CHAPTER ONE

Macromolecular Trafficking and Immune Evasion in African Trypanosomes

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Contents

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Abstract

Intracellular trafficking is a major mechanism contributing to maintenance of the surface composition in most eukaryotic cells. In the case of unicellular eukaryotic pathogens, the surface also represents the host–parasite interface. Therefore, the parasite surface is both a critical player in immune recognition, from the host's point of view, or in immune evasion, from the pathogen's point. The African trypanosomes are remarkable in dwelling throughout their period in the mammalian host within the bloodstream and tissue spaces, and have evolved several mechanisms that facilitate chronic infection. Here, we discuss current understanding of intracellular trafficking pathways of trypanosomes, and relate these processes to immune evasion strategies by the parasite and avoidance of immune responses from the host.

Key Words: Antigenic variation, Immune evasion, Intracellular trafficking, Protein sorting, Trypanosome.

1. General Overview of the Trypanosome Life Cycle

The lifestyle of *Trypanosoma brucei,* the African trypanosome, is venturesome, and unlike the vast majority of endoparasites, has exploited mechanisms allowing survival without sequestration within host cells. While thriving in the body fluids of vertebrate hosts, the parasites are continuously attacked by the immune system and also endure harsh physicomechanical conditions that prevail in the mammalian circulation.

The mammalian host is infected when a tsetse fly carrying trypanosomes (*Glossina* spp.) takes a blood meal (Vickerman, 1985; Vickerman et al., 1988) (Fig. 1.1). The insect's bite inoculates the parasites into the host's subcutaneous tissue, where they rapidly proliferate. Metabolic products, or other undefined factors, cause local inflammation of the skin at the bite site, producing a so-called chancre, which is the first sign of infection. From here, rapidly dividing cells enter the draining lymphatic system and are

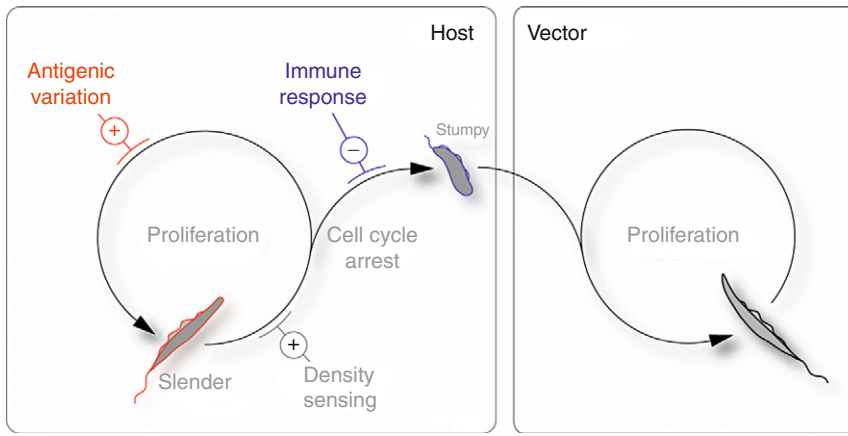

Figure 1.1 Partial life cycle of trypanosomes, emphasizing the transition from the mammalian host to the insect vector. Left box (host): Proliferative slender forms (gray) survive within the host bloodstream, lymphatics, and issue spaces. These cells exhibit antigenic variation as the primary route to immune evasion, facilitating chronic infection. Additionally, a density sensing mechanism, mediated by stumpy induction factor, causes the slender forms to exit from the cell cycle, preventing overgrowth of the host by excessively high parasite numbers. Cell cycle exit is accompanied by morphological changes, resulting in the stumpy form, as well as additional underlying metabolic and proteome changes, preadapting the parasite to life in the tsetse fly. These cells are sensitive to immune killing and do not exhibit antigenic variation. Right box (vector): On entry to the tsetse midgut, stumpy form parasites reenter the cell cycle and begin to proliferate. A fuller version of the parasite life cycle is detailed in Vickerman (1969).

flushed into the circulation. An early symptom of infection is enlarged cervical lymph nodes of the neck, the Winterbottom's sign, but both of these symptoms fade rapidly and have limited diagnostic use (Ormerod, 1991).

The parasites are well prepared for life in the mammalian host. The quiescent, nondividing metacyclic stage, which awaits transmission in the insect salivary glands, expresses a special version of the most conspicuous weapon that trypanosomes have evolved to defend against host attack: A dense layer of a single protein species, the variant surface glycoprotein (VSG), shields the entire cell surface (Pays, 2006). In the skin tissue, following injection the metacyclic trypanosomes reenter the cell cycle and proliferate as long slender trypomastigotes (Fenn and Matthews, 2007). Energy metabolism in this form is simplified and relies completely on nonoxidative consumption of glucose, which is constantly provided by the host. In the bloodstream, the trypanosome population oscillates between proliferative slender forms and cell cycle-arrested stumpy forms. The parasites secrete an, as yet, undefined low-molecular-weight factor which triggers differentiation to the stumpy stage in a cell-density-dependent manner. As parasitemia rises, more stumpy induction factor (SIF) accumulates and the cells become quiescent (Reuner et al., 1997). Thereby, population density is limited, allowing the parasites to persist for significant periods without killing the host. Thus, one could argue that the stumpy stage acts altruistically in sacrificing itself for the sake of population survival. In fact, stumpy forms survive only for a rather short period (a few days) and appear to be efficiently eliminated by the humoral immune response. However, the stumpy stage is crucial for life-cycle progression as only they can successfully establish an infection in the transmitting insect vector (Fig. 1.1). Stumpy forms are preadapted to a life within the fly, including increased mitochondrial activity, reorganization of the endomembrane system, and hypersensitivity to environmental cues such as cold shock and citrate/*cis*-aconitate (Engstler and Boshart, 2004).

When the fly ingests stumpy form trypanosomes, the parasites immediately encounter a loss of temperature homeostasis. This has been suggested to cause upregulation of the major insect stage surface proteins, procyclins (Engstler and Boshart, 2004). The expression of procyclins is accompanied by rapid loss of the VSG coat. How exactly the VSG is lost is not fully understood, but the participation of both a metalloprotease and phospholipase appears likely (Gruszynski et al., 2003). The expression of distinct procyclins during the process of adaptation to the insect environment has also been described in a series of elegant experiments, however, the function of the new cell-surface coat remains unclear (Acosta-Serrano et al., 2001). In fact, procyclins appear to be dispensable both *in vitro* and their expression is not essential for successful passage through the tsetse fly (Vassella et al., 2009).

In the insect, trypanosome metabolism switches from glycolysis to cytochrome-mediated oxidative respiration. After establishing a midgut infection, trypanosomes migrate to the fly salivary glands, to complete the life cycle. Little is known about this journey and how trypanosomes navigate through the fly. Having reached the salivary glands, the epimastigote parasites attach to the microvilli of epithelial cells (Urwyler et al., 2007). This anchoring is mediated by the trypanosome flagellum through an, as yet, unknown mechanism. The density of the actively dividing epimastigotes within the salivary glands can become rather high. Finally, the attached epimastigotes give rise to quiescent (i.e., nondividing) metacyclic trypanosomes, which reacquire the VSG coat. This completes the trypanosome life cycle.

Although this basic itinerary for *T. brucei* transmission through host and vector has been known for decades, the signals and molecular responses underlying the alterations between proliferative and quiescent stages remain enigmatic. While considerable effort and progress in understanding the slender-to-stumpy and stumpy-to-procyclic differentiation events has been made recently, and a molecular marker, PAD1, has now been identified for the stumpy stages, very little is known about transition between fly stages (Dean et al., 2009). This is in part due to the fact that fly resources are limited and few laboratories are experienced in maintaining and manipulating tsetse. This extensive research effort should be devoted to understanding the fly–parasite interaction and development stages within the tsetse fly is underlined by exciting recent findings showing that meiosis and the exchange of genetic material occurs only within the fly; hence the tsetse fly is formally the definitive host.

2. Immune Evasion Mechanisms

2.1. VSG and antigenic variation

The ability to survive in the vasculature of the mammalian host, despite constant exposure to a highly sophisticated immune response, was a major challenge to understanding of trypanosome virulence mechanisms. It is now 30 years since seminal work identified the VSG surface coat as the basis for antigenic variation (Cross, 1977).

A 15 nm thick layer of 5×10^6 identical VSG homodimers covers each trypanosome cell. This amazingly high concentration of a single species of plasma membrane protein is unprecedented, and represents $\sim 90\%$ of cell-surface protein. The spacing between individual VSG dimers is 3–5 nm, which effectively shields most of the VSG epitopes from antibody recognition (Overath and Engstler, 2004). The classical view proposes that other

surface proteins are buried within the VSG coat and that the plasma membrane is virtually untouchable by the immune system. However, a full description of the mammalian host immune response remains to be achieved and it is possible that additional determinants are recognized.

Evolution may have shaped the molecular structure of VSG for maximum efficiency as an immunological shield. Two extended α-helices project perpendicular to the cell surface and provide the molecule with an extended conformation (Freymann et al., 1990). VSG proteins generally comprise two domains, a larger externally disposed N-terminus of 350–400 residues and a smaller C-terminal domain 40–80 residues that is proximal to the plasma membrane (Chattopadhyay et al., 2005). Once packed into the surface coat, only a restricted number of amino acids are accessible to external probes. The exposed amino acid positions are highly variable, while more conserved sites, especially several cysteines required for disulfide bond formation and hence secondary structure, are less accessible (Field and Boothroyd, 1996). While the crystal structure of two N-terminal domains was solved some 20 years ago, the structure of the relatively conserved C-terminal was described only recently (Chattopadhyay et al., 2005). NMR analyses revealed that C-terminal domains display related core structures consisting of two α- or 3_{10}-helices and two antiparallel β-sheets. However, the length of the secondary structure elements and the loops connecting these elements vary between different C-terminal domains. Up to now no complete VSG structure has been solved, and the structure of the connection between the two domains remains unknown. More importantly, we still have little structural appreciation of the linkage between the VSG C-terminal domain and the hallmark of VSGs, the glycosylphosphatidylinositol (GPI) anchor.

The GPI anchor is rapidly added posttranslationally to the C-terminus of VSG and ultimately anchors the protein to the outer leaflet of the plasma membrane (Martin and Smith, 2006). The lipid anchoring of VSG has many important implications for the physicochemistry of the trypanosome cell surface and VSG sorting. A great many GPI-anchored proteins (GPI-APs) with various functions have been described in many organisms (Field and Menon, 1993). However, compared to *trans*-membrane proteins, GPI-APs are rare or of low abundance in most taxa. As the insect stage dominant antigens, procyclins and BARP, are also GPI anchored, African trypanosomes link all dominant surface proteins via a GPI anchor to the plasma membrane independent of the life-cycle stage (Engstler et al., 2004; Urwyler et al., 2007).

The unique structure of VSG and its ability to support an extreme form of molecular crowding on the cell surface would not help the parasites to survive in the mammalian bloodstream unless coupled to antigenic variation. In fact, the immune system would actually become hyperactivated by an almost crystalline array of protein epitopes, accurately displayed on the

surface of the trypanosome cell, and VSG is known to be an effective immunogen. During rising parasitemia the majority of trypanosomes belong to a particular antigenic type, giving rise to a strong antibody response, which kills the large majority of trypanosomes bearing that VSG. These parasites are apparently cleared from the bloodstream. However, antigenic variation replaces the VSG on the cell surface of a small proportion of trypanosomes with another, and immune selection determines if these parasites expressing a new VSG can survive. Laboratory strains switch rarely, with a rate of 10^{-6}–10^{-7} per cell generation. Recent work suggests that the antigenic switching rate in natural isolates is much higher, up to 10^{-2}–10^{-3} (Lythgoe et al., 2007). A repertoire of hundreds of different VSGs that can potentially be expressed on the cell surface indicates that the host immune response always lags behind, allowing trypanosomes to survive for prolonged periods.

VSGs are transcribed from one of 20 telomeric expression sites (ESs) (Cross, 1996; McCulloch, 2004; Pays, 2006). The VSG gene is located at the end of a long polycistronic transcriptional unit, and transcribed by RNA polymerase I. This transcription unit contains more than 10 genes, which are termed as expression site associated genes (ESAGs). The function of many ESAGs is still unknown. The active ES is localized to an extranucleolar structure that is known as the ES body (Navarro and Gull, 2001). Besides the ES-linked copies, the majority of VSG genes locate as nontranscribed basic copies to either large arrays in central regions of megabase chromosomes or as single copies on mini chromosomes. The basic copies are abundantly flanked by repeats that facilitate homologous recombination with the active ES.

