies (MVBs) (Raiborg *et al.*, 2003; Gruenberg and Stenmark, 2004). Endocytosed tracers that are destined to be degraded reach MVBs after being segregated from recycling receptors, but before reaching the lysosomes. Whether MVBs are a form of late endosomes or transport intermediates between early and late endosomes is a matter of definition or believe. In fact, all endosomes along the degradation pathway contain multivesicular elements, including regions of the early endosome (Piper and Luzio, 2001). In trypanosomes only very few MVBs can be observed and those are either Rab5- or Rab7-positive (Engstler, unpublished). While the early endosomal MVB(s) appears as circular structure(s), the late endosomal MVBs are irregularly shaped. Thus, once again, in trypanosomes, these compartments can be distinguished simply by morphology.

Internalization and endosomal sorting of GPI-anchored proteins

GPI-anchored proteins are plasma membrane proteins and subject to internaliza-

tion, downregulation and degradation (Skretting *et al.*, 1999; Muniz and Riezman, 2000; Butikofer *et al.*, 2001; Chatterjee and Mayor, 2001; Mayor and Riezman, 2004). Similarly to soluble luminal proteins, GPI-anchored proteins cannot be recognized by cytosolic components and are believed to either interact with transmembrane proteins, or to be sorted via their biophysical properties. Current knowledge about endosomal trafficking and sorting of GPI-anchored proteins is still unsatisfactory, at least in part due to their low abundance. Just 0.5% of surface proteins in mammalian cells are GPI-anchored and there are up to 200 different GPI-proteins. In trypanosomes a single GPI-anchored protein (VSG) covers the plasma membrane of bloodstream stage cells, and a small family of procyclins dominates the insect stage cell surface. GPI-anchored proteins are found in various endosomes, which has been interpreted as evidence for the existence of several internalization pathways (see Fig. 9.2). Thus, it appears to be finally accepted that there is no specialized internalization mechanism for GPI-proteins. The same is true for trypanosomes, simply because there is only one endocytic pathway. *T. brucei* restricts the internalization of plasma membrane and embedded proteins to clathrin-mediated endocytosis (Allen *et al.*, 2003; Grünfelder *et al.*, 2003; Overath and Engstler 2004). Rather large clathrin vesicles (CCV class I) abundantly bud from the FP. Every second six new CCVs are rapidly transported by an actin-dependent mechanism to early endosomes, which locate approximately 4 microns away from the pocket. In the steady state, about 60–70 CCV class I can be found in the posterior part of the cell (Overath and Engstler, 2004). Thus, the high abundance of GPI-anchored proteins, the localized and exclusive mechanism of plasma membrane internalization, and the amazingly high capacity of the endocytosis machinery, provide good opportunities for studying trafficking and sorting of lipid-anchored proteins.

Recently, new mechanisms for internalization of GPI-anchored proteins from the plasma membrane have been described (Sabharanjak *et al.*, 2002). A dynamin-independent internalization pathway has been postulated, which is distinct from clathrin- or caveolin mediated uptake. This GEEC (GPI-anchored-protein-enriched early endosomal compartment) pathway involves trafficking of GPI-anchored proteins to RAB5-negative early endosomes. At least in bloodstream stage *T. brucei*, there is no unambiguous evidence for the existence of a GEEC pathway or specialized early endosomes. Using VSG as a prototypic GPI-anchored protein, the acid phosphatase *Tb*MBAP1 as a resident transmembrane protein and fluorescent lipids as marker for the plasma membrane, no differences in the kinetics of delivery to Rab5-positive early endosomes or divergences in the early localization of the reporters could be detected (M. Engstler, unpublished).

In mammals and yeast GPI-anchored proteins are internalized with low efficiency. The GPI anchor itself has been suggested as the retention determinant of GPI-anchored proteins in RE (Chatterjee and Mayor, 2001). Furthermore, GPI-anchored proteins appear to be recycled significantly slower than transmembrane proteins or lipids. How a lipid modification could retain a protein more efficiently in a membrane compartment than a bulky trans-membrane domain is not easily comprehensible. Theoretically, GPI-anchored proteins should be internalized from the cell surface and recycled at a similar rate or even faster than membrane proteins that

lack sorting motifs. This is in fact true for trypanosomes. The GPI-anchored VSG is endocytosed with the same (extremely fast) kinetics as the plasma membrane dye FM 1–43FX. Furthermore, a mutated membrane-bound acid phosphatase (Engstler *et al.*, 2005), lacking the C-terminal acidic cluster sorting motif, is endocytosed and recycled with similar kinetics as VSG (M. Engstler, unpublished). Recycling endosomes appear to be the main sorting station of GPI-anchored proteins. However, unlike yeast or mammalian cells, trafficking through recycling endosomes in *T. brucei* is not rate-limiting. In fact, the delivery from early endosomes follows biphasic kinetics, with one part of VSG or reporter proteins being delivered very rapidly to the recycling endosome, while the other half arrives significantly later. The reason for this is a detour of part of the internalized membrane and embedded proteins to late endosomes. Interestingly, this material is not delivered to the lysosome but is re-directed to recycling endosomes, albeit with comparatively slow kinetics. Since the density of VSG on the cell surface is essential for survival of the parasite *in vivo* and *in vitro*, the variant surface glycoprotein must be concentrated on recycling to the FP. Lipid-rafts have been suggested as a driving force behind the recycling of GPI-anchored proteins (Mayor *et al.*, 1998; Varma and Mayor, 1998; Lipardi *et al.*, 2000; Ikonen, 2001; Brown, 2002; Sharma *et al.*, 2002; Paladino, 2004; Polishchuk *et al.*, 2004; Sangiorgio *et al.*, 2004). In *T. brucei* GPI-anchored proteins are probably concentrated in recycling endosomes by withdrawal of membrane in the form of clathrin-coated vesicles. This has been shown for VSG (Grünfelder *et al.*, 2003; Engstler *et al.*, 2004), but is also true for the transferrin receptor and even for reporter proteins such as GPI-anchored GFP (K. Bayer and M. Engstler, *submitted*).

Furthermore, the number of GPI anchors per protein can influence the fate of membrane proteins. Native dimeric VSG (two GPI anchors) is efficiently excluded from lysosomal degradation and is extremely stable ($t_{1/2}$ >30 h) (Bulow *et al.*, 1989; Seyfang *et al.*, 1990), while transferrin receptor (1 GPI anchor) is slowly directed to the lysosome ($t_{1/2}$ ~1.5 h) (Mussman *et al.*, 2004; Schwartz *et al.*, 2005). Soluble VSG mutants (0 GPI anchor) are rapidly delivered to the lysosome and degraded ($t_{1/2}$ ~45 min) (Triggs and Bangs, 2003). Recombinant reporters with one GPI anchor apparently segregate between cell surface delivery and lysosomal degradation (Chung *et al.*, 2004; Schwartz *et al.*, 2005). How this parsing occurs in not clear but is probably a function of the physical properties of each reporter as it cycles through the system. Interestingly, if a single GPI anchored reporter is able to access the external plasma membrane it can be shed with an intact GPI anchor, probably as a result of the use of the short chain fatty acid myristate ($C_{14:0}$) in bloodstream form GPI anchors (Ferguson *et al.*, 1985). The homodimeric structure of VSG provides two GPI anchors insuring that the coat is firmly cell associated in intact trypanosomes.

How GPI-proteins are excluded from entry into budding vesicles remains to be elucidated. Endocytosed lipids are routed to different intracellular locations (van Meer and Simons, 1988; Koval and Pagano, 1989; Kobayashi *et al.*, 1998; Edidin, 2003; Gruenberg, 2003; van Meer and Sprong, 2004; Holthuis and Levine, 2005), and can be recycled to the cell surface through an itinerary that is indistinguishable from rapidly recycling proteins.

This is especially evident in trypanosomes supporting the hypothesis that most internalized molecules do not require positive sorting signals for recycling to the cell surface. The chemical properties of both the head groups and the hydrophobic portions of lipids could act as sorting determinants (Helms and Zurzolo, 2004) and the lipid composition of membranes can influence protein sorting.