Multiple mechanisms for mobilizing VSG genes have been described. Activation of a silent ES displaying another VSG is known as an *in situ* switch. This form of switching not only activates another VSG, but also a new array of ESAGs. It has been postulated that activation of alternative ESAGs could be involved in adaptation to various host environments (Gerrits et al., 2002). Telomere exchange between ESs or gene conversions that introduce all or part of a basic copy VSG into the active ES are also known. The potential for creation of mosaic VSG sequences from multiple basic copy ORFs extends the repertoire hugely, while gene conversion most probably dominates in field infections, and provides access to this near limitless repository.

2.2. Membrane dynamics and antibody clearance

The VSG coat is endocytosed with unprecedented speed and fidelity (Engstler et al., 2004). An area equivalent to the entire plasma membrane is internalized and recycled once every 10 min. Compared to membrane recycling in other organisms this is amazingly fast kinetics, especially when

taking into account that all membrane traffic is routed via the tiny flagellar pocket, which only accounts for ~2% of surface membrane (Field and Carrington, 2009). In trypanosomes, internalization of plasma membrane and embedded proteins is restricted to clathrin-mediated endocytosis (CME). Comparatively large clathrin vesicles, termed CCV class I, abundantly bud from the flagellar pocket. Every second six new CCVs are rapidly transported by an actin-dependent mechanism to early endosomes, which are located between 2 and 4 μm away from the pocket. About 60–70 CCV class I can be found in the posterior part of the cell. CCV I fuse to early endosomes that are easily detectable in trypanosomes as mostly circular cisternal structures (Grünfelder et al., 2002). In bloodstream forms this also appears independent of dynamin, but in the procyclic it is possible that dynamin is required (Morgan et al., 2004; Chanez et al., 2006).

The next step on the itinerary of endocytosed membrane is the recycling endosome, which appear to be the main sorting station of GPI-APs. 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 lyosome but is redirected to recycling endosomes, albeit with comparatively slow kinetics (Engstler et al., 2004). In trypanosomes, recycling endosomes are most prominent endomembrane structures. In this "recycling factory," ligands uncouple from their receptors and are sorted together with endocytosed fluid-phase cargo into smaller clathrin-coated vesicles (CCV class II), which abundantly bud from the rims of the cisternal recycling endosome (Grünfelder et al., 2003). CCV class II have two destinations within the cell: late endosomes and the lysosome. They are devoid of VSG, transferrin receptor (TfR), and some *trans*-membrane proteins (e.g., invariant surface glycoproteins (ISGs)). Thus, by a negative mechanism, namely withdrawal of membrane, GPI-APs are passively (i.e., by default) concentrated (Overath and Engstler, 2004). How GPI-proteins are excluded from entry into budding vesicles remains to be elucidated. The subregion of the recycling endosome that carries the concentrated VSG eventually gives rise to small, disk or cup-shaped carriers that fuse with the flagellar pocket. These exocytic carriers (EXCs) are profusely found within the posterior part of the cell (Grünfelder et al., 2003).

Why have trypanosomes evolved such a sophisticated and highly active plasma membrane recycling machinery? The selective pressure for uptake of sufficient nutrients from host blood is one obvious answer. However, it has been shown that the vast majority of fluid-phase cargo is actually not transported to the lysosome, but is excreted by the cells (Engstler et al., 2004).

More than 20 years ago, it was reported that antibodies bound to the trypanosome cell surface are internalized and most probably routed to the lysosome. These early studies suggested that host antibodies are cleared from the cell surface of bloodstream trypanosomes within 15–30 min (Russo et al., 1993). A more recent report has revealed that VSG-bound IgGs (VSG-IgG) accumulate at the flagellar pocket region within 20–40 s. VSGs and bound antibodies are rapidly internalized via CME (Allen et al., 2003; Engstler et al., 2007). 3D-fluorescence microscopy and quantitative colocalization analyses with organelle-specific marker proteins have confirmed that a significant amount of antibody is routed via late endosomes to the lysosome, while VSG is recycled to the cell surface.

The internalization of antibody-bound VSG from the trypanosome cell surface comprises three consecutive steps, each displaying distinct temperature sensitivities. Initially, VSG–IgG complexes accumulate at the posterior pole of the cell. The kinetics of this process is comparatively independent of temperature; even at 12 °C rapid antibody accumulation is observed. In contrast, the rate of entry of VSG–IgG complexes into the flagellar pocket is significantly decelerated at 24 °C, and at 12 °C the process almost halts. Once arrived in the flagellar pocket, VSG–IgG is internalized by bulk membrane uptake. The endocytosis of IgG-bound VSG exhibits similar temperature sensitivity as the entry into the flagellar pocket. Hence, the three-step antibody clearance process involves posterior accumulation of antibodies as immediate event and passage through the flagellar pocket as rate-limiting step. Posterior accumulation of VSG–IgG does not result from VSG shedding and is independent of endocytosis.

Downregulation by RNAi of clathrin heavy chain causes a block of all endocytic traffic. Although in clathrin-depleted cells endocytosis is stalled, VSG–IgG accumulates at the flagellar pocket in a similar manner as in control cells. Posterior accumulation of VSG–IgG requires energy as the glycolytic inhibitor 2-deoxyglucose decreases posterior accumulation of VSG–IgG complexes. When cellular motility is stalled by ATP depletion, no antibody accumulation is observed, but cells remained uniformly coated with immunoglobulin, suggesting a correlation between cellular motility and antibody clearance (Engstler et al., 2007).

Bloodstream forms of *T. brucei* swim with an average speed of 20 μm s^{-1}. A directional, spiral trajectory is mediated by a single flagellum, which emerges from the flagellar pocket, attaches to the cell body, and extends beyond the anterior pole of the cell (Fig. 1.2). FlaI is required for the connection of the flagellum to the cell body, and downregulation of FlaI results in detachment of the flagellum and loss of directional motility. FlaI-depleted trypanosomes retain their VSG coat and VSG-bound antibodies are internalized from the flagellar pocket with similar kinetics as in control cells. Strikingly, obstruction of directional swimming coincides with a loss of accumulation of antibody-bound VSG at the posterior cell surface.

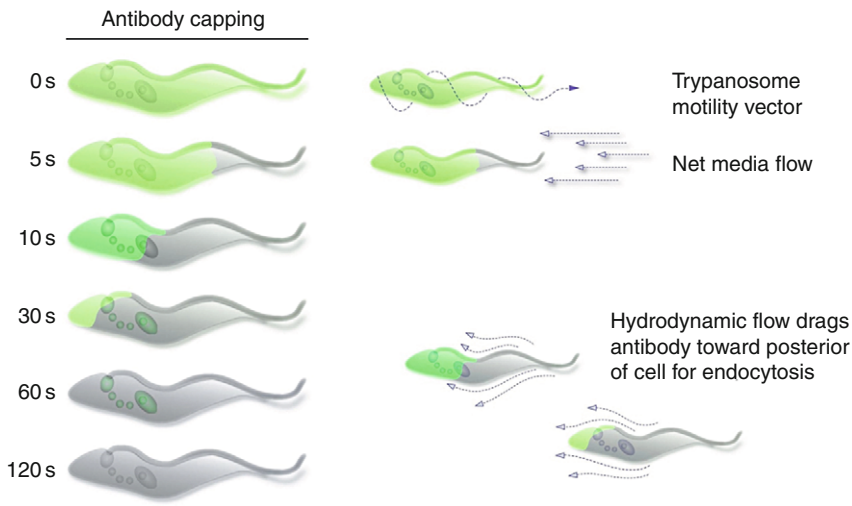

Figure 1.2 Immune evasion by antibody capping; a role for hydrodynamic flow. Left column: Antibody recognizing the surface variant surface glycoprotein (VSG) is rapidly capped toward the posterior of the cell. The high rate of endocytosis facilitates efficient uptake of the antibody, which is ultimately degraded; the uptake process, even in the presence of high antibody titers, can be completed within 2 min. Right column: Trypanosomes continuously swim, and thereby generate directional flow fields on their cell surface. These flow forces become more significant when the surface VSG is recognized by immunoglobulins. Antibody–VSG complexes are pulled by hydrodynamic forces toward the rear of the cell, where they are endocytosed. This implies that purely physical forces can sort proteins in the plane of the plasma membrane. The schematic shows antibody in green coating VSG in gray. Note that the mechanism for capping, or delivery of antibody to the flagellar pocket, is likely distinct from that which operates in metazoan cells.

Conversely, a marked reduction in antibody concentration is observed in the flagellar pocket area, which is explained by continuing membrane recycling in the absence of antibody accumulation. Clearly, there is direct involvement of cell motility and endocytosis in antibody clearance (Engstler et al., 2007).

The size, but not the nature of the protein bound to VSG, is critical for accelerated removal from the cell surface; for example, IgM is removed much more rapidly than Fab fragments. Mammals respond to trypanosome infections with elevated levels of VSG-specific IgM rather than IgG. Both immunoglobulins are internalized in a concentration-dependent manner but the overall kinetics of IgM-uptake is significantly faster. This observation is difficult to explain on the basis of specific recognition of antibody-bound VSGs by cytoplasmic adapter proteins as VSG is anchored via a GPI-anchor.

A provocative alternative possibility is that hydrodynamic flow acts on swimming trypanosomes and specifically drags VSG–Ig complexes toward the flagellar pocket (Fig. 1.2). The most direct evidence for

hydrodynamic drag arises from downregulation of the dynein arm intermediate chain DNAI1, which reverses the trypanosome swimming direction. Hydrodynamic drag predicts that the immunoglobulins would be pushed toward the other cell pole in the DNAI1-supressed cells, and is exactly what is observed (Engstler et al., 2007).

However, the role of antibody clearance for parasite survival *in vivo*, that is, in the natural host, remains to be elucidated. Antibody removal could be crucial for parasite survival during early parasitemia, when antibody titers are still low, after differentiation to the cell cycle-arrested stumpy stage, which is critically required for the successful completion of the parasite life cycle or for removal of immunoglobulins recognizing invariant epitopes. All of these possibilities remain to be fully investigated.

2.3. Cellular immune responses

The trypanosome lifestyle is provocative in its complete exposure to the mammalian host, yet, at the population level they prosper in the face of massive immune attack. VSG is highly immunogenic and trypanosomes produce it in huge amounts. The immune system, when confronted with a wave of parasites, aims at rapidly clearing the trypanosomes, and paradoxically this mechanism is essential for chronic infection. Rapid growth of trypanosomes can overwhelm the host within days, and one mechanism to avoid this is the continuous secretion of SIF, triggering differentiation from long slender to stumpy stage. The importance of SIF is underscored by the observation that strains that have become unresponsive to SIF kill their host within a few days. Further, generation of VSG-specific antibodies is crucial to early host survival and B cell-deficient mice poorly control trypanosome infections (Baral et al., 2006).

Antibody-opsonized trypanosomes will be phagocytosed and destroyed by hepatic Kupffer cells. The paracrystalline structure of the VSG coat, with homogeneously organized epitopes, may explain why the early immune response is largely T cell independent (Mansfield, 1994). The VSG-specific antibody response is crucially insufficient for efficient protection during early phases of infection and IFN-γ appears to be required. The majority of IFN-γ is produced after T cell stimulation through antigen presenting cells (Magez et al., 2006). The absence of MHC-II diminishes IFN-γ production and parasite control. Also natural killer cells have been proposed to be involved in the production of IFN-γ (Mansfield and Paulnock, 2005).

In the beginning, it was believed that IFN-γ would bind directly to trypanosomes stimulating their proliferation. Now it appears clear that *in vivo* IFN-γ is responsible for a proinflammatory reaction, which attacks the parasites (Namangala et al., 2001). Mice deficient of either IFN-γ or IFN-γ receptor reveal high-level parasitemia and die earlier than control animals. The IFN-γ-mediated control involves classic macrophage

activation. The effector cells produce a range of antitrypanosome compounds, including tumor necrosis factor alpha (TNF-α) as well as reactive oxygen species, which cause the so-called host oxidative burst. Reactive nitrogen, NO, is also involved. Interestingly, the role of NO as inflammatory effector appears to be distinct for infections with different trypanosome species (Vincendeau et al., 1992). Toll-like receptors are thought to be involved in the activation of macrophages by T cell-derived IFN-γ and endogenous TNF-α. The toll-like receptors represent a steadily growing family of proteins that can recognize conserved pathogen signatures, such as the bacterial lipopolysaccharide, nonmethylated DNA, or GPI structures (Coller et al., 2003). During the cyclical destruction of large trypanosome populations, massive amounts of these molecules are released into the bloodstream. Consequently, parasite control involves toll-like receptors that recognize the VSG-GPI and free nonmethylated CpG DNA sequences.