Many aspects of the trypanosome secretory and endocytosis systems render these parasites attractive objects for studies on the molecular determinants and mechanisms that underlie membrane trafficking, including the shear abundance of GPI-anchored VSG, the rapid clathrin-dependent endocytosis occurring exclusively at the FP membrane, the well-defined endosomal architecture and the singular major organelles.

Early secretory pathway, ER chaperones, folding, quality control and exocytosis

The major products of the secretory systems of the TriTryp Kinetoplastida are highly unique, both from each other and higher eukaryotes. For *T. brucei* both major life stages are dominated by GPI anchored proteins. VSG probably accounts for 90% of polypeptide delivered to the cell surface, and the VSG fold is probably conserved in several other surface antigens, specifically the invariant surface glycoproteins (ISGs) and the transferrin receptor ESAG6/7. By contrast *Leishmania* invests considerably in GPI-anchored complex glycolipids and glyoconjugates (LPG) in addition to surface proteins. *T. cruzi* expresses a family of heavily glycosylated mucin molecules; again these proteins are attached to the cell surface via a GPI anchor (see Chapter 10).

The first step of the secretory pathway, regardless of final protein topology or destination, is the import of nascent polypeptides into the ER lumen. Thus sequestered from the reducing environment of the cytosol newly synthesized polypeptides are operated on by an integrated machinery to achieve the correct folding state, disulfide formation, and specific modifications, e.g. addition of *N*-glycans or GPI anchors, to the final mature protein. Only when the protein attains the native fold is it available for transport from the ER. Proteins that fail to pass this quality control process are typically diverted into a degradative pathway referred to as ER-associated degradation (ERAD). Functional studies and database searching reveals that trypanosomes are likely able carry out all of these processes similarly to other eukaryotes. However, certain key players present in other systems are absent, and in this regard trypanosomes may provide insight into what is a minimal secretory synthesis machine.

Synthesis and ER import

In eukaryotes import of secretory proteins can be co-translational or post-translational and is mostly dependent on amino-terminal signal sequences that are removed during translocation (Rapoport *et al.*, 1999; Fewell *et al.*, 2001). Co-translational import involves recognition of the signal sequence by signal recognition particle (SRP), a cytosolic ribonucleoprotein complex of 7SL RNA and six conserved proteins. The trypanosomal SRP is unusual in that it is composed of 7SL RNA and four of the conserved proteins (SRP72, SRP68, SRP54 and SRP19), whilst the missing Alu-binding subunits are apparently replaced by a tRNA-like molecule (Lustig *et al.*, 2005). Post-translational transport

utilizes cytoplasmic chaperones of the HSP70 gene family to maintain fully translated proteins in an unfolded state prior to translocation. In either case the signal sequence, in conjunction with SRP or alone, mediates interaction with the translocon, an oligomeric protein translocation pore composed of Sec61αβγ and Sec62/63. Of these factors, orthologues for only Sec61α, Sec 61γ and Sec63 are clearly present in the trypanosome genome. This may indicate that a minimal translocon exists in divergent eukaryotes or, given that several of these factors are very small hydrophobic membrane proteins, divergence has occurred to the point where identification by BLAST fails. Nevertheless, both SRP-dependent and -independent translocation are known to occur in trypanosomes. RNAi knockdown of SRP54 is lethal but surprisingly does not block the initial translocation of endogenous reporters that represent soluble, type I transmembrane, and GPI-anchored topologies (Liu *et al.*, 2002). Similarly, knockdown of the SRP-independent pathway components Ssa1 and Sec71p (also known as Sec66) is lethal but does not block translocation of EP procyclin (S. Michaeli, personal communication). EP procyclin was also translocated in the SRP54 knockdown indicating that secretory proteins in trypanosomes may have some latitude in utilization of these two distinct pathways.

Folding and quality control

During translocation into the ER most secretory glycoproteins are modified by addition of preformed $Glc_3Man_9GlcNAc_2$ oligosaccharides to *N*-linked glycosylation sites (Helenius and Aebi, 2004). Once translocated into the lumen secretory polypeptides encounter a complex machinery of molecular chaperones that function to catalyze protein folding in a process generally referred to as quality control (Trombetta and Parodi, 2003; Kleizen and Braakman, 2004). The first component is the HSP70 family member BiP, which acts as a gatekeeper on the luminal side of the translocon and thus is the first chaperone encountered. It binds nascent proteins via exposed hydrophobic segments of the unfolded polypeptide chain. BiP then passes the folding substrate onto enzymes that catalyze folding, GRP94 (endoplasmin), and disulfide formation, protein disulfide isomerase (PDI). An alternative mechanism for holding unfolded polypeptides are the related ER lectins, membrane-bound calnexin and soluble calreticulin, which bind to monoglucosylated *N*-linked glycans generated by the action of ER resident glucosidases I and II. Bound proteins are released and enter into a cycle of further deglucosylation and reglucosylation mediated by glucosidase II and UDP-Glc: glycoprotein glucosyltransferase (UGGT) that is specific for unfolded glycoproteins (Fewell *et al.*, 2001; Trombetta and Parodi, 2003; Helenius and Aebi, 2004). Chaperones and the PDI homologue Erp57 associated with the lectin–substrate complex assist in folding. This cycle continues until the substrate protein is either folded, and therefore no longer recognized by UGGT, or is targeted as a terminally misfolded. The best-documented route to ERAD is further *N*-glycan trimming by ER α1,2-mannosidase I to generate a $Man_8GlcNAc_2$ structure that is recognized by another ER lectin called EDEM. Proteins with such structures are then targeted for retrotranslocation via the Sec61 translocon for degradation by the cytoplasmic proteasomal system. ER trimming of *N*-glycans also occurs on properly folded proteins, so how misfolded proteins are selectively targeted for ERAD is not clear.

Trypanosomes have many of the components of this machinery. A BiP homologue was the first characterized marker for the early secretory pathway in trypanosomes and, characteristic of molecular chaperones, it interacts transiently with newly synthesized VSG (Bangs *et al.*, 1993; Bangs *et al.*, 1996). Recent evidence indicates that BiP is essential (Subrahamin *et al.*, 2005). There is a clear GRP94 homologue that interestingly was first identified in *Leishmania* in a screen for defects in LPG synthesis (Descoteaux *et al.*, 2002). A PDI homologue (PDI-2, originally called BS2) was identified in the pre-genomic era in a screen for developmentally regulated proteins (Hsu *et al.*, 1989), but has subsequently been characterized as a lysosomal/endosomal protein, as has a second homologue, PDI-1 (Rubotham *et al.*, 2005). However, this localization is not exclusive because both homologues also prominently localize to the ER (JD Bangs, unpublished observations), suggesting possible dual functions, and consistent with the presence of a C-terminal tetrapeptide ER retrieval signal (KQDL) in PDI-2 (discussed below) (Bangs *et al.*, 1996). The *T. brucei* genome contains three additional PDI-like sequences (Tb927.7.5790, Tb927.4.2450, Tb927.5.1020), all of which have likely N-terminal signal sequences, but only a single W**C**GH**C** redox motif and thus are more analogous to thioredoxins than PDI or Erp57. One of these (Tb927.5.1020) has an apparent type I membrane topology with a cytoplasmic domain that ends in the sequence KKRN. This sequence conforms to the KKXX motif established as an ER retrieval signal for type I transmembrane proteins in other systems (Pelham, 1995). No KKXX motif has been identified or validated in African trypanosomes, and if this proves functional it will reveal that these parasites have adopted an unusual strategy for achieving the standard eukaryotic profile of ER chaperones. There is also a clear Ero1 homologue in *T. brucei*. For both HSPs and PDIs functional analysis of these gene families will be required to determine which have important roles in protein folding, either by RNAi or by direct biochemical approaches.

Trypanosomatid protozoa, including African trypanosomes, also have a functional lectin-mediated quality control system, albeit with some interesting variations (Parodi 1998). Indeed, these variations were critical in the discovery of the calnexin/calreticulin cycle. First, trypanosome lipid-linked *N*-glycan donors are non-glucosylated and only after the oligomannose structure is transferred to protein is a single glucose attached by UGGT (Parodi *et al.*, 1983). It is precisely because the trypanosome precursor was known to be non-glucosylated that subsequent glucosylation of protein-linked oligosaccharides, i.e. UGGT activity, was unequivocally demonstrated. In all other systems addition of a glucose residue cannot be readily distinguished from incomplete trimming by ER glucosidase II (Parodi *et al.*, 1993). Second, since only a single glucose residue is added there is no need for an ER glucosidase I activity, which is absent in trypanosomes. There is the expected glucosidase II activity (Jones *et al.*, 2005), consistent with the observation that ER glucosidase inhibitors disrupt glycoprotein synthesis and trafficking in trypanosomes (Kelley *et al.*, 1995; Jones *et al.*, 2005). VSG isolated from these cells retained additional glucose residues but no major growth defect or trafficking phenotype was reported. Finally, trypanosomes have two calreticulin genes, but no homologue of calnexin is present in any of the TriTryp genomes, indicating that a single lectin:chaperone system is sufficient. Furthermore, knockout of calre-

ticulin in *T. cruzi* had comparatively minor effects on secretion of cruzipain suggesting that there is considerable flexibility in the protein folding pathways of these parasites (Conte *et al.*, 2003). Overall these studies suggest that disruption of the transient glucosylation quality control system within the trypanosome ER is tolerated, at least in culture.

Does ER quality control feed into ERAD in trypanosomes? Trypanosomes have a well-defined cytosolic proteasomal system (Li *et al.*, 2002), and querying the database with the yeast ER α1,2-mannosidase I and EDEM sequences identifies the same array of four highly related genes (Tb927.8.2910–2940). All are currently annotated as ER mannosidase I, however, three considerations argue that they are actually EDEM homologues. First, the match is much stronger when queried with EDEM than mannosidase. Second, like bona fide EDEM, the trypanosomal homologues are missing two cysteine residues that form an intramolecular disulfide bond essential for catalysis (Lipari and Herscovics, 1996; Hosokawa *et al.*, 2001; Jakob *et al.*, 2001). Third, these proteins have putative N-terminal signal sequences and are likely to be ER luminal proteins like EDEM (Olivari *et al.*, 2005), rather than type II membrane proteins like ER mannosidase I. Thus, it is likely that trypanosomes have an ERAD system, although functional data are currently lacking (but see below).

Detailed investigations into VSG exocytosis have provided an exceptionally well-documented itinerary for this protein, as well as suggesting the presence of a quality control system. VSGs are synthesized in the ER where they are subject to rapid N-glycosylation and GPI-addition. (Bangs *et al.*, 1986, Ferguson *et al.*, 1986, Duszenko *et al.*, 1988). As mentioned above, VSG transiently interacts with BiP (Bangs *et al.*, 1996), and also rapidly dimerizes within the ER (McDowell *et al.*, 1998; Triggs and Bangs, 2003). It then trafficks rapidly to the Golgi ($t_{1/2}$ ~15 min) where it is subject to processing of both N- and GPI-glycans, followed by rapid delivery to the surface. Transport to the Golgi requires both Rab1 and Rab2 isoforms (Dhir *et al.*, 2004). Interestingly, a VSG bearing several point mutations designed to disrupt the central alpha helices is retained as an aggregate in the ER when expressed in procyclic trypanosomes, and is degraded in a proteasomal inhibitor-sensitive manner (Triggs and Bangs, unpublished data). Also deletion mutations predicted to affect VSG secondary structure, and point mutations designed to block GPI addition, result in decreased expression and reduced surface transport (Wang *et al.*, 2003, Boehme *et al.*, 2002). Collectively these results are consistent with an operative quality control system, including ERAD, in African trypanosomes. The specific role of N-glycans in VSG quality control is likely to differ between specific VSGs. Mutation of the single N-glycan addition site in MITat1.4 VSG suggested that N-glycosylation is required for efficient expression (Wang *et al.*, 2003), whereas a more systematic mutation analysis of several VSGs (including MITat1.4) indicates that N-glycosylation is dispensable for surface routing of VSG (N. Jones M. Mues, I. Subota, M. Carrington and M. Engstler, in preparation). The latter results are broadly consistent with earlier studies with the inhibitor tunicamycin that pointed against an essential role for N-glycosylation in VSG secretion (Ferguson *et al.*, 1986; Bangs *et al.*, 1986).

ER localization machinery

Luminal ER proteins must themselves be deliberately targeted to the compartment where they perform their functions. The

general scheme of this process has been documented in yeast and animal cells (Pelham 1991; Pelham 1995). Luminal proteins have C-terminal tetrapeptide sequences that conform to the canonical motif XDEL (X is any amino acid). In mammals there is some flexibility but typically it is KDEL; in yeast it is exclusively HDEL. When proteins bearing such motifs escape from the ER they encounter a specific XDEL receptor (ERD2p) that has steady-state localization in post-ER compartments. Binding to the receptor induces the complex to enter a retrograde trafficking pathway to the ER.

Available evidence suggests that the same mechanism functions in trypanosomes. The C-terminal tetrapeptide sequences of BiP, GRP94, calreticulin and PDI-2 are respectively, MDDL, AGDL, KSDL, and KQDL. The BiP and PDI-2 signals both mediate retention of transgenic secretory reporters, as does the mammalian signal, KDEL, suggesting that trypanosomes are promiscuous in signal recognition (Bangs *et al.*, 1996). However, as discussed above PDI-2, which has a functional tetrapeptide, may also be located in the lysosome suggesting that other undefined forward trafficking signals may over-ride ER retrieval. Trypanosomes also have an ERD2 homologue, and though its activity has not been well characterized, RNAi knockdown is lethal and results in a modest secretion of ER reporter constructs (Peck and Bangs, unpublished observations).

Exit from the ER

GPI addition is essential for the subsequent transport of most GPI-anchored proteins (Field *et al.*, 1994). During export from the ER, both GPI and TM proteins are recruited into COP II-coated transit vesicles; for yeast evidence suggests that GPI and TM proteins are packaged into distinct vesicle populations (Muniz *et al.*, 2001). Sorting of GPI and TM proteins depends on several factors including the GTPase Ypt1p, members of the SNARE family (Morsomme *et al.*, 2003) plus Lag1p and Dgt1p (Barz and Walter, 1999, Ferguson-Yankey *et al.*, 2002, Muniz and Riezman, 2000), two related proteins recently identified in *S. cerevisiae* and implicated as having a specific role in transport of GPI-anchored proteins from the ER. A clear Lag1p/Dgt1p orthologue is also present in *T. brucei*, but only a single family member, suggesting a simpler system in the trypanosome. Comparison between the secretion kinetics of GPI and soluble proteins has been studied in trypanosomes and the GPI acts to increase the rate of secretion (Bangs *et al.*, 1997, McDowell *et al.*, 1998, Triggs *et al.*, 2003). The mechanism of this rate enhancement is not clear.

Coat proteins

In higher eukaryotes, the various components of the endomembrane system can be discriminated based on the presence of distinct coat proteins that serve to stabilize structure and facilitate formation of transport intermediates.

Coatomer

Both the coatomer complexes (COP I and COP II) responsible for intra-Golgi and ER to Golgi transport respectively are fully conserved. COP I is composed of seven protein subunits ($\alpha\beta\beta'\gamma\delta\varepsilon\zeta$), together with ARF1, a GTPase and members of the emp47 family, and has been described experimentally (Maier *et al.*, 2001); very high conservation is seen for the $\alpha\beta'$ orthologues, with lower conservation for $\gamma\zeta$ and δ and less still for the β and ε subunits. Homologues of the emp47 family are not detectable by BLAST. For COP II, again

all of the subunits are present (sec23, 24, 13 and 31), as is the controlling GTPase, Sar1. Interestingly, there are two copies of the Sec13 and Sec23 orthologues, which may suggest the capacity for some differentiation of ER-derived transport vesicles. Similarly, members of the emp24 family have been implicated in collaborating with the COP II coat to recruit specific cargo subsets; all trypanosomatids have multiple members of this family, three in *T. brucei* and *L. major*, whilst *T. cruzi* has two. Again, this may reflect a capacity for functional specialization.