Highly inflammatory reactions govern the initial phase of infections with African trypanosomes. The long-lasting type I immune response leads to many of the pathological signs associated with sleeping sickness, such as anemia, fever, splenomegaly, liver damage and neurological disorder and cachexia, from which any infected human or animal will finally die. Therefore, having survived the first wave of parasitemia, the infected host needs to remodel its immunological landscape to enhance survival chances. Although TNF-α knockout mice are not able to control parasitemia efficiently, they reveal prolonged survival (Magez et al., 1999). This is also true for mice treated with anti-IFN-γ antibodies. The initial proinflammatory phase ends by secretion of cytokines and generation of alternatively activated macrophages. Interleukin 10 inhibits classically activated macrophages. The alternatively activated macrophages display anti-inflammatory properties and could dampen the pathological consequences of type I immune response.

In addition to the induction of various immune pathological damage in different tissues, immunosuppression is yet another consequence of trypanosome infection and T cell suppression is severe. Obviously, this leads to frequent opportunistic superinfections, which additionally weaken the host. Early on, immunosuppression appears to be mediated by NO, prostaglandin E2, and TNF-α from classically activated macrophages (Mansfield and Paulnock, 2005; Sternberg and Mabbott, 1996). At later stages, the immunosuppression is mediated by alternatively activated macrophages, but our knowledge about the mechanisms behind this process is rudimentary.

African trypanosomes are capable of manipulating and controlling the highly sophisticated immune system of their vertebrate host. Apparently, this hijacking can occur at very different levels. While some trypanosome species kill their host rather quickly, other infections can last for months or even years. In any case, most hosts will not survive their encounter with trypanosomes.

2.4. Innate immunity

Fascinating exceptions to the fatal outcome are humans and primates, which are fully protected against most trypanosome species. Human innate immunity against African trypanosomes has been known for more than a century. However, only recent extensive work of several laboratories has finally shed light on the molecular mechanisms behind the trypanosome resistance.

Normal human serum (NHS) *in vitro* is cytotoxic for *T. brucei*. Neither immunoglobulins, the complement activation pathway nor the blood clotting system are responsible. Furthermore, NHS is not activated by trypanosome-derived factors. Many different substances and macromolecules have been put forward as candidate trypanosome lytic factor (TLF), one of which, high-density lipoprotein (HDL), was postulated as early as 1960 to be involved in trypanosome killing. The first systematic study on the nature of TLF, demonstrated the involvement of HDL and revealed that trypanosome lysis is a two-step process (Rifkin, 1978). Initially trypanosomes are motile and intact but start swelling. After about an hour, lysis commences. The kinetics of trypanosome killing depends on human serum concentration and temperature; at 4 °C the process is totally inhibited but is optimal at ~37 °C (Rifkin, 1984). Although this key concept of trypanosome lysis by TLF was formulated in the mid-1980s, it required 20 years to identify TLF, to understand the molecular mechanism of lysis and to unravel the secret of resistance to TLF by human pathogenic trypanosomes, namely *T. brucei rhodesiense*.

Since it was known that TLF was an HDL component, the obvious candidate for the toxic factor was the major constituent of HDL itself, namely apoA-1 (Gillett and Owen, 1991; Hajduk et al., 1989). However, biochemical characterization of HDL revealed that TLF is only a minor subset of human HDL. Furthermore, transgenic mice expressing human apoA-1 were not significantly protected against trypanosome infection (Rifkin, 1991). Fractionation of HDL and reconstitution revealed that more than one protein was required for the assembly of the lytic particle, suggesting that distinct TLF components may act cooperatively (Hajduk et al., 1989). A more detailed analysis of TLF proteins suggested paraoxonase–arylesterase and haptoglobin-related protein (Hpr) as potential toxins. Since paraoxonase–arylesterase is present in nonlytic human HDL, it could be excluded. The haptoglobin-related protein, on the other hand, was selectively enriched in TLF and antibodies directed against the protein were shown to inhibit lysis in a dose-dependent manner (Smith and Hajduk, 1995).

Hpr is restricted to primates (Maeda, 1985). Although the physiological function is unknown, it is evolutionarily derived from haptoglobin, an abundant acute-phase protein forming a high-affinity complex with free hemoglobin. Haptoglobin is critically involved in clearance of hemoglobin

from the circulation after intravascular hemal lysis. The haptoglobin–hemoglobin complex is recognized by macrophage surface receptors and then degraded. Hpr is a dimer, and in contrast to haptoglobin, is associated with apoA-1. Interestingly, although it was generally accepted that TLF was part of HDL, reports suggesting an additional trypanolytic activity appeared.

This second lytic factor had a higher molecular mass than TLF and was termed TLF-2 (Tomlinson et al., 1997). It was reported to be a lipid-poor complex containing mainly IgM together with apoA-1 and Hpr, while TLF-1 contained essentially apoA-1 and Hpr. Haptoglobin is a potent inhibitor of TLF-1, but does not interfere with TLF-2-meditated lysis. Apparently, the specific lytic activity of TLF-1 was much higher than that of TLF-2 (Raper et al., 1999). Confusingly, haptoglobin levels below physiological concentrations were shown to be sufficient to completely inhibit TLF-1. However, purified TLF-1 protected mice in a dose-dependent manner against trypanosome infections, and it was generally accepted that Hpr was the actual toxin. Further, phylogenetic analyses of the primate lineage revealed a direct correlation between the presence of Hpr and trypanolytic potential. While sera from several old world monkeys contained trypanolytic activity, this was not the case for chimpanzee serum. The Hpr gene of chimpanzees was thought to be a pseudogene, but recent data suggest that the coding region is intact (Lugli et al., 2004).

Trypanosome lysis by TLF/TLF-2 was initially believed to be initiated at the plasma membrane (Rifkin, 1984). However, a detailed cellular study showed that TLF rather affects the endocytic pathway after receptor-mediated endocytosis (Hager et al., 1994). There are about 350 high-affinity TLF binding sites within the flagellar pocket, and electron microscopy revealed that gold-labeled TLF is endocytosed and transported to the lysosome, where it accumulates and causes disintegration of the organelle. The release of lysosomal proteases into the cytoplasm would be lethal to the parasite. It was also suggested that free radical generation through the Fenton reaction at low pH could cause peroxidation of lysosomal membrane lipids, contributing to membrane disruption (Bishop et al., 2001). A further mechanism was proposed whereby Hpr, or part of it, disrupted the membrane directly (Molina Portela et al., 2000). The generation of transgenic mice expressing Hpr added yet another level of complexity to the problem. Although Hpr was properly incorporated into HDL, those mice were not protected against trypanosomes (Hatada et al., 2002).

Two *T. brucei* subspecies are resistant against TLF and are the causative agents of human sleeping sickness. It was known that TLF-resistant strains become susceptible when maintained for long periods in mice, and when exposed to human serum, resistant parasites arise from sensitive populations at low frequency. This apparent high-frequency reversal was difficult to reconcile with classical genetic views of resistance. In fact, it appears that antigenic variation and resistance to human serum are linked processes.

The underlying gene responsible for TLF resistance was identified and termed serum resistance associated gene (SRA) (de Greef and Hamers, 1994). Curiously, the SRA sequence closely resembles VSG, although the region encoding surface exposed loops was deleted from SRA. Thus, SRA is likely a truncated VSG (Campillo and Carrington, 2003). However, unlike VSG, SRA is not expressed on the cell surface, but appears to be restricted to endosomes and lysosome (Vanhamme et al., 2003). The exact localization within the endosomes and the rate of recycling remains to be elucidated. Importantly, when SRA was expressed in human serum sensitive trypanosomes, the parasites became human infective (Xong et al., 1998).

SRA is expressed from just one expression site as an ESAG. The exact mechanism by which SRA confirms human serum resistance is still not unambiguously clear. Mutation analyses suggest that an N-terminal helix could be involved in neutralizing the TLF toxin (Vanhamme et al., 2003). Through interaction with immobilized SRA a fraction from normal human serum was isolated. Surprisingly, Hpr did not bind to SRA, but apoL-1 revealed specific and strong binding. ApoL-1 is associated with HDL and its physiological function is still unclear. Recombinant apoL-1 revealed an SRA-dependent trypanolytic potential. ApoL-1 is found in both TLF-1 and TLF-2, and consequently, this lipoprotein was suggested to be the only trypanosome toxin in human serum (Shiflett et al., 2005). Like Hpr, apoL-1 can be found in many primates, but is absent from the chimpanzee genome (Poelvoorde et al., 2004).

It remains an open question whether apoL-1 and Hpr have to act synergistically for full protective immunity, but reconstitution experiments suggest that both proteins contribute to human serum resistance. While apoL-1 is necessary and sufficient for lysis, Hpr may mediate binding of TLF to a cognate receptor at the trypanosome cell surface. This receptor has recently been identified as a glycoprotein modified by poly-*N*-acetyllactosamine (pNAL) residues and located in the flagellar pocket (Vanhollebeke et al., 2008). In mice, the receptor binds the haptoglobin–hemoglobin (Hp–Hb) complex with high affinity and is responsible for the uptake of sufficient heme for incorporation into trypanosome hemoproteins. *T. brucei* apparently cannot discriminate between Hp–Hb and TLF1–Hpr–Hb complexes. Consequently, in human blood Hb-charged TLF1 complexes are targeted to the trypanosome cell. Thus, it appears that Hpr is required for high-affinity TLF-receptor binding, while apoL-1 is the actual toxin.

The exact mode of apoL-1-mediated lysis is still a matter of debate. However, it appears likely that it acts by generating pores in the lysosome membrane (Perez-Morga et al., 2005; Vanhollebeke et al., 2007). ApoL-1 reveals surprising homology to the pore-forming domain of bacterial colicins. In fact, it was shown that the N-terminal domain of apoL-1 can generate ionic pores *in vitro* and *in vivo*. Furthermore, a pH-sensitive domain is thought to be involved in membrane targeting. Thus, a possible scenario

for trypanolysis by human serum can be summarized as follows. In the flagellar pocket, TLF1–Hpr–Hb particles bind to the TLF-receptor via Hpr. Passage of the internalized complex through the endocytic pathway is accompanied by progressive acidification. This induces conformational changes in the apoL-1 membrane-tethering domain, which could facilitate sequestration of apoL-1 from the HDL carrier. Free apoL-1 inserts into the membrane of the acidic lysosome. Within the membrane, the two central hydrophobic helices of the apoL-1 pore-forming domain open a pore, leading to depolarization of the lysosomal membrane. Influx of chloride ions from the cytoplasm into the lysosome generates osmotic pressure that triggers lysosome swelling. The depletion of cytoplasmic chloride results in compensatory chloride uptake from the extracellular environment. Eventually, the developing intracellular pressure compromises the plasma membrane and the trypanosome is killed. While there are certain aspects of the model that still await experimental confirmation, it provides the so far most complete explanation of trypanolysis in human serum.

3. Endocytic Pathways

The endocytic system is an important component of the intracellular trafficking system that modulates the composition of the cell surface through the sorting of internalized proteins, lipids, and glycoconjugates into recycling or degradative pathways, and thus play a crucial role in many biological functions, including maintenance of the cell surface, immune modulation, signal transduction, and nutrition (Piper and Katzmann, 2007). Study of the uptake of various ligands and surface proteins, coupled with development of subcompartment-specific markers has allowed considerable delineation of the major endocytic routes in *T. brucei* and the organism probably has the best characterized endocytosis apparatus of any protist. Light and electron microscopy have revealed the presence of morphologically distinct populations of early and late endosomes, based on the kinetics in which they are loaded with material and the specific sets of markers they contain (Engstler et al., 2004; Grünfelder et al., 2002, 2003). Further, the later endocytic compartments, the multivesicular body and lysosome have also been defined, and in part functionally investigated (Leung et al., 2008; Peck et al., 2008).