Clathrin and adaptins

Clathrin has been described in trypanosomes and comparatively well studied in *T. brucei*. Orthologous genes are present in *T. cruzi* and *L. major*. During the formation of transport vesicles coat proteins assemble on the cytoplasmic leaflet to shape the emerging vesicle and to select the cargo. Clathrin assembles on different membranes with distinct but closely related adaptor protein (AP) complexes for sorting at the *trans*-Golgi network (TGN) (AP1) or endocytic events at the plasma membrane (AP2). AP-1 and AP-2 are the better characterized, however two other AP complexes (AP-3 and AP-4) have recently been identified in various eukaryotes. AP-3 is proposed to function in sorting from the early endosome to later organelles in the secretory pathway whilst AP-4 targets proteins from the TGN to the basolateral plasma membrane (reviewed Bonifacino and Traub, 2003). All AP complexes are heterotetramers, composed of two large chains (one each of γ/α/δ/ε and β1–4, respectively, 90–130 kDa), one medium-sized chain (μ1–4, ~50 kDa), and one small chain (σ1–4, ~20 kDa) (Bonifacino and Traub, 2003). The probable evolutionary emergence of the four AP complexes has previously been inferred from phylogenetic analysis (Boehm and Bonifacino, 2001). The precursor of the modern-day AP-3 was first to diverge from the proto-AP complex, which was followed by the AP-4 complex and finally distinct AP-1 and AP-2 complexes evolved, initially sharing a single β subunit. The *T. brucei* genome encodes adaptin genes to form the AP-1, AP-3, and AP-4 complexes. The absence of the AP-2 complex (Morgan *et al.*, 2002) may be due to saturating levels of GPI-anchored VSG at the cell surface, which contains no cytoplasmic sorting signal. The *L. major* genome contains adaptin subunits for the AP-1, AP-2 and AP-3 complexes, but the sequences for the AP-4 large subunits (ε and β4) are absent and μ4 gene appears to be a pseudogene. It is unclear if the σ4 subunit is residual or has adopted a new function in the context of the remaining adaptors. The *T. cruzi* genome encodes subunits for all four complexes with the exception of the σ1 adaptin. The σ1 function may be performed by another σ subunit, as there are 4 copies of the σ3 subunit in the *T. cruzi* genome. Overall it is likely that the genome of the kinetoplastid last common ancestor encoded four AP complexes. The lineage-specific loss of AP-2 and AP-4 implies fundamental differences in the mechanisms of endocytosis between the kinetoplastids.

AP-2 binds numerous endocytic accessory adaptors, including epsin, AP180/CALM, Dab2, HIP and numb, ARH, and regulatory proteins, including amphyphysins, AAK1, GAK, auxilin, RME-8 and synaptojanin through the appendage domains of the α and β2 subunits. The Kineoplastida genomes encode homologs of HIP, epsin N-terminal homology domain proteins (but not epsin itself, Field *et al.*, 2005), AAK1, RME-8 and synaptojanin. Numerous genes also contain

BAR, FYVE and VHS domains that facilitate interaction with membranes. The scission of clathrin coated vesicles at the plasma membrane and TGN is facilitated by dynamin or the dynamin related protein VPS1. The genomes of *L. major* and *T. cruzi* encode a single dynamin related protein, whilst *T. brucei* contains two copies which are ~98% identical and likely a result of a recent tandem duplication of a short chromosomal region. The *T. brucei* gene product is involved in mitochondrial fission, and not endocytosis (Morgan *et al.*, 2004). The absence of AP2 and dynamin potentially suggests the presence of novel mechanisms for cargo recognition, sorting, recruitment of endocytic factors to the plasma membrane and for vesicle scission. It is likely that identification of any such novel process will require the direct biochemical isolation of factors and subsequent functional characterization.

Absent coats

All of the domains defined in adaptin subunits of other organisms by pfam are present in the kinetoplastida homologues with the exception of the large subunit ear domains, which is only present in the γ subunit. The related Golgi-localizing, γ-adaptin ear homology, ARF-binding proteins (GGAs) and stonins which share partial homology with the adaptins (Bonifacino and Traub, 2003) are absent from kinetoplastid genome and are Opistokhonta restricted (Field *et al.*, 2005). Given the high preponderance of GPI-anchored molecules in trypanosomatids, and the association of these proteins with caveolin-mediated trafficking in metazoans and yeast, it is of significance that caveolin is absent from the trypanosome genomes. Again, caveolin is evolutionarily restricted and is metazoan specific. Overall the trypanosomes appear to have a rather minimal vesicle coat system, but the configuration has been remarkably stable between the kinetoplastids.

Sorting signals, ubiquitin, multivesicular bodies and the lysosome

Sorting signals

The direct identification of sorting signals incorporated into *trans*-membrane domain proteins is not very advanced in the trypanosomatids. For endocytosis a recent study of an acid phosphatase from *Leishmania* identified the C-terminal cytosolic region (MEVWRRYMKFKNKQSEAIIV) as containing potential sorting signals related to the conventional tyrosine and dileucine-containing signals from the higher eukaryotes, including a requirement for the very C-terminal IIV motif for endocytosis (Weise *et al.*, 2005). A similar motif is present in the lysosomal protein p67 (see below).

A second endocytosis signal, a requirement for at least one of three conserved lysine residues within the cytoplasmic region of ISG65 has been identified (Chung *et al.*, 2004). This is highly suggestive of a role for monoubiquitination in tagging of *trans*-membrane proteins for endocytosis. Trypanosomes possess much of the ubiquitination machinery for processing endocytic cargo, but similar to the clathrin system, there are several significant absentees.

Ubiquitination and the multivesicular body

Ubiquitin is activated by covalent attachment to ubiquitin ligase in an ATP-dependent reaction. Initially uniquitin is conjugated to an E1 class ligase, and the ubiquitin is passed to an E2 and finally an E3 ligase; only the E3 ligases appear capable of direct transfer to the ultimate substrate.

For modification of endocytic cargo, two E3 ligases have been found to be particularly important, Rsp5 and c-abl; these two enzymes appear to be responsible for monoubiquitination of a variety of plasma membrane proteins that are subsequently internalized (Hicke and Dunne, 2003). Neither of these ligases are found in the trypanosome genome, despite the presence of the majority of the downstream system, the ESCRT complexes, which are responsible for subsequent recognition of ubiquitinated cargo and, sorting and delivery to the MVB (Bowers *et al.*, 2004; Fig. 9.3). MVBs are also clearly identifiable structures within the trypanosome by electron microscopy and the machinery required for deubiquitination is highly conserved, indicating that the ESCRT system likely processes ubiquitinated proteins. Significantly expression of a dominant negative allele of Vps4 is lethal (Sutterwala and Bangs, unpublished). It is therefore likely that trypanosomes use a novel system for ubiquitination of endocytic cargo.

The lysosome

The lysosome has historically been defined as a static terminal degradative compartment of the endocytic pathway (Kornfeld and Mellman, 1989). It is operationally defined as a discrete organelle of high density (ρ = 1.10 g/ml) and low pH ($\leq$ 5.0), containing a unique complement of structural membrane glycoprotein markers (lysosome-associated membrane proteins; LAMP) and acid hydrolases (lipase, proteases, phosphatases and glycosidases). However, in recent years it has been recognized that the lysosome is a more dynamic organelle that is in constant communication with the Golgi, other endosomal and pre-lysosomal compartments, and even the plasma membrane (Andrews, 2002; Luzio *et al.*, 2003).