3.1. Clathrin-mediated endocytosis

All endocytosis in African trypanosomes is CME and occurs solely at the flagella pocket (FP; Allen et al., 2003; Overath et al., 1997) (Fig. 1.3). CME involves assembly of receptor-bound cargo and GPI-anchored membrane

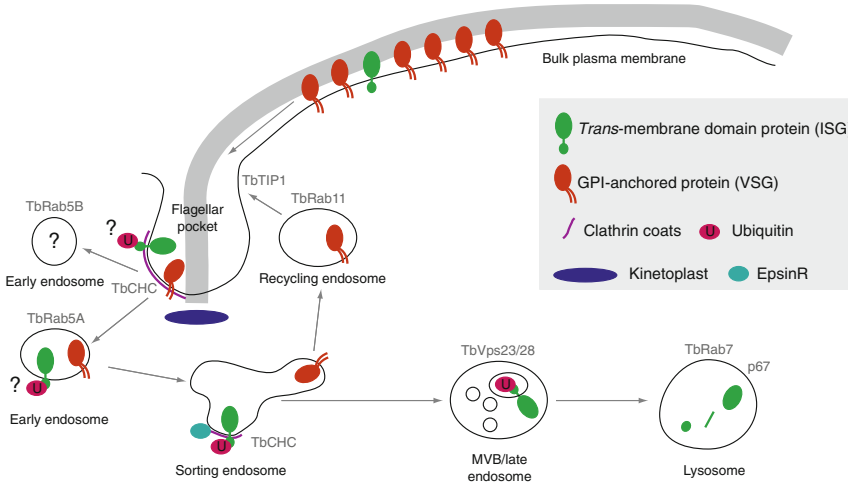

Figure 1.3 A model of sorting of GPI-anchored and *trans*-membrane domain proteins in trypanosomes. *Trans*-membrane domain proteins such as ISGs (green) are present at low density at the cell surface in comparison to the dominant GPI-anchored VSG (red). There is presently no evidence that there is selective partitioning of GPI versus *trans*-membrane anchored proteins at the cell surface. Endocytosis requires the function of clathrin (purple) at the flagellar pocket, and for ISG65 and VSG, this serves to target the molecules to the Rab5A-positive early endosome. VSG is segregated at the sorting endosome, and is excluded from a clathrin-tagged membrane microdomains; it is hypothesized that clathrin may actively sort *trans*-membrane domain proteins at this location, via recognition of ubiquitylated cargo (pink lozenge); this may involve the trypanosome epsin-related protein, which interacts with clathrin (cyan lozenge). Recycled molecules are returned to the cell surface via a Rab11-dependent pathway that also involves a coiled-coil Rab11-interacting protein that likely serves as a docking site at the flagellar pocket. *Trans*-membrane domain cargo is delivered to the lysosome via the multivesicular body, and degraded. This latter pathway depends on functioning of the ESCRT complex, including TbVps23. The site(s) where ubiquitin is added are unknown. Also the model assumes that all GPI-anchored proteins are recycled and all *trans*-membrane domain proteins are directed to the lysosome—this is unlikely to be the case, but data concerning trafficking of additional factors are not available at this time. Finally, the function of the Rab5B endosome remains mysterious as besides the presence of lactosamine-repeat determinants, the identity of the molecules transported via this route are unidentified.

components into clathrin-coated pits, which are formed at the site of endocytosis by recruitment and polymerization of assembly units of the cytoplasmic protein, clathrin, composed of three heavy chains (CHC), each tightly associated with a single light chain (CLC; Kirchhausen and Harrison, 1981). This structural organization results in a basket-like polyhedral lattice (Brodsky et al., 2001) that assists in deformation of the underlying membrane (Conner and Schmid, 2003) into coated pits. Subsequently, these pits invaginate and pinch off, forming class I CCVs (Engstler et al., 2004;

Grünfelder et al., 2003; Overath and Engstler, 2004). Class I CCVs rapidly shed their coat and the resulting vesicles dock and fuse with an early endosomal compartment. Subsequent intracellular sorting of components designated for degradation occurs via a negative sorting mechanism (Overath and Engstler, 2004). Here, class II CCVs containing components for lysosomal degradation bud from early and recycling endosomes while VSGs are concentrated in Rab11-positive, flat, disc-like structures designated EXCs that fuse with the FP (Grünfelder et al., 2003).

Mammalian forms of African trypanosomes exhibit extremely rapid endocytosis and recycling compared to the insect forms. This upregulation of ~10-fold (Natesan et al., 2007) is suspected to be involved in immune evasion in bloodstream forms (Morgan et al., 2002). A similar variation is exhibited in clathrin expression levels (Morgan et al., 2001; Natesan et al., 2007), evidence for developmental regulation of endocytosis. Clathrin ablation is lethal due to a direct arrest of vesicular traffic from the FP leading to an enlarged FP or "BigEye" phenotype (Allen et al., 2003; Hung et al., 2004). This underscores the unique role of clathrin and CME as the sole endocytic mechanism in African trypanosomes.

CCV-formation machinery in *T. brucei* is unusual (Fig. 1.4). First, pit formation does not involve a concentration step (Grünfelder et al., 2003), a hallmark of CME in other eukaryotes. Second, since the *T. brucei* genome does not code for the key adaptor protein, AP-2 (Morgan et al., 2002), the mechanism of clathrin recruitment to the plasma membrane is unknown, despite clear ultrastructural evidence of clathrin-coated pits and CCVs (Engstler et al., 2004). AP-2 is a heterodimeric complex that binds cargo receptors and recruits clathrin in addition to providing a platform for assembly of accessory proteins that stabilize the activated receptor/AP-2/clathrin coat interaction. Reasons of this loss or its replacement are unknown but its loss may be due to the extremely high levels of cell-surface VSG (Field et al., 2007b) precluding further concentration. Significantly, the absence of AP-2 is common to all salivarian trypanosomes, that is, those possessing a VSG-mediated antigenic variation mechanism.

3.2. Cargo adaptors and sorting

In the absence of an AP-2 complex, it is unclear how clathrin is specifically targeted to the flagellar pocket membrane or how the system interacts with cargo (Fig. 1.4). One candidate for the latter is the trypanosome epsinR. Various epsin isoforms have been characterized (Chen et al., 1998; Rosenthal et al., 1999; Spradling et al., 2001) and epsinR or epsin-related proteins (Ford et al., 2002; Hirst et al., 2003), also called *cl*athrin *int*eracting protein, Clint (Kalthoff et al., 2002) or enthroprotin (Wasiak et al., 2002) are also implicated in transport processes. The epsin family shares a similar domain organization and mainly interacts with membrane lipids, clathrin,

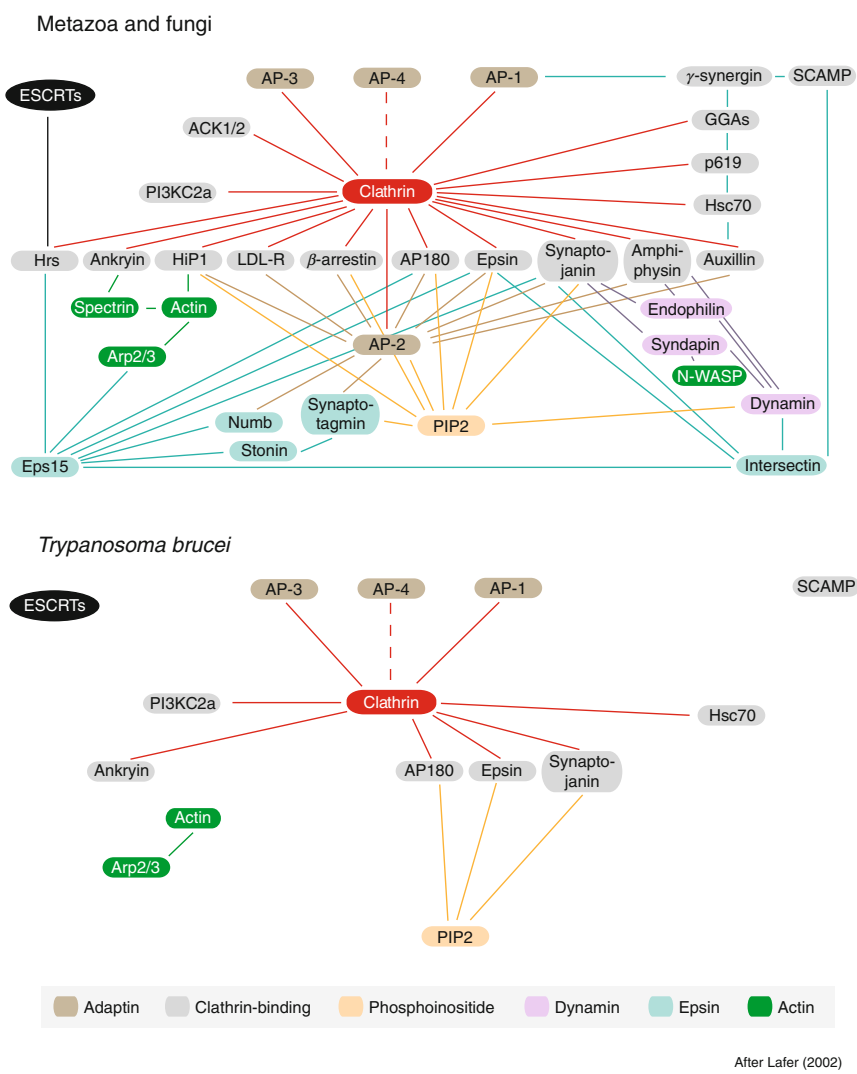

Figure 1.4 Presence and connectivity between gene products in trypanosomes and metazoa. In metazoan cells clathrin mediates only one of several endocytosis routes, while in trypanosomes all evidence indicates exclusive use of the clathrin pathway. A clear extensive level of connectivity is evident between the polypeptides of the clathrin endocytosis pathway in metazoa, and many of these factors also participate in additional clathrin-independent processes. In trypanosomes, the majority of these factors are absent from the genome, or experimental evidence also precludes a direct role in clathrin-mediated endocytosis. Moreover, the majority of connections shown are hypothetical and have not been experimentally determined. Red, clathrin; black, ESCRTs; brown, adaptins; gray, direct clathrin-binding partners; green, actin; orange, phosphoinositide phosphates; purple, others. The diagram is based on the data originally given by Lafer (2002).

and recruits additional proteins. At the N-terminus is a phosphatidylinositol (4,5)-bisphosphate ($PtdInsP_2$) binding epsin N-terminal homology (ENTH) domain of ~150 amino acids (de Camilli et al., 2002). The remaining largely unstructured region includes an ubiquitin-interacting motif (UIM), clathrin-binding box, and AP/GGA-binding motifs in the central region. The epsinR forms lack the UIM but retain most of the other sequence features.

The *T. brucei* genome has one ENTH-family gene, TbEpsinR; the sequence has conserved lipid-binding residues in the ENTH domain, lacks NPF and UIM motifs and multiple DPW motifs are replaced by DxF, and together with phylogenetic reconstruction, placing this clearly as an epsinR subfamily member (Gabernet-Castello et al., 2008). Like other endocytic factors, it is located between the kinetoplast and nucleus, and ablation is lethal. RNAi knockdown indicates a clear role in endocytosis, turnover of ISGs, and a conserved interaction with clathrin. However, knockdown of TbEpsinR does not prevent clathrin recruitment to the membrane or emergence of a BigEye, which suggests that while TbEpsinR and clathrin clearly function together in endocytosis, it is probable that other factors help load clathrin onto the membrane. Further, TbEpsinR is not a player in the mechanism of endocytosis *per se*, and clearly implicated as a cargo adaptor. A related ENTH/ANTH domain protein, TbAP180, has so far not been studied, and there is essentially no data on roles or locations of phosphoinositides in trypanosomes.

3.3. Early and recycling endocytic compartments

Endocytic compartments progressively mature into degradative of recycling pathways with a concomitant change in the markers they possess. Much work in higher eukaryotes has focused on using Rab GTPases of the Ras superfamily as such markers due to their integral roles in the regulation of vesicle transport, thereby allowing the spatial coordination of vesicle targeting, docking, and possibly budding (Zerial and McBride, 2001). A similar approach has also been applied to trypanosomes.

While endoyctosis in trypanosomes has a single mode of entry for material, that is, CME (Allen et al., 2003; Overath and Engstler, 2004), up to 16 Rabs are present in the *T. brucei* genome, reflecting an inherent level of sophistication within the trafficking system (Ackers et al., 2005). Sorting of material most likely occurs within internal endocytic compartments and not at the plasma membrane. This is supported by the presence of distinct, punctuate, early endocytic structures differentiated by the presence of two Rab5 homologues, TbRab5A and TbRab5B, and containing distinct cargo molecules (Field et al., 1998; Pal et al., 2002a). Selectivity is apparent at this initial stage of uptake as the Rab5A endosome contains IgG, transferrin, VSG, and ISG65, a type I *trans*-membrane protein

(Chung et al., 2004; Field et al., 1998; Hall et al., 2004a,b; Pal et al., 2002a). In contrast, the only cargo to be localized to the TbRab5B compartment is recognized by polyclonal antisera to ISG100, suggesting a differential function between the two endosomal populations (Field et al., 1998). How this discrimination occurs is unclear, but the mode of membrane attachment may function to direct cargo to specific early endosome compartments, and is supported by the presence of carbohydrate-mediated endocytic targeting in trypanosomes (Nolan et al., 1999; Pal et al., 2002a). Both TbRab5A and 5B clearly participate in endocytosis, levels of TbRab5A and 5B protein correlate with clathrin expression levels and knockdown leads to loss of clathrin expression and the BigEye enlarged flagellar pocket phenotype, suggesting a direct interaction between these factors (Hall et al., 2004a; Koumandou et al., 2008).