In African trypanosomes the lysosome is a single discrete organelle located at a perinuclear position in the posterior end of the cell. Morphologically it is indistinguishable between the procyclic and bloodstream stages. Given that endocytosis is so greatly upregulated in the bloodstream parasites it is no surprise that lysosomal activities are also upregulated; specifically elevated expression of phosphatase and proteolytic activities relative to levels found in insect stage (Langreth and Balber, 1975; Pamer *et al.*, 1989; Mackey *et al.*, 2004). This difference likely reflects issues of nutrient acquisition for each stage. Procyclic cells can rely on the hydrolytic environment of the tsetse midgut to provide solutes for transport, while the bloodstream stage must take up and digest for itself host serum macromolecules.

In bloodstream parasites the lysosome can be labeled by uptake of fluorescent and electron-opaque markers of both fluid phase and receptor-mediated endocytosis (Langreth and Balber 1975; Alexander *et al.*, 2002; Engstler *et al.*, 2004; Hall *et al.*, 2004). Most biologically relevant in terms of receptor-mediated uptake is transferrin, which has a well-defined receptor in the FP (Ligtenberg *et al.*, 1994; Overath *et al.*, 1997), and trypanolytic factor (TLF) (Shimamura *et al.*, 2001). Some evidence suggests that transferrin is delivered to the lysosome and degraded for nutritional purposes, however some of this may be recycled. TLF is an unusual subclass of human HDL that is toxic for non-human infectious African trypanosomes. It has an unidentified high affinity receptor in the FP (Drain *et al.*, 2001), and upon delivery to the lysosome is activated to kill the parasite. Killing is dependent on lysosomal pH and proteolytic activity (Bishop *et al.*, 2001). Interestingly, killing may also be dependent on interchain disulfide re-

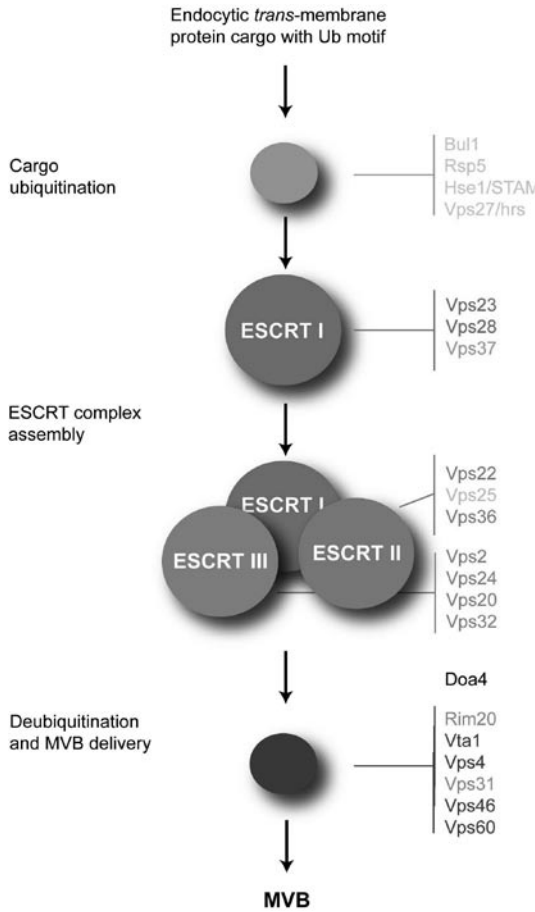


Figure 9.3 The ESCRT complex system. In higher eukaryotes incoming cargo molecules are either ubiquitinated at the plasma membrane or by the Rsp5 complex. None of the components of this complex are found in trypanosomes and in particular Vps27/hrs is restricted to the Opisthokonta (Field *et al.*, 2005). Ubiquitinated proteins are then recognized by ESCRT I and this complex then assembles with two further ESCRT complexes (II, and III). Removal of ubiquitin, in an ATP-dependant manner and delivery to a multivesicular body requires the function of Doa4 together with the ESCRT III-associated complex). Factors not identified in the trypanosome genome are shown in faded text and all factors are shade-coded according to the complex to which they associate. The figure is based on data from Bowers *et al.* (2004), together with additional interrogations of the trypanosome database.

duction of haptoglobin-related protein, an essential component of TLF for cytotoxicity (Shimamura *et al.*, 2001; Shiflett *et al.*, 2005).

Luminal lysosomal proteins

In cell fractionation studies typical acid hydrolase activities used in other systems, including protease, glycosidase, lipase and phosphatase, have been taken as markers for the lysosome in trypanosomes (Steiger *et al.*, 1980; Opperdoes and Van Roy, 1982). However, few of these activities have been characterized at the molecular level. Two activities that have been are the cysteine protease homologues, cathepsin-L

(trypanopain) and cathepsin-B. The former has been known for a long time (Mottram *et al.*, 1989; Pamer *et al.*, 1989), that latter has only been recently identified by database searching (Mackey *et al.*, 2004). Selective membrane-permeable inhibitors of cysteine proteases are toxic for bloodstream trypanosomes (Scory *et al.*, 1999; Troeberg *et al.*, 1999) and trypanopain was initially believed to be the target. However, RNAi studies indicate that cathepsin-B, but not trypanopain, is essential for trypanosome viability *in vitro* suggesting that cathepsin-B is the target of these potentially pharmacologically useful inhibitors (Mackey *et al.*, 2004).

Both enzymes are synthesized as inactive pro-proteins that are activated upon arrival in the lysosome by proteolytic removal of N-terminal prodomains. In trypanosomatids there is no *N*-linked mannose-6-phosphate (Cazzulo *et al.*, 1990), the ligand for the classic receptor-mediated targeting of lysosomal hydrolases in animal cells (Kornfeld and Mellman 1989), and it is presumed that the peptide prodomains contain the essential targeting information. In *T. cruzi* the prodomain of cruzipain, a trypanopain orthologue, was able to deliver GFP to the lysosome and targeting was disrupted by mutation of a specific sequence with homology to a lysosomal targeting motif in mammalian cathepsin L (Huete-Perez *et al.*, 1999). However, similar GFP fusions with the trypanopain prodomain in *T. brucei* failed, and mutation of the putative targeting motif led to ER localization of trypanopain suggesting retention of misfolded protein (S. Sutterwala and J.D. Bangs, unpublished observations). Misfolding is perhaps not surprising since amino acid residues within the putative targeting motif are also required for proper folding of mammalian cathepsin L (Chapman *et al.*, 1997).

The only other characterized luminal lysosomal components are the PDI-1 and PDI-2 proteins described above (Rubotham *et al.*, 2005). Their presence in the lysosome is unusual, particularly for PDI-2, which as discussed previously, has a functional ER retention tetrapeptide. On the other hand, lysosomal/endosomal localization of these PDI homologues could account for observed disulfide bond reduction of haptoglobin-related protein when TLF enters the endosomal compartment of susceptible trypanosomes (Shimamura *et al.*, 2001).

Lysosomal membrane proteins

The lysosomal membrane of animal cells typically contains abundant glycoproteins, the best characterized of which, LAMP1 and LAMP 2, are type I transmembrane proteins bearing multiple *N*-glycans extensively modified by terminally sialylated poly-*N*-acetyllactosamine (Hunziker and Geuze, 1996). LAMPs are thought to form a protective glycocalyx on the luminal face of lysosomes (Granger *et al.*, 1990).

A single protein, p67, has been identified in trypanosomes with the basic properties of mammalian LAMPs (Brickman and Balber 1994; Kelley *et al.*, 1999). p67 was originally identified by biochemical approaches and the genome reveals two closely related genes. p67 has no sequence homology with known vertebrate lysosomal proteins, but its overall structure is analogous to LAMP-1 and LAMP-2, in that has a type I topology, is highly glycosylated (14 potential *N*-glycosylation sites), and in bloodstream trypanosomes is extensively modified by addition of poly-*N*-acetyllactosamine. As with mammalian LAMPs, the function of p67, other than to provide a convenient marker for the lysosome, is not clear. However, RNAi studies indicate that it is essential (R.F. Peck and J.D. Bangs,

unpublished observations). Targeting to the lysosome in procyclic stage cells is dependent on two di-leucine motifs within the p67 cytoplasmic domain (Alexander *et al.*, 2002) (N.N. Tazeh and J.D. Bangs, unpublished observations). Presumably these motifs interact with components of the clathrin/adaptin coated vesicle machinery as has been demonstrated for di-leucine motifs in mammalian systems (Hunziker and Geuze, 1996; Robinson and Bonifacino, 2001). Surprisingly, in bloodstream parasites deletion of the entire cytoplasmic domain has no effect on lysosomal targeting (Alexander *et al.*, 2002), suggesting that alternative targeting information is a stage specific manner, or alternatively that the lysosome is simply the default destination in this endocytically active stage of the life cycle.