Rab5-positive early endosomes interface with components of the recycling systems, predominantly controlled by TbRab11 (Field et al., 1998; Hall et al., 2004a,b; Jeffries et al., 2001; Pal et al., 2003). VSG and the TfR are extensively recycled in BSF and quickly enter a highly fenestrated, TbRab11-positive compartment after uptake, separating them from soluble fluid-phase cargo (Overath and Engstler, 2004; Pal et al., 2002a, 2003) (Fig. 1.3). The recycling pathway appears to be under extensive developmental regulation as TbRab11 is rather more highly expressed in BSF, and while TbRab4 is involved in fluid-phase lysosomal transport, its participation in recycling appears restricted to procyclic culture forms (PCFs) (Hall et al., 2004b, 2005a,b). Importantly, PCF cells do not separate fluid-phase cargo from surface proteins in the same manner as BSF, and the endocytic system appears to be less diversified in the insect life stage, supported by the fact that in PCF TbRab5A and TbRab5B are located on the same vesicular structures (Field et al., 1998; Hall et al., 2005a,b). More emphasis is placed on recycling pathways in the BSF, whereas in PCFs the endocytic system is committed to lysosomal routes (Hall et al., 2005a,b). Undoubtedly, differences in the regulation of endocytosis between life stages are indicative of altered requirements for survival in the insect and mammalian host (Natesan et al., 2007).

3.4. Multivesicular bodies and late endocytosis

In higher eukaryotes, multivesicular bodies (MVBs) receive input from both the endocytic pathway, and also the post-Golgi network. Membranes intended for lysosome degradation are incorporated into intralumenal vesicles (ILV) that bud from the MVB-limiting membrane. Fusion of these vesicles with the lysosome results in degradation of the vesicles along with their contents. The pathway is proposed to mediate the degradation of damaged proteins, perhaps including exocytic cargo misprocessed in the Golgi complex, and also proteins that require efficient downregulation, for

example, activated receptor-type tyrosine kinases (Katzmann et al., 2002; Piper and Katzmann, 2007).

At the ultrastructural level, MVB architecture appears conserved in *T. brucei* and the similarity extends to positioning of the organelle in terms of relationships with the early endosome and the lysosome (Allen et al., 2007). At present, the highly dynamic structure is undefined; however, trypanosomes do posses the signals and molecular machinery necessary to direct cargo into MVB via ubiquitylation and action of the ESCRT complexes, albeit with some lineage-specific differences (Leung et al., 2008) (Figs. 1.3 and 1.5). Although purely speculative, one could assume that the extensive remodeling of the cell surface during parasite differentiation might be accompanied by a massive expansion in MVB activity. Analysis of MVB function during differentiation could be informative and studies in PCF are warranted, especially due to the apparent lack of sorting within the earlier endocytic compartments.

In addition, MVB internal vesicles do not always mark proteins for degradation and different ILV may be cordoned off into distinct endosomal carrier vesicles in mammalian cells (Chow and Mellman, 2005; Gu and Gruenberg, 1999; van Niel et al., 2006). Whether this occurs in *T. brucei* is unknown, but could go some way to explain the pleiotropic effects upon knockdown of proteins involved in the endocytic system, for example, clathrin and AP-1 (Allen et al., 2003, 2007).

3.5. Lysosome

Juxtaposed to Rab5 endosomes in an area close to the terminal endocytic compartment are the morphologically distinct late endosomes. Rab7 defines these structures and mediates delivery of material to the lysosome

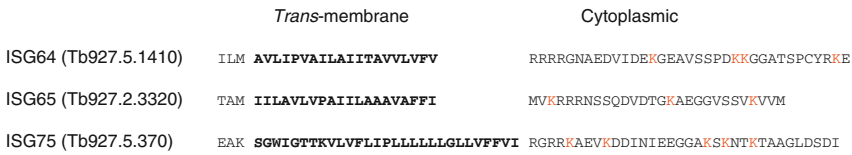

Figure 1.5 Putative ubiquitylation sites in trypanosome *trans*-membrane domain surface proteins. The extreme predicted C-terminal domain sequences of representative members of three of the invariant surface glycoproteins, ISG64, ISG65, and ISG75, are shown. The hydrophobic *trans*-membrane domain is shown underlined, and lysine residues in the short cytoplasmic domains are highlighted in red. Note the rather short length of these cytoplasmic domains and the absence of a canonical dileucine or tyrosine-based endocytosis motif. Only the two C-terminal-most lysine residues in ISG65 have been experimentally shown to be capable of covalent modification by ubiquitin *in vivo* (Chung et al., 2008), while the three C-terminal lysines in ISG75 are implicated empirically in endocytosis and turnover (K.F. Leung and MCF, manuscript in preparation). Accession numbers are given in parenthesis.

(Overath and Engstler, 2004). Protein degradation occurs within the lysosome via the concerted action of acid hydrolases (Kornfeld and Mellman, 1989), and the organelle plays a central role in nutrient acquisition and immune evasion, directly contributing to parasite pathogenesis. Internalized immune complexes are rapidly trafficked to the lysosome for degradation, and the structure is the site at which the antiparasitic effect of TLF is manifested in susceptible species (Balber et al., 1979; Barry, 1979; Hager et al., 1994; Pal et al., 2003; Pays et al., 2006; Raper et al., 2001; Shimamura et al., 2001).

In trypanosomes, the lysosome is a single-membrane-bound vacuole-like organelle, and is in a constant state of flux due to the dual flow of material from the cytosol and from other endocytic compartments (Liu et al., 2000; Pal et al., 2003; Shimamura et al., 2001). Studies on a resident glycoprotein of the trypanosome lysosome, the p67 protein, have furthered our understanding of lysosomal function, but its role in maintaining the integrity of the lysosomal membrane was uncovered only recently (Peck et al., 2008). p67 is potentially analagous to the LAMP and LAMP-like proteins of mammalian lysosomes, and shares the type I topology and extensive *N*-glycosylation of the mammalian proteins as well as a dileucine-based cytoplasmic targeting signal (Allen et al., 2007). However, there is no evidence for sequence similarity or common ancestry between p67 and higher eukaryote LAMPs. Precisely how p67 participates in maintaining the lysosome remains unclear, but evidence suggests the protein may play a role in recycling or export of proteins across the lysosomal membrane (Peck et al., 2008).

Most work has focused on the transport and processing of p67, revealing the conservation of dileucine signal-based lysosomal targeting and some evidence for a saturable cytosolic component (Allen et al., 2007; Tazeh and Bangs, 2007). Trafficking of p67 is regulated in a stage-specific manner and occurs independently of AP-1 complex action, signifying the presence of divergent lysosomal trafficking mechanisms in trypanosomes (Alexander et al., 2002; Allen et al., 2007).

The stage-specific trafficking of p67 and the potent phenotype following ablation in BSFs correlate with higher lysosomal activity in this life stage, and serves to illustrate that lysosome morphology and activity is developmentally regulated (Alexander et al., 2002; Kelley et al., 1995; Langreth and Balber, 1975; Mackey et al., 2004; Pamer et al., 1989; Peck et al., 2008). However, there is no evidence for significant alterations in the expression level of the p67 protein or alterations to lysosome morphology or positioning in any life stage (Natesan et al., 2007). However, endocytosis and lysosomal activity in trypanosomes are coupled; downregulation of nutrient acquisition in the insect life stage is associated with downregulation of the major thiol protease, trypanopain (Caffrey et al., 2000; Mbawa et al., 1992). Hence, while the overall structure as defined

by p67 may appear equivalent throughout the life cycle, functionality is likely to be developmentally controlled.

In other eukaryotic systems, transient and stable fusion of endosomes with the lysosome can result in the generation of hybrid organelles (Bright et al., 2005). Formation of such hybrids is dependent on the presence of Syntaxins 7 and 8, Vti1B, NSF, SNAPs, and Rab7 (Antonin et al., 2000a,b, 2002; Mullock et al., 1998; Pryor et al., 2004), while lysosomal reformation is probably mediated by the retromer complex (Arighi et al., 2004; Seaman, 2004). Trypanosomes have orthologues of all of these factors, indicating potential conservation of the machinery required to direct these restructuring events despite the fact that direct fusion of MVB/late endosomes and lysosomes has not yet been reported (Berriman et al., 2005; J.B. Dacks, MCF, and V. Koumandou, unpublished data).

3.6. Involvement of the cytoskeleton in endocytosis

In higher eukaryotes, the actin and tubulin cytoskeletons have clear roles in endocytosis and movement of vesicles. In trypanosomes, the apparent absence of a cytoplasmic tubulin population, apart from the subpellicular corset, raises the issue of how the various structures are positioned, and the mechanisms by which vesicles move between them. This remains an unaddressed area at the present time. Rather more is known concerning the role of actin. Endocytosis requires cell cortex remodeling (Engqvist-Goldstein and Drubin, 2003; Qualmann et al., 2000), and hence actin is implicated. The involvement of actin polymerization in endocytosis has been very clearly demonstrated but varies between cell types (Fujimoto et al., 2000). Though there is distinct variation between organisms and study is almost exclusively of Opitstokhonta (Lanzetti et al., 2004; Merrifield, 2004), African trypanosomes present a specifically interesting case. In BSFs actin is essential for efficient endocytosis, evident in polarized localization at the endocytic site and an enlarged FP observed on knockdown (García-Salcedo et al., 2004); the phenotype is remarkably similar to that obtained by RNAi against clathrin (Allen et al., 2003; Hung et al., 2004), Rab5 (Hall et al., 2004a,b), Rab 11 (Hall et al., 2005a,b), and ARF1 (Price et al., 2007), suggestive of endocytic blockade.

The precise role of actin in trypanosome endocytosis has not been determined but addressing certain questions could improve our understanding of the process greatly. Firstly, how does actin polymerization occur in the two forms? The actin-related protein Arp2/3 complex, which is important in driving actin polymerization, is present in the trypanosome genome (Berriman et al., 2005), but the Arp2/3 activators and eps15 are lacking, suggesting that a novel process or scaffold protein could be involved. Secondly, how do endocytic factors influence or initiate actin polymerization or F-actin promote endocytosis? A possible candidate involved in actin

remodeling, and which is developmentally regulated, is Rab5 (Lanzetti et al., 2004; Pal et al., 2002a) and in higher eukaryotes possibly acts through an actin-binding effector molecule; significantly Rab5 expression levels influence clathrin protein copy number (Koumandou et al., 2008), suggestive of direct control of endocytosis through Rab5. However, a large number of other candidates are also present in the genome, and many could aid cross-linking of soluble actin into an F-actin network at the plasma membrane facilitating endocytosis in BSF. Recent data from our laboratory has implicated a myosin IB in endocytosis, based on colocalization with clathrin and emergence of an enlarged flagellar pocket on RNAi knockdown (VOA and MCF, unpublished data). A better understanding of the role of actin, and the overall control of endocytic activity will likely require direct characterization of the protein–protein interactions that subtend this system.

3.7. Complexity of endsomal sorting

The extent of Rab protein functional complexity and hence the potential number of independent transport steps within the trypanosome endocytic system remains unknown, and is further complicated by the fact that additional potential endocytic Rab proteins have recently been discovered (JHL, M. Ali, and MCF, unpublished data). Promiscuity is evident as the activity of several Rabs can likely be modulated by a single GTPase activating protein (GAP) (Field and O'Reilly, 2008). This suggests a level of integration within the endosomal system comparable to other eukaryotes. Furthermore, orthologues of several proteins involved in retrograde trafficking pathways in higher eukaryotes can be found within the genome. The presence of syntaxin 16 and Vps45 indicate the probable presence of a highly developed endosome to *trans*-Golgi network trafficking pathway (Dacks and Doolittle, 2004; Koumandou et al., 2007) and the retention of the COPI trafficking coat complex suggests functionality of both an intra-Golgi anteriograde and Golgi to endoplasmic reticulum (ER) retrograde route (Berriman et al., 2005). However, the presence and functional importance of retrograde transport in trypanosomes has yet to be addressed experimentally.