Although other lysosomal membrane proteins have not been characterized in trypanosomes, one protein that is most certainly present is a V-type proton ATPase (V-ATPase) required for endosomal and lysosomal acidification (Nishi and Forgac, 2002). V-ATPases are composed of two multisubunit domains, a cytosolic V_1 ATPase domain (eight subunits) and a transmembrane V_0 proton translocation domain (five subunits). Putative homologues of all subunits are present in the *T. brucei* genome database.

GTPases controlling pathways

The Rab and Arf GTPase subfamilies are mainly involved in membrane trafficking and closely related functions, most significantly in vesicle tethering and fusion for specific steps within the endomembrane system. The specificity of Rab proteins for subcellular compartments makes them highly attractive as markers for compartments of the exocytic and endocytic systems and has been exploited for the trypanosomes (Morgan *et al.*, 2003, Cappai *et al.*, 1993, Mendoca *et al.*, 2000).

Rab proteins

There are 16 Rabs encoded by the *T. brucei* genome, 18 in *L. major* and 22 in *T. cruzi* (Berriman *et al.*, 2005). For a unicellular organism this likely reflects a comparatively sophisticated vesicular transport system. The large number of Rabs in the trypanosomatids, particularly in *T. cruzi*, may reflect substantial flexibility within the vesicular transport system and in particular the endocytic apparatus. In contrast to *Saccharomyces cerevisiae*, but in common with mammals, not all of these genes encode a protein with a dicysteinyl C-terminal motif and hence there is potential for heterogeneity in the prenylation of trypanosomatid Rabs (Ackers *et al.*, 2005).

Core functionality, i.e. ER to plasma membrane exocytosis, endocytosis, recycling and lysosomal delivery are controlled by Rabs 1, 2, 4, 5, 7 and 11—all of which are fully conserved in the trypanosomatids. Interestingly, Rab4 is absent from the malaria parasite, and presumably all recycling is mediated in this organism by a Rab11-dependent pathway. Additionally Rab6, which is responsible for retrograde transport through the exocytic system and Rab18, which has a poorly characterized role in Golgi and post-Golgi transport, are also conserved in trypanosomes and *Plasmodium* suggesting that, for protozoa at least, these pathways are part of the minimal Rab complement required for viability. Exocytosis is comparatively simple in the trypanosomes, with the only suggestion of functional complexity being the presence of two Rab1 isoforms in *T. brucei*. Significantly, none of the kinetoplastida have a Rab3 or Rab27 homologue, suggesting the absence of a regulated se-

cretion pathway. However, it is possible that highly specific systems have evolved as Rab3 and Rab27 are also absent from *Plasmodium*, which clearly has several regulated exocytic systems, including the rhoptries, dense granules and micronemes. By contrast, the endocytic system may be more varied between the trypanosomatids. Two Rab5 isoforms are well conserved between the three organisms, and some evidence from *T. brucei* suggests that these GTPases control pathways that transport distinct cargos (Pal *et al.*, 2002), whilst *T. cruzi* possesses a third Rab5 gene (Araripe *et al.*, 2005, Ramos *et al.*, 2005). Further, *T. brucei* possesses a single Rab11 gene, whilst *Leishmania* and *T. cruzi* possess two and three Rab11 genes respectively—however, two of the *T. cruzi* Rab11 genes are almost identical and hence may have essentially the same function, whilst the final Rab11 isoform in *Leishmania* and *T. cruzi* is highly divergent, and may have a unique function (Berriman *et al.*, 2005). Some developmental alterations in expression of the single *T. brucei* Rab 11 isoform have been reported and are suspected to underpin stage-specific changes in endocytic rates (Jeffries *et al.*, 2001). A number of additional Rab genes are also present in the genomes of the kinetoplastida that fall outside of basic core functions. This group have clear homologues in the higher eukaryotes. Represented are Rab14, 21, 23, 28 and 32, with only *T. cruzi* possessing a Rab32 homologue (which has been implicated in mitochondrial function) and *T. brucei* lacking both Rab32 and Rab14 isoforms. The limited data available on Rab14 suggest a potential role in phagocytosis and lysosomal delivery, suggesting a potential additional pathway in *Leishmania* and *T. cruzi* compared to *T. brucei* (Harris and Cardelli, 2002). Recent analysis of TbRAB23 suggests that this protein is present in the nucleus, although studies of the mammalian Rab23 indicate a location on cytosolic membranes (Dhir and Field, 2004; Evans *et al.*, 2003). Phylogenetic analysis also indicates that TcRab32 and TbRab1B are distantly related to other trypanosome Rabs, and hence will require direct investigation for assignment. A detailed comparative analysis between *T. brucei* and higher eukaryotes identified three trypanosome Rab genes, designated X1, X2 and X3 with no clear higher eukaryote homologues (Ackers *et al.*, 2004). Both X1 and X2 are associated with the exocytic system, but their precise function has not been described (Field and Field, 1997; Field *et al.*, 1999, 2000). Further, RabX2 is unusual in that it lacks GTPase activity; this has been verified experimentally for *T. brucei* (Field *et al.*, 2000). Clearly the presence of these genes in trypanosome genomes, and their absence from both the higher eukaryotes and *Plasmodium*, suggests a specific and important function.

Rab proteins operate in concert with a large number of additional proteins, including those that modulate the GTPase cycle directly (e.g. GTPase activating proteins, GAPs, and G-nucleotide exchange factors, GEFs), others responsible for vesicle fusion and tethering (including the SNARE proteins and tethering factors) and several phosphatase activities. There are insufficient genes in the genome for each trypanosome Rab to be partnered by a specific GAP, and this reflects the integration of the system also observed in higher eukaryotes.

Other GTPases involved in transport

The cellular functions of the ARF protein family can be broadly classified as regulation of membrane traffic and of the cytoskeleton. The ARF family appears to func-

tion by a distinct mechanism, undergoing a conformational change on GTP hydrolysis that is distinct from that used by the Rab/Ras proteins (Pasqualato *et al.*, 2002). ARF genes may be divided into several subfamilies, including the ARFs, ARF-like proteins (ARLs) and Sar1 family. The latter is an important mediator of recruitment of the COP II coat to the ER, and is present in all three kinetoplastida. The Sar1 family is rather simple, with a single member being retrieved from the *T. brucei* and *T. cruzi* genomes, whilst *L. major* has two genes which are ~98% identical, and presumably the result of a recent gene duplication.

In strong contrast to the ancient emergence of a core set of Rab proteins, which are shared between most eukaryotes, the ARF family appears to be less universal. Phylogenetic reconstruction of the kinetoplastida ARF/ARL families suggests that there are only two distinct ARF isoforms in the trypanosomes and three in *Leishmania*, but the point of divergence is so deep that the protozoan genes have clearly evolved from a single precursor that predates speciation. Significantly, all of the trypanosome ARF family retain the conserved myristylation signal at the N-terminus, and therefore are likely membrane associated. Therefore the last common ancestor of trypanosomatids and metazoans most likely possessed a single ARF gene; in consequence prediction of function of trypanosome ARF genes based on sequence homology is likely to be uninformative. Interestingly, for one isoform there are four distinct ORFs in the *T. brucei* and *T. cruzi* genomes—for *T. brucei* these four constitute a tandem array on chromosome 9. Interestingly, the ARF6 protein is associated with clathrin-independent endocytosis (Donaldson, 2003), which is a pathway that appears completely absent in the African trypanosome—in agreement with this observation, no ARF6 homologue is present in the genome. The importance of this gene family in trypanosomes is further underscored by recent studies demonstrating that several members of the family are essential in *T. brucei* and indeed play a role in membrane transport (D.F. Smith, personal communication). The ARL gene family in the kinetoplastida is even more divergent than the ARFs, but again most evidence from other systems supports a role mainly involved with vesicle transport, membrane dynamics and associated cellular systems. *H. sapiens* has eight distinct ARL isoforms, but by phylogenetic reconstruction only two of these, ARL2 and ARL6, are shared with the trypanosomatids; the conservation of ARL2 is particularly interesting as this protein is potentially a very important factor in membrane dynamics in higher eukaryotes and is also conserved in yeasts and nematodes. In addition, the ARF4, 7, 5, 6 and 8 are not found in *S. cerevisiae*, further evidence for independent evolution of the ARL family in distinct lineages. The clear absence of ARL1 and ARL3 orthologues may also reflect differences in the Golgin family in trypanosomatids—only a single member of this family, related to Golgin 55, is present in the genome, but this isoform does not possess a GRIP domain (Dhir *et al.*, submitted).

The remaining human ARLs have distinct lineages from the protozoan sequences. A large family of ARLs are present in the kinetoplastida, several subfamilies of which are shared by two or more of the genomes. Only two ARL proteins have been studied in any depth in trypanosomatids, an ARL3 homologue in *Leishmania* and an ARL designated ARL1 in *T. brucei*. For the *Leishmania* gene product more extensive phylogenetic reconstruction using the

whole genome suggests that this assignment is incorrect as this sequence is in fact not very similar to ARL3 from higher eukaryotes and is designated as ARLD (Berriman *et al.*, 2005); the *Leishmania* protein appears to have numerous affects, with a principal function in flagella biogenesis (Cuvillier *et al.*, 2000). The *T. brucei* ARL1 has a clear role in membrane transport, with a defect in exocytosis—however the phenotype is pleomorphic and a precise role has not yet emerged for this molecule (Price *et al.*, 2005). The degree of divergence in the phylogenetic relationships is highly suggestive of lineage-specific evolution, and indicates the acquisition of a large family of GTPases in the parasitic systems that likely have no true equivalent in the higher eukaryotes.

SNARE proteins

The SNARE family comprises a number of coiled-coil transmembrane proteins that are responsible for vesicle fusion. These proteins form a four-helical bundle that promotes membrane apposition and eventual fusion, facilitating delivery of vesicle contents to an acceptor membrane. SNAREs function in collaboration with Rab proteins, and in common with Rabs, exhibit specificity in their subcellular localization. SNAREs are difficult to identify based on sequence data alone.

Analysis of the *T. brucei* genome for SNARE proteins identifies 20 ORFs that conform to minimal criteria, having a molecular weight between 15 and 30kDa and containing a SNARE-like coiled coil. These sequences populate SNARE family clades for proteins implicated in transport to early endosomes (Snc2, TLG), Golgi to late endosomes (Ykt), ER to Golgi (Sec22), *trans*-Golgi network (syntaxin 6) and intra-Golgi (syntaxin 5). There are also several trypanosomatid SNAREs that do not cluster with a specific higher eukaryote SNARE clade; these ORFs may indicate novel transport steps, or more likely sequence divergence precluding assignment. Overall, the trypanosome SNARE proteins suggest a conserved core in the endomembrane system, which is consistent with the data from analysis of Rab proteins (above).

Developmental regulation of trafficking systems

The observation of a major difference in endocytic rates between the bloodstream and procyclic forms of *T. brucei* is comparatively old. Recent work is beginning to uncover the molecular basis underpinning this difference and to suggest potential biological reasons for the developmental regulation. Various estimates of rate differences between BSF and PCF stages put the increase in endocytosis at between 10- and 100-fold (Langreth and Balber, 1975; Pal *et al.*, 2002, Engstler *et al.*, 2005). Recent work now reveals that the precise stage-specific differences may vary between trypanosome strains. While the monomorphic strain *T. brucei* MITat1.2 (427) reveals a 12-fold difference in endocytosis rates between procyclic and bloodstream stages, the endocytic upregulation in *T. brucei* AnTat1.1 is 23-fold when procyclic cells are compared to the proliferating slender bloodstream stage, and 68-fold for procyclic and stumpy cells. This is in agreement with a measured three- to fourfold augmentation in endocytosis rate between slender and stumpy bloodstream stage trypanosomes (Engstler *et al.*, in preparation).

Several important protein factors of the endocytic system have differential expression, specifically the clathrin heavy chain and Rab11, whilst differences in location have been described for the two Rab5

homologues (Pal *et al.*, 2002; Morgan *et al.*, 2001; Jeffries *et al.*, 2001). Upregulation of clathrin and Rab11 in the BSF are clearly consistent with an increased endocytic rate, and to maintain membrane homeostasis a similar augmentation in the recycling arm is also required. Further, differentiation of the BSF early endosome population into Rab5A and Rab5B subpopulations (the two markers colocalize in the PCF) may also indicate a stage-specific requirement for differential sorting of endocytic cargo; however both pathways appear essential, and Rab5A appears to mediate VSG, immunoglobulin and transferrin receptor endocytosis, while knockdown of Rab5B also inhibits endocytosis, transferrin uptake and the distribution of poly-*N*-acetyllactosamine-containing glycoproteins (Pal *et al.*, 2002; Hall *et al.*, 2004).

Analysis of clathrin function in BSF and PCFs by RNAi knockdown indicates a constitutive role in endocytosis (Allen *et al.*, 2003; Hung *et al.*, 2004). An additional function is uncovered in the PCF stage, specifically in delivery of *trans*-membrane proteins to the surface as well as a role in post-Golgi transport; it is however likely that this function is also constitutive but simply masked in the BSF by the extreme effect on endocytosis (Allen *et al.*, 2003; Hung *et al.*, 2004).

In terms of recycling, it appears that the roles that Rab4 and Rab11 play in this process, as well as some of the basic features of the system, are under developmental control. For example in BSF the majority of internalized surface protein (VSG) is recycled but by contrast the equivalent material is delivered to the lysosome in the PCF (Hall *et al.*, 2005a). Further, in BSFs Rab4 plays a role primarily in delivery of fluid phase endocytic cargo to the lysosome and has a minimal function in receptor-mediated endocytosis or recycling, whilst Rab11 appears required for receptor-mediated endocytosis as well as lysosomal targeting of surface-bound lectins and recycling, but not fluid phase endocytosis (Hall *et al.*, 2004, 2005a). In the PCF, however, Rab11 is required for fluid phase endocytosis, a function of Rab4 in BSFs, and internalization of surface proteins. Rab4 also contributes to these processes in the PCF as well as having a role in recycling (Hall *et al.*, 2005B). Clearly, these findings suggest that extensive remodelling of the endocytic system accompanies differentiation.

There is little evidence for major remodelling of the exocytic system. As the two major life stages of *T. brucei* have similar cell surface areas and doubling times, it is unlikely that there is a major requirement for modulation of exocytosis. The major ER chaperone, BiP, is moderately upregulated in the BSF (Bangs *et al.*, 1993), which may be required for folding of the structurally more complex VSG compared to the simpler procyclins, whilst the Golgi complex appears more extensive in BSFs at least by staining with antibody to Rab31, possibly to facilitate the synthesis of complex class *N*-glycans (Field *et al.*, 2000). A major difference in the cellular response to depletion of exocytic cargo was uncovered recently by RNAi knockdown of VSG (Sheader *et al.*, 2005); significantly depletion of VSG resulted in rapid cell cycle arrest and rounding up of cells but in contrast knockout studies of procyclins have demonstrated that PCFs remain viable without their major coat proteins (Vasella *et al.*, 2003).