3.8. Surface receptors and endocytic pathways

As true parasites, trypanosomes are reliant on the host for many metabolic needs, and these have to be acquired. This poses a specific problem to the bloodstream stage trypanosomes, as they have to shield receptors and transporters from the immune system. Only low-molecular-weight molecules can pass across the surface coat and thus reach plasma membrane channels, receptors, or transporters. Macromolecules, however, must be

recognized by conventional receptors, and many are thought to be restricted to the flagellar pocket. Critically, the flagellar pocket is occluded from the surface and hence not accessible to many immune effectors, including lymphocytes (Overath et al., 1997). Further, rapid uptake and degradation, as discussed earlier for immunoglobulins, likely extends to additional factors, including complement.

Very few trypanosome receptor proteins have been characterized. The TfR is vital for trypanosomes since it provides the parasites with iron. In vertebrates, TfRs are *trans*-membrane proteins, but the trypanosome receptor is an example of convergent evolution and is unrelated to the metazoan receptor. The trypanosome TfR is a heterodimer, and the product of two ESAG genes, 6 and 7 (Salmon et al., 1994). The complex is anchored to the plasma membrane via a single GPI-anchor attached to ESAG6, and has structural similarity to VSG. *S*witching between ESs allows expression of different TfRs with distinct binding properties, which may facilitate adaptation to the distinct transferrin proteins encountered in different hosts (Gerrits et al., 2002). However, the biological significance of transferrin-based selective pressure to antigenic variation is a matter of debate, and the comparatively high concentrations of transferrin in mammalian sera also question the significance of comparatively small differences in affinity. Significantly, the ESAG repertoire itself is quite variable between different ESs, with the exception that all contain ESAG6 and 7, underscoring the importance of this receptor to the trypanosome.

Despite structural similarity between the trypanosome TfR and VSG, TfR appears not to be expressed on the cell surface (Mussmann et al., 2004). Instead most is retained by the flagellar pocket and the endosomal system, which may assist in ensuring that the TfR is not recognized by the immune system. While this may also be true for other receptor molecules, few have been identified unambiguously. Both the HbHp receptor and SRA, which are also GPI-anchored, appear to share a similar location to the TfR; it remains to be seen how general this phenomenon will be.

The mechanism by which receptors are sequestered to the flagellar pocket remains to be addressed. The number of lipid anchors has been suggested to play a role in TfR localization; while the ESAG6/7 dimer contains one GPI anchor, VSGs have two and are readily routed to the cell surface (Schwartz et al., 2005a). However, it is unclear how such a mechanism would operate, and importantly overexpression of TfR leads to the receptor spilling out onto the surface. The exclusive use of short GPI anchors (myristate, C14:0), which should more easily flip out of the membrane, is a bloodstream stage-specific feature. The exact roles of the GPI anchor, ectodomain sequences, and possibly also N- and O-glycans in flagellar pocket retention of proteins remain to be systematically evaluated.

Accumulation of host steroids is essential for trypanosomes, as they have a limited synthetic capacity. Bloodstream stage trypanosomes take up

low-density lipoprotein (LDL) significantly faster than fluid-phase cargo and this process is saturable and inhibitable. Two classes of receptor-binding site have been described, a low-copy high-affinity and a high-copy low-affinity site (Coppens et al., 1991). These sites are likely manifestations of the same receptor as manipulation of copy number has equivalent effects on both high- and low-affinity copy number. The LDL receptor itself has not been identified.

Appreciation of the organization, identity, and chemical nature of trypanosome flagellar pocket proteins remains in its infancy, and the absence of a validated flagellar pocket or endosomal proteome presents a considerable barrier to further investigation. Most significantly, high-molecular-weight pNAL-type N-glycans fill the pocket lumen and most probably contribute considerably to the gel-like matrix that fills the structure (Atrih et al., 2005). Many pNAL-containing proteins are clearly residents of the trypanosome endosomal system, which includes the flagellar pocket membrane (Nolan et al., 1999). It is likely that these high-molecular-weight glycans contribute to the trafficking of the glycoproteins that bear them, but again, until a full list of pNAL-bearing factors is forthcoming, it remains difficult to evaluate the full importance of this posttranslational modification to trypanosome endocytosis.

4. Developmental Remodeling and Signaling

In the transition between hosts the parasite must adapt to very different conditions, including nutrient sources, defense requirements, and temperature. This is facilitated by a differentiation program including at least two distinct phases. A preadaptation step involves differentiation to nonproliferative forms, metacyclic, or stumpy stages, which exhibit partial or full expression of final differentiated stage proteins. Differentiation is completed by full remodeling of gene expression and reentry to the cell cycle.

In terms of macromolecular trafficking, two features stand out that accompany these transitions. First is exchange of the major surface antigens, VSG expressed in the bloodstream form, and procyclins in the procyclic stage. A further surface switch also accompanies the transition to epimastigotes within the tsetse fly, where the procyclin coat is exchanged for BARP. Interestingly all of these proteins possess GPI anchors (Roditi and Lehane, 2008; Urwyler et al., 2007). The second aspect is the very large difference in endocytic capacity between the high rate in the bloodstream form and the much lower level in the procyclic stage.

At least in general terms, changes to the surface coat can be meaningfully interpreted. VSG is required for antigenic variation, and indeed is reactivated in the metacyclic stage in readiness of introduction into a

vertebrate host. Procyclins are highly charged and with a repetitive protease-resistant C-terminus. These proteins were proposed to mediate defense against trypanolytic factors in the tsetse fly, and while the ability of procyclin-null parasites to complete the insect portion of the life cycle clearly indicates that they are nonessential, it is likely that an important contribution is made, and that these procyclin-nulls are at a selective disadvantage (Vassella et al., 2009).

The augmentation of endocytic activity in the bloodstream stage is presumed to be an important component of immune evasion (Overath and Engstler, 2004; Field and Carrington, 2004), and this is supported both by the clear ability of the system to remove and degrade immune effectors from the surface (Engstler et al., 2007; Field and Carrington, 2004), as well as the observation that reactivation of the high endocytic rate is a component of metacyclogenesis, that is, part of the preadaptation of insect stage parasites (Natesan et al., 2007).

Developmental regulation of trypanosome trafficking does appear mainly restricted to endocytic pathways, and there is little evidence for changes to expression of factors associated with either ER exit, anterograde Golgi transport, or late exocytic factors between the two life stages, albeit with restriction of such analysis to mRNA levels only (Koumandou et al., 2008). While several endocytic factors are upregulated, including clathrin, Rab11 and RME8, a clear view of how the endocytic pathway is controlled does not emerge from transcriptome analysis. This may reflect the absence of transcriptional control, but is also likely a result of regulation by control of protein levels (Koumandou et al., 2008) and the participation of multiple enzymes and transient complexes in modulation of membrane transport. Significantly, the unfolded protein response, a pathway stimulated by increased concentrations of unfolded nascent chains in the ER, and which is mediated by increased transcription of key ER chaperones, including BiP, is also absent from *T. brucei* (Koumandou et al., 2008). Clearly, this is an area where further exploration is warranted.

5. Sorting Signals

5.1. Targeting at the ER

Retention of lumenal ER-resident proteins in higher eukaryotes requires the presence of a conserved C-terminal tetrapeptide sequence motif, mediating interaction with the Erd2 receptor (Lewis et al., 1990). The precise sequence varies in a species- and protein-specific manner, and in African trypanosomes the motif is divergent from the canonical XDEL motif, where X is any amino acid (Bangs et al., 1996). For example, the retention signal of TbBiP is MDDL and that of an ER-located protein disulfide isomerase

(PDI) is KQDL. Mutant forms of BiP where the native MDDL signal is replaced by KQDL or the mammalian signal sequence KDEL are efficiently retained in the trypanosome ER, indicating considerable flexibility in the sequence requirements for recognition by the trypanosome Erd2. The significance of this is unclear.

Trypanosome ER membrane proteins of trypanosomatids, for example, the ER Ca^{2+}-ATPase, also contain a C-terminal motif directing ER localization (McConville et al., 2002). This is also highly related to the higher eukaryote signal, and consists of the sequence KKXX in the cytosolic domain. While full exploration of the sequence requirements/restrictions for retention of either lumenal or membrane-spanning ER proteins in trypanosomes has not been achieved at this point, it is significant that the systems are so similar to higher eukaryotes, indicating a very early establishment of these mechanisms in eukaryotic evolution.

5.2. Endocytic and lysosomal targeting signals

In higher eukaryotes, several categories of sorting signal have been identified and include dileucine-based [DE]XXXL[LI] signals that promote interaction with adaptins, together with tyrosine-based NPXY/YXXØ motifs, lysine residues that provide sites for the addition of ubiquitin and addition of mannose-6-phosphate as a lysosomal marker (Braulke and Bonifacino, 2009). It appears that only part of this system of signals is conserved in trypanosomes. Most prominently, the mannose-6-phosphate system is absent, and no evidence for the residue, enzymatic activity for its synthesis, or genes corresponding to the biosynthetic enzymes is present.

Sorting of proteins to the lysosome can occur via a [DE]XXXL[LI]-related signal. However, whereas the dileucine motif in higher eukaryotes consists of an acidic cluster, with no specified amino acid sequence, followed by two leucine residues, the trypanosomal lysosomal protein p67 requires the two acidic amino acids DE to immediately precede the dileucine. The DELL sequence is both necessary and sufficient for lysosomal targeting as shown by the generation of a chimeric protein in which the C-terminus of the major lysosomal protein p67 was added to the ER-resident BiP, followed by mutagenesis of the DE or the LL motifs (Allen et al., 2007).

Endocytosis of *trans*-membrane proteins from the cell surface remains comparatively unexplored, and a full understanding of this topic remains to be achieved. A small selection of surface *trans*-membrane proteins have been studied, principally the ISG65 and 75 families and TbMBAP1, a membrane-bound acidic phosphatase (Chung et al., 2004; Engstler et al., 2005). ISG65 resides both on the cell surface and within early endosomes, but lacks dileucine or tyrosine-based signals. Targeting is instead achieved through ubiquitylation of cytoplasmic lysine residues (Fig. 1.5). TbMBAP1

localizes mainly to the endosomal compartment of *T. brucei*, where it is readily found in early, late as well as recycling endosomes. Stepwise localization to the flagellar pocket, the flagellum, and the pellicular cell surface can be achieved by increasing expression levels. It is unclear how sorting of this protein is achieved as again classical sorting signals are absent (Engstler et al., 2005).

5.3. Endomembrane sorting based on posttranslational modification

Two additional mechanisms may be operating in trypanosomes, and it is unclear if, or how, these are related to higher eukaryotic systems. pNAL N-glycans have been discussed earlier, and suggested to act as a sorting signal for endocytosis (Nolan et al., 1999); this is likely a rather unique mechanism specific to trypanosomes, but these high-molecular-weight N-glycans do resemble those found on many metazoan lysosomal membrane glycoproteins. Interaction with a specific, but yet unidentified, protein with pNAL-binding specificity in the flagellar pocket or endosomes could explain the retention of pNAL-containing proteins in the flagellar pocket thus restricting access to the cell surface. Secondly, GPI anchors have been implicated as sorting signals in African trypanosomes with protein localization dependent on GPI valence, whereby proteins containing two GPI anchors show a cell-surface localization, proteins with one GPI anchor are localized in the flagellar pocket and proteins with no GPI anchor are rapidly degraded in the lysosome (Schwartz and Bangs, 2007; Schwartz et al., 2005b). Full evaluation of these mechanisms must await further and more systematic study, and it is unclear how general either of these processes may be in terms of the range of trypanosome proteins that are sorted using one or other of these mechanisms.

5.4. Sorting to the flagellum

While not *sensu stricto* a component of the endomembrane system, it is becoming increasingly apparent that there is both a mechanistic and evolutionary connection between the endomembrane system and flagellar biogenesis. Targeting of flagellar proteins can occur via a tripeptide sequence comprising the amino acid residues HLA (Ersfeld and Gull, 2001). Deletion of this signal in the flagellar proteins PFRA and ARP severely compromises flagellar targeting of these proteins. However, not all flagellar proteins contain this tripeptide signal, and may reflect the presence of different pathways for import into the flagellum or of preformed protein complexes that require only one protein of the complex to carry the HLA signal. As many nonflagellar as flagellar proteins contain the HLA motif, suggesting

that the sequence is not sufficient for exclusive transport to the flagellum and that the structural context of the HLA motif likely makes an important contribution to function as a flagellar targeting signal. A further signal for targeting of proteins to the flagellum was discovered when two flagellar adenylate kinases, TbADKA and TbADKB, present in *T. brucei* were studied (Pullen et al., 2004). These two proteins contain an additional 55 amino acids at their N-terminus compared to other known adenylate kinases that are usually localized in the cytoplasm or mitochondria. Deletion of the N-terminal extension from the adenylate kinase and addition of the 55 amino acids to GFP demonstrated that the sequence is both necessary and sufficient for flagellar targeting. The extension contains a conserved motif comprised of the amino acids $YLX_4IPXLXE$, followed by two conserved proline residues. The receptor proteins that recognize these motifs have yet to be identified.

5.5. Sorting to the glycosome

In trypanosomes, enzymes of the glycolytic pathway are targeted to a peroxisome-related organelle, the glycosome (Sommer and Wang, 1994). This compartment is abundant within the cytoplasm of the bloodstream form, and retains an import system that is clearly derived from classical peroxisomal targeting pathways. Glycosomal import is mediated by two main peptide targeting signals, PTS1 and PTS2, that are well characterized and conserved across species (Hettema et al., 1999). PTS1 is a C-terminal tripeptide motif. In *T. brucei* functional tripeptides conform to the motif [S/A][K/S/H/R]L. This is comparable to the first C-terminal tripeptide motif SKL identified in firefly luciferase, which mediates peroxisomal targeting (Sommer et al., 1994). PTS2 is a nonameric peptide found at or in close proximity to the N-terminus. The consensus sequence is [R/K][L/V/I]X_5[H/Q][L/A/F] and *T. brucei* aldolases are imported into the glycosome via PTS2 (Chudzik et al., 2000). Additional targeting signals that work independently or in conjunction with PTS1 or PTS2 have been suggested. Studies on *T. brucei* PEPCK suggested the possibility of an additional internal signal about 40 amino acids upstream of the C-terminal SRL signal sequence (Sommer et al., 1994). Again, the level of conservation of these sequence motifs between trypanosomes and higher eukaryotes is remarkable, reflecting a common mechanism that has likely been maintained under strong evolutionary selective pressure. Significantly, while peroxisome/glycosome targeting signals are highly conserved, the function of the peroxisome has been dramatically altered in trypanosomes, and glycosomes appear to be restricted to the trypanosomatids, and clearly not present in metazoa or fungi.

6. Secretory Protein Folding and Exocytosis

6.1. Membrane protein biosynthesis

Production of the protein molecules that constitute the cell surface is initiated at the ER, and hence in this context the trypanosome ER is responsible for synthesis of the VSG coat, antigenic variation, and the host parasite interface. The ER is the site of *N*-glycosylation, disulfide bond formation, addition of GPI anchors, and protein folding into appropriate tertiary and quaternary structures. In addition, the ER possesses a mechanism for sorting appropriately folded proteins from incompletely or malfolded polypeptides and resident ER proteins, a process generally called quality control (Ellgaard et al., 1999; Sitia and Braakman, 2003). Recent work has unexpectedly found that if synthesis of the VSG polypeptide is interrupted by RNAi, then trypanosomes rapidly fall out of the cell cycle (Sheader et al., 2005). This is of particular interest, as one possible explanation is that the trypanosome possesses a specific checkpoint or quality control sensor that is able to monitor ongoing VSG synthesis, potentially placing a novel burden on the trypanosome ER.

6.2. Polypeptide delivery

Sorting of secretory and most membrane protein residents of the endomembrane system begins with targeted import into the ER. Translocation into the ER lumen or membrane is mediated by a cleavable N-terminal signal peptide (SP) or an internal signal sequence and occurs by either of two pathways. Interaction of the SP of the nascent protein with the signal recognition particle (SRP) leads to the ribosome complex being targeted to the translocon, through which the protein traverses the ER membrane cotranslationally. In the second SRP-independent pathway proteins are translocated posttranslationally from the cytoplasm into the ER. Here the SEC complex, that spans the ER membrane, interacts with the translocon to facilitate engaging of the signal sequence thus guiding the nascent polypeptide through the translocon. Both pathways require the active participation of the major ER-resident chaperone and ATPase BiP, which ensures directional movement of the protein into the ER.

In higher eukaryotes, nascent polypeptide delivery to the ER can be cotranslational or posttranslational (Abell et al., 2004; Kutay et al., 1995; Stefanovic and Hedge, 2007). In mammalian cells, SP-containing proteins are translocated exclusively by either one of these two pathways with the SRP pathway being predominant. In *Saccharomyces cerevisiae* posttranslational translocation is likely as prominent as the SRP-dependant translocation. In the former, entry is initiated by binding of a ribonucleoprotein complex,

composed of the 7SL RNA and six polypeptides comprising the SRP, to the SP of the nascent chain in an active ribosome (RNC) forming a SRP–RNC complex (Schwartz and Blobel, 2003; Walter and Johnson, 1994) in which translation is slowed or arrested. In a GTPase-dependent cycle (Bacher et al., 1996), the SRP–RNC complex is targeted to a membrane-bound SRP receptor (SR; Keenan et al., 2001; Seiser and Nicchitta, 2000) and SRP subsequently released. Translation resumes and the polypeptide is translocated through a translocation pore/translocon composed of three proteins forming the Sec61 complex, Sec61α, β, and γ (Rapoport et al., 1996). In posttranslational translocation, extensive folding and aggregation of the nascent polypeptide must be prevented and delivery of ribosome-free preproteins is by a cytosolic chaperone pathway, mainly involving the Hsp70 family (Egger et al., 1997; Wickner and Schekman, 2005; Yam et al., 2005). For proteins whose carboxy-terminal hydrophobic *trans*-membrane domains act as a signal, delivery is via multiple pathways (Rabu and High, 2007).

Downregulation of key components of each of these two pathways by RNAi in *T. brucei* indicates that most SP-containing proteins can be translocated by either pathway, though subsequent processing may be influenced by the pathway taken. Exceptions to this promiscuity are polytopic proteins, which are solely translocated by the cotranslational pathway, and GPI-APs, which are predominantly imported using an SRP-independent pathway. The preference of GPI-APs may be due to a less hydrophobic SP, mirroring the situation found in *S. cerevisiae*. However, studies here are at an early stage, and RNAi is an indirect method for characterizing such processes; the recent demonstration of reconstitution of ER translocation in a fully *in vitro* system in *T. brucei* will likely facilitate further biochemical dissection of this system. Significantly, although many eukaryotic signal sequences function in heterologous systems, the exact nature of the interaction between the SP and the translocation machinery may retain species-specific features. In particular, kinetoplastid signal sequences, though reliably predicted using standard *in silico* algorithms, frequently fail to direct translocation of secretory and membrane proteins when expressed in higher eukaryotic systems.

Polypeptide ER delivery, maturation, and quality control in the kinetoplastids is of evolutionary interest, and not simply due to divergence. These organisms have surfaces dominated by GPI-anchored molecules. While *T. brucei* has VSG, procyclin, and a heterodimeric TfR (Schell et al., 1991), the American trypanosome *T. cruzi* has abundant surface GPI-anchored mucins (Di Noia et al., 1998; Pollevick et al., 2000). In *Leishmania* lipophosphoglycan (LPG), free GPI lipids (GIPLs; Beverley and Turco, 1998) and GPI-anchored glycoproteins (Bahr et al., 1993) all contribute to surface architecture. These molecules are efficiently and rapidly synthesized and translocated to the surface, hence protein

maturation and ER function may be endowed with unique features in these organisms.

Trypanosomastids do have an unusual SRP complex. First, in addition to the ubiquitous 7SL RNA, they possess a short tRNA-like molecule, sRNA-76 in *T. brucei* (Béjà et al., 1993), and sRNA-85 in *Leptomonas collosoma* (Liu et al., 2003). Second, of the six polypeptides (Srp9/21, 14, 19, 54, 68, 72) of the higher eukaryote SRP, only Srp19, 54, 68, and 72 are present in *T. brucei* (Liu et al., 2003; Rosenblad et al., 2004). Interestingly, sRNA-76 binds the Alu domain or domain I of the 7SL RNA to which the absentees (Srp9/21 and 14) bind in other systems (Lustig et al., 2005). Knockdown of individual trypanosome SRP proteins is lethal and results in mislocalization due to sorting defects, but nascent polypeptides still transverse the ER (Liu et al., 2003; Lustig et al., 2005) possibly by a posttranslational chaperone pathway. In common with mammalian and yeast systems, the SRP is assembled in the nucleolus, but Srp68 and Srp72 are also involved in XPO1 assisted nuclear export (Biton et al., 2006). Putative genes encoding subunits of the nascent polypeptide-associated complex (NAC), a factor that works in concert with SRP in maintenance of high fidelity in ER targeting (Lauring et al., 1995; Wiedmann et al., 1994), are present in the trypanosomatid genomes, suggesting that this aspect is common between trypanosomes and higher organisms.

Data mining for members of the protein translocation apparatus in the *T. brucei* genome suggests multiple missing factors. For example, while the mammalian system has a heterodimeric SR (SRα and SRβ; Schwartz and Blobel, 2003; Tajima et al., 1986), only an SRα homologue is clearly present in *T. brucei,* inferring a minimal requirement for SR as in prokaryotes. However, the distant similarity of SRβ to GTPases of the Sar1/ARF family (Miller et al., 1995) may suggest that other uncharacterized trypanosome GTPases could fill the role of SRβ. Recently, Lustig et al. (2007) showed that RNAi of TbSRα caused accumulation of SRP–RNC complexes and inhibition of spliced-leader RNA synthesis, subsequently resulting in decreased mRNA levels. The trypanosome also encodes only two of the three Sec61 ER-translocon subunits (Sec61α and Sec61γ), while of the accessory proteins that function with the Sec61 complex in the higher eukaryote chaperone-dependent pathway, Sec62p, 63p, 71p, and 72p, only Sec63p and Sec71p are present in trypanosomes (Goldshmidt et al., 2008). However, several of these factors possess the Sec7 domain and several novel Sec7 domain-containing proteins are also present in the trypanosome genome, rising the possibility that a divergent factor or factors could function in concert with the translocon. While our knowledge here remains rather fragmentary, these highlighted variations suggest significant divergence between ER-targeting mechanisms in kinetoplastids and higher eukaryotes and could explain the poor translocation competence of *T. brucei* proteins when expressed in heterologous systems (Al-qahtani et al., 1998).

6.3. Polypeptide folding and maturation

Oligosaccharyltransferase (OST) is a multimeric enzyme complex that transfers a preassembled dolichol-linked core glycan, $Glc_3Man_9GlcNAc_2$, to asparagine residues exclusively within NXS/NXT glycosylation sequons within the nascent polypeptide during translocation (Chavan et al., 2005; Helenius and Aebi, 2001, 2004; Moremen and Molinari, 2006). The process is thus a component of both cotranslational and posttranslational pathways. The glycan moiety imposes a conformational constraint that retains the polypeptide in solution (Braakman, 2001) and its modification by the concerted action of numerous factors is important in subsequent folding and sorting events (Helenius and Aebi, 2001; Trombetta and Parodi, 2005).

Folding of the glycosylated polypeptides is carried out by ER-resident chaperones and other proteins such as lectins including; calnexin (CNX) and calreticulin (CRT), binding protein (BiP/GRP78), endoplasmin (GRP94), PDI, peptidyl-prolyl *cis-trans* isomerase (PPI), ERp72 and ERp57. In a CNX–CRT chaperon pathway, glucosidase I and II (GI and GII), sequentially remove the first and second glucose residues of the polypeptide-bound glycan (Moremen and Molinari, 2006). The resultant $GlcMan_9GlcNAc_2$ is recognized by membrane-bound CNX and/or its soluble homologue CRT forming a complex that prevents aggregation, premature oligomerization, and formation of nonnative disulfide bonds. While bound to CNX–CRT, the glycoprotein is presented to a thiol oxidoreductase homologue of PDI, ERp57, forming short-lived disulfide bonds, and consequently catalyzes folding through formation and reshuffling of intra- and intermolecular disulfide bonds before the monoglucosylated glycan–polypeptide intermediate is released (Cai et al., 1994; Hebert and Molinari, 2007). Based on biophysical properties such as exposed hydrophobic patches, unpaired cysteines, immature glycans (Meusser et al., 2005), a folding sensor UDP-glucose:glycoprotein glucosyltransferase (UGT1) scans through the glycoprotein directing nearly native misfolded intermediates for reglucosylation and into another CNX–CRT cycle, while misfolded intermediates are transported to the cytosol for degradation by the proteasome (Caramelo and Parodi, 2007; Hebert and Molinari, 2007). Conformers are finally deglucosylated by GII prior to aided selection by a type I lectin, ERGIC-53 (Appenzeller et al., 1999; Klumperman et al., 1998) and exported to the Golgi apparatus. BiP is a master ER regulator involved in multiple functions (Hebert and Molinari, 2007) and acts at least in two ways in the folding process. First, sequentially through binding and preventing aggregation and secondly through delivery of nascent polypeptides to additional chaperones such as glucose-regulated protein 94 (GRP94), a glycoprotein involved in late folding steps. BiP recognition of the hydrophobic domains of nascent polypeptides and subsequent release is

mediated through repetitive cycles of ATP hydrolysis and ADP exchange (Gething, 1999) while participation in complexes composed of Grp94, ERdj3, Grp170, lectins, and additional factors serves to bring such factors into proximity with folding polypeptides (Shen and Hendershot, 2005; Tatu and Helenius, 1997; Zhang et al., 1997).