Membrane transport and virulence

Rapid endocytosis is a major feature of the bloodstream stage of *T. brucei* that is absent from the procyclic form. It is unlikely that

there is a heightened requirement for endocytosis of nutrient receptors as BSF and PCF trypanosomes have similar growth rates; calculations suggest that, for transferrin (iron) at least, there are an excess of receptors (Steverding, 2003) plus the parasite likely has mechanisms for controlling the expression level of receptors to ensure sufficient nutrient uptake (Mussman *et al.*, 2004; Hall *et al.*, 2005a). Classical models of the surface of the African trypanosome have emphasized the extreme abundance of VSG, and the near-saturating concentration of the protein at the plasma membrane (Overath *et al.*, 1997, Mehlert *et al.*, 2002); these models propose that the VSG monolayer acts as an effective barrier against antibody recognition of invariant surface determinants. This view is consistent with concentrative mechanisms that appear to maintain the level of VSG at the surface (Grunfelder *et al.*, 2002) and a requirement for continual VSG expression to maintain cell viability and normal morphology (Shreader *et al.*, 2005). However, these models are essentially static and VSG is highly mobile within the lateral plane of the cell surface. The concept that VSG shields invariant determinants has been challenged by the demonstration that antibodies can recognize members of the abundant 65 kDa invariant surface glycoprotein (ISG_{65}) family (Ziegelbauer *et al.*, 1992; Jackson *et al.*, 1993) on living trypanosomes (Chung *et al.*, 2005). Further, antibodies recognizing ISG_{65} can be detected in the sera of chronically infected bovines (M. Carrington, personal communication) whilst antibody to ISG_{65} (termed ISG_{70} in that study) was detected in the serum of a calf infected with *T. theileri* (Doherty *et al.*, 1993). Both of these studies raise the possibility that the host's immune system can recognize invariant determinants on living trypanosomes, and asks how, with this happening, a chronic trypanosome infection can be maintained. Anti-VSG immunoglobulin bound to the surface of living parasites in *in vitro* cultures is rapidly internalized, degraded and recycled, whereas the stability of the VSG is unaltered (Barry, 1979; O'Beirne *et al.*, 1998; Pal *et al.*, 2003). This internalization of immune complexes can afford trypanosomes rapid protection from antibody-dependent destruction of trypanosomes during an immune response (Balber *et al.*, 1979), and may well also operate for antibody that is bound to non-VSG surface determinants.

Recently significant progress has been made towards understanding the molecular basis for host range restriction of *T. brucei brucei*, which is not human infective and is rapidly killed by trypanolytic factor in human serum, as opposed to *T. brucei rhodesiense*, that is highly human infective. The basis for this resistance is the serum resistance associated gene (SRA), which encodes a VSG-like membrane protein (Xong *et al.*, 1998). SRA interacts in some manner with trypanosome lytic factor via its apolipoprotein L1 subcomponent (Vanhamme *et al.*, 2003), but there is conflicting evidence concerning the cellular itinerary of SRA, and consequently how it prevents lysis by TLF is unclear. One line of evidence suggests that SRA protein resides in the lysosome and acts directly in this location to block TLF activity (Vanhamme *et al.*, 2003). The alternative view is that SRA resides in earlier endosomal compartments and acts by preventing delivery of TLF to the lysosome where the lytic process initiates (S. Hajduk, personal communication).

Recent work has shown that the procyclin coat undergoes a programmed maturation within the tsetse fly, and that this process may be modeled in *in vitro* cul-

ture by specific modification to the growth medium (Acosta-Serrano *et al.*, 2001; Vassella *et al.*, 2004). It is not at all clear what the role of these changes may be in ensuring development and progression through the insect vector, but very recent data have shown that manipulation of recycling pathways in procyclic forms can lead to specific alterations to the location, surface display and abundance of procyclins (Hall *et al.*, 2005b). As procyclin is a highly stable protein, specific degradation of procyclin isoforms may be required to facilitate the developmental progression that is seen in the insect vector, and potentially this could be achieved via membrane transport events.

Evolutionary aspects of membrane transport

The availability of over 20 completed genomes from a wide range of eukaryotic taxa now makes a rational exploration of evolution of the endomembrane system feasible. The addition of three trypanosomatid genomes specifically broadens understanding of intracellular transport systems as trypanosomes represent the first genomes for organisms of the Excavata, a rather poorly defined and undersampled clade (Simpson and Roger, 2003). Further, *T. brucei* is an attractive organism on account of distance from the crown eukaryotes (the Opisthokonta), excellent RNAi technology and a comparatively well-structured endomembrane system. In the broadest of terms, the new data confirm and extend the existing paradigm that the major gene families responsible for vesicle transport are ancient and universally present (Dacks and Field, 2004; Field *et al.*, 2005; Table 9.1). The entire vesicle fusion system (including syntaxins, NSF, SNAPs etcetera), Rab and ARF family, coatomers, adaptins and clathrin-associated proteins are fully represented by members of all eukaryotic

Table 9.1 Evolutionary distributions of major vesicle transport factors**.** Genomic distribution, by BLAST analysis of multiple completed genomes, for numerous members of transport systems was performed and phylogenetic reconstruction used to identify systems likely present throughout the eukaryota and those restricted to specific kingdoms (informatics details in Field *et al.*, 2005). Factors are listed as universal, Opisthokonta-specific (includes metazoa and fungi) and metazoa only. Owing to a lack of direct investigation (for example by forward genetics, proteomic, etc.) it is unclear if novel coats, fusion systems or other mechanisms are present within trypanosomes

Universal	Opisthokonta-specific	Metazoa-specific
Rab	epsin	Stonin
SNAREs	eps15	Caveolin
GARP	vps27/hrs	Dab2
ESCRT	GGA	
Clathrin		
Adaptins		
Retromer		
epsinR		
eps15R		
COP I		
COP II		

taxa as are genes encoding components of the GARP, COG, ESCRT and retromer systems. The implications of these findings are wide-ranging, indicating that the last common eukaryotic ancestor possessed a highly sophisticated membrane transport system. Given the great divergence times between the taxa (e.g. estimated at 3×10^9 years before present for the trypanosomatid/Opisthokonta split) these findings, together with measurements of genetic distance, also suggest that the root of the Eukaryota may have been evolutionarily distant from the last common ancestor that gave rise to the radiation of eukaryotic kingdoms (Simpson and Roger 2003). Does this represent a particularly spectacular example of punctuated equilibrium, whereby the long root is in fact a reflection of a very rapid period of evolution, which in temporal terms may have been rather short (Eldridge and Gould 1972), or was there a prolonged period of stasis prior to a rapid period of speciation at the deepest level?

A number of Opisthokonta-specific factors have arisen, for example caveolin, which mediates a clathrin-independent endocytic pathway, is found only in metazoa. Further, dab2 and stonin, which function with clathrin as cargo adaptors, are also metazoa specific (Field *et al.*, 2005). Further, the GGA family, vps27 and epsin and eps15 are also found only in the Opisthokonta. The latter three factors may indicate a fundamental difference in the manner in which endocytic cargo are handled in the crown eukaryotes compared to the protists; specifically epsin, eps15 and vps27 all interact with ubiquitinated proteins, and their absence suggests either a lack of a role for ubiquitin in endocytosis or the presence of distinct mechanisms in trypanosomes. Interestingly, when considering domains as opposed to entire proteins, there is a high representation of PH domains within the trypanosome genome and an absence of SH2 domains, suggesting a considerable bias towards phosphoinositide signalling and away from kinase-mediated pathways (El-Sayed *et al.*, 2005). As both kinase and phosphoinositides contribute to regulation of trafficking in higher eukaryotes, this finding is potentially highly significant for understanding the integration of transport in trypanosomes.

Perspectives

The study of membrane transport in trypanosomes has advanced considerably in the last 15 years, in part due to the substantial progress made in model systems that has served to enable identification and characterization of markers and pathways in trypanosomes at the molecular level. More recently exploration of trypanosomal systems in their own right has begun, and the advent of RNAi together with the full genome has facilitated broader approaches as well as more detailed interrogation of specific systems. Over 500 ORFs are likely associated with transport processes in some manner, representing ~15% of the protein coding capacity of the genome. In general trypanosomes retain the core endomembrane functions, but clearly the many absentee factors imply novel mechanisms. Determining these trypanosomal mechanisms together with definition of the likely role of membrane transport in virulence remain as exciting challenges for the years ahead.

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