The domination of the trypanosome surface by GPI-anchored molecules and the emphasis on a limited number of superabundant antigens prompts expectations of evolution of highly selective and efficient folding pathways, modification, and export mechanisms. Indeed, some clear differences with higher eukaryotes do exist, starting with the missing gene products discussed earlier. Further, the otherwise universal dolichol-linked N-glycan precursor varies from other eukaryotes (Labriola et al., 1999), among trypanosomatids and between life stages (de la Canal and Parodi, 1987; Parodi, 1993). Trypanosomes transfer an unglucosylated glycan, $Man_{9–5}GlcNAc_2$ and additional highly unusual biantennary mannose-based structures; specifically $Man_{3–4}GlcNAc_2$ in *T. brucei* (Jones et al., 2005; Zamze et al., 1991), $Man_{7–9}GlcNAc_2$ in *T. cruzi*, and $Man_{6–7}GlcNAc_2$ in *Leishmania* spp. as opposed to $Glc_3Man_9GlcNAc_2$. This appears to be due, at least in part, to an inability to synthesize the glucosyl donor dolichol-P-Glc (de la Canal and Parodi, 1987) and is possibly due to loss of biosynthetic gene products (Manthri et al., 2008) while intraspecies variation may be an aspect of developmental regulation.

The subunit composition of OST ranges from one to eight polypeptides across the eukaryota; trypanosomastids retain a single subunit, the catalytic Stt3p (Kelleher and Gilmore, 2006). The absence of OST compositional complexity is suggested as a reason for the accumulation and transfer of a single dominant glycan moiety (Castro et al., 2006). The unglucosylated glycan-polypeptide is first bound by BiP, an essential protein in *T. brucei* (Subramaniam et al., 2006). BiP's cochaperone, a GRP94 homologue, is also present, while additional BiP cofactors, including ERdj3, have homologues present in *T. brucei*. Some of the over 20 putative DnaJ chaperone proteins encoded in the genome could also be involved in folding of nascent polypeptides in the trypanosome ER.

The trypanosome lectin chaperone system has only CRT (Labriola et al., 1999). Though CRT and CNX share a common ancestry through gene duplication (Navazio et al., 1998), the presence of only CRT in trypanosomes suggests CRT as a minimal requirement for protein folding. A recent study demonstrated variation in glycoprotein dependence on CNX/CRT in higher eukaryotes and importantly, the ability of CRT to effectively substitute for CNX in certain circumstances (Molinari et al., 2004). Moreover, the observation that loss of either lectin alone did not compromise protein folding supports the model that CRT represents a fully functional system in trypanosomes. Interestingly, protein folding in CRT-deficient cells is compromised only for specific proteins (Knee et al., 2003); this could

suggest that evolutionary selection may have been sufficient to remove the need for both CNX and CRT from the trypanosomatid lineage.

Polypeptide disulfide bonds can be formed by both class I and II PDIs and several PDI-like proteins are present in trypanosome genomes. Earlier studies showed downregulation of a class II TbPDI in PCF. Similarly observations on both class I and II PDIs as well as an unexpected endosomal localization and inessentiality on knockdown have also been made (Hsu et al., 1989; Rubotham et al., 2005). Since cysteine residues that form disulfide bonds in mature VSGs are highly conserved (Carrington et al., 1991), PDI knockdown could be expected to result in anomolous VSG folding; the lack of such a phenotype potentially implicates other PDI-like proteins and/or oxidoreductases present in the genome as being capable of assisting VSG to fold.

Partially folded proteins are detected by trypanosome UGT and subsequently glucosylated into monoglucosylated polypeptide intermediates for refolding (Conte et al., 2003; Trombetta et al., 1989). Consequently, trypanosomastids lack GI activity but have retained GII for deglucosylation of fully folded polypeptides. Functional analysis of *T. brucei* GII revealed unique features in trypanosome folding and possible roles for the variant N-glycan dolichol-linked precursors (Jones et al., 2004; Jones et al., 2005). Trimming of high mannose forms could be carried out by any of several α-mannosidase I proteins in *T. brucei* (Tb927.82910-40) while a putative mannose-specific lectin (Tb11.02.1680) could have a role in recognition of specific oligomannosyl structures. Appropriately folded/assembled proteins are subsequently trafficked to post-ER compartments.

6.4. ER exit to the Golgi complex or ER-associated degradation (ERAD)

Precision in sorting and export of nonresident ER proteins and retrieval of escaped resident factors is key to maintenance of a constant ER composition. Defects in these processes can be catastrophic and are associated with specific disease states in mammals (Castro-Fernández et al., 2005). Natively folded proteins and/or appropriately assembled multimeric complexes are selectively segregated from resident ER proteins at organized membrane domains called ER exit sites (ERES) or transitional ER. Here, cargo molecules are packaged and trafficked in COPII coated vesicles to the Golgi complex (Antonny and Schekman, 2001; Barlowe, 2002). Sar1p, a small GTPase, coordinates cargo and COPII cytosolic coat complexes recruitment at the ERES. Sar1p is initially activated by conversion from the GDP- to GTP-bound state by an ER-bound *trans*-membrane guanine nucleotide exchange factor (GEF) Sec12p (Barlowe and Schekman, 1993; Nakano et al., 1988). Sar1p-GTP inserts into the ER membrane (Huang et al., 2001), recruits the Sec23/24p heterodimer, forming a cargo selecting

prebudding complex (Kuehn et al., 1998) that recognizes export signals. The Sec13/31p subcomplex, a driver for coat polymerization, membrane curvature, and activator of the Sec23p GTPase activity, is subsequently added to the cargo complex (Barlowe, 2002; Kuehn et al., 1998; Lederkremer et al., 2001) generating coated vesicles. Other factors have also been implicated in this assembly (Sato and Nakano, 2007).

Irreversibly misfolded proteins are destined for ERAD. Some are quiescently retained by the ER or transported to the Golgi and later retrieved to the ER (Hammond and Helenius, 1994; Kincaid and Cooper, 2007; Klausner and Sitia, 1990; Young et al., 1993) while others are retrotranslocated to the cytosolic proteasome or vacuole (Hong and Kaiser, 1996). Selection is based on exposed hydrophobic peptide patches, signal sequences, or *trans*-membrane domains. Distinction from folding intermediates is achieved by a slow-acting enzyme, mannosidase I which removes terminal mannose residue (Jacob et al., 1998) and trims only persistently misfolded proteins (Ellgaard and Helenius, 2001). The resultant intermediate is recognized by a further lectin, ER degradation-enhancing α-mannosidase-like protein (EDEM), that also interacts with CNX and possibly releases aberrant proteins from the cycle (Molinari et al., 2003; Oda et al., 2003; Wang and Hebert, 2003). Other mechanisms of segregation have been proposed (Caramelo and Parodi, 2008). BiP and two interacting partners, Scj1 and Jem1, are also involved in directing misfolded soluble proteins for degradation (Nishikawa et al., 2001; Plemper et al., 1997). Retro-translocation occurs through the Sec61 channel (Pilon et al., 1997; Tsai et al., 2002; Wiertz et al., 1996).

Homologues of the COPII exit machinery are clearly present in trypanosomes (He et al., 2004). As in other eukaryotes, the trypanosome translocon could be involved in retro-translocation of misfolded proteins. A functional ERAD system most likely is also functioning since four homologues of mannose-binding proteins, potentially representing mannosidase I and EDEM, are present in the genome. In addition, ER retention and subsequent degradation of structurally faulty VSGs (Böhme and Cross, 2002; McDowell et al., 1998; Wang et al., 2003) or mistargeting (Triggs and Bangs, 2003) are clear indications of an active ER-based quality control and ERAD machinery. DnaJ domain-containing proteins, potential cochaperones of BiP including Scj1 and Jem1 are also present.

The high abundance and rates of synthesis and traffic of many surface proteins and extensive previous work on protein traffic make further exploration of trypanosome exocytosis feasible. With most candidates of this system experimentally uncharacterized, much more investigation is required to fully understand trypanosome protein folding, and especially on the possible roles this process may play in parasite survival in the mammalian host.

7. Golgi Apparatus; Functions and Replication

The Golgi apparatus is a highly organized and dynamic structure, reflected by the role of the Golgi complex as a site for glycan elaboration and macromolecular sorting, producing a huge array of glycan structures essential for parasite cell survival, but whilst also receiving constant input from anterograde and retrograde trafficking pathways. *T. brucei* contains a single Golgi apparatus comprising flattened cisternal membranes arranged in a stacked configuration, proximate to an ERES (Grab et al., 1997). The overall architecture of the organelle is instantly recognizable by electron microscopy and is highly similar to the structure as described for higher eukaryotes. The entry face of the organelle, the *cis*-Golgi network, most likely receives the entire cohort of proteins and majority of lipids derived from the trypanosome ER. Material is then processed before being sorted to final destinations from the exit face, the *trans*-Golgi network, again homologous to the higher eukaryote structure (Mellman and Warren, 2000). However, our understanding of the detailed functions resident within the trypanosome Golgi apparatus is not very advanced, and certainly we cannot yet evaluate the presence and importance of any trypanosome-specific functions or organizational features.

7.1. Golgi replication by binary fission

The environment encountered by trypanosomes in the mammalian host appears to dictate that a fully functional exocytic system is required, exemplified by a block in cytokinesis upon knockdown of VSG expression (Lillico et al., 2003; Sheader et al., 2005). It is unclear if this is a direct result of sensing of loss of VSG molecules, emptying of the exocytic pathway (VSG accounts for the major bulk of biosynthetic exocytic polypeptide), or some more complex mechanism unrelated to exocytosis. Evidence from higher eukaryotes indicates that the Golgi complex cannot arise completely *de novo* (Shorter and Warren, 2002), and hence the presence of a single Golgi apparatus in the trypanosome has the consequence that faithful replication and segregation during mitosis is essential to avoid creation of daughter cells lacking the Golgi organelle. Contrary to mammalian cells, Golgi biogenesis in trypanosomes occurs early in the cell cycle (Field et al., 2000) and probably by binary fission; fragmentation as observed in mammalian cells is never seen (Shorter and Warren, 2002). The process occurs in a highly coordinated and regulated manner, just after basal body (BB) duplication. The signals for initiation of replication are currently unknown, however, the order in which the trypanosome Golgi complex is assembled and the mode of inheritance

have recently been elucidated in considerable detail, and may indicate some novel aspects to Golgi biogenesis in trypanosomes.

Visualizing a GFP fusion of the trypanosome paralogue of the Golgi reassembly stacking protein, GRASP55/65, revealed that the new Golgi apparatus appears independently of the preexisting Golgi complex concurrent with emergence of a new ERES during early stages of mitosis. Interestingly, although the new Golgi appears *de novo*, photobleaching experiments using the Golgi-resident enzyme *N*-acetylglucosamine transferase B as a probe show that the ERES is not the sole source of new Golgi material (He et al., 2004). Membrane components traffic from the old to the new Golgi complex highlighting the role of the old organelle and raising the question as to the identity of the transfer carriers. More recent work using additional markers indicates that growth of the new Golgi complex is at least a two-stage process; structural and enzymatic components are laid down first, followed by those necessary to move and sort cargo in transit (Ho et al., 2006).

7.2. Role of the cytoskeleton in Golgi complex replication

Centrins, calcium-binding proteins associated with microtubule organizing centers including the BB, are also implicated in trypanosome Golgi biogenesis. The trypanosome orthologue of centrin-2 marks a bilobed Golgi-associated structure throughout the cell cycle, and is essential for Golgi duplication. This centrin-2 containing structure partially overlaps the new ERES and may serve to position the exit site in a process coupled to microtubule dynamics. A further possibility is that the structure defines a physical link between the new Golgi apparatus and the associated ERES, thus defining the location where Golgi biogenesis occurs (He, 2007; He et al., 2005). Indeed, depletion of polo-like kinase (TbPLK), essential for replication of the centrin-2 structure, results in a malformed bilobe and numerous dispersed Golgi-like structures throughout the cell. However, although their exact structure and function remains obscure, the appearance of satellite Golgi-like structures without an associated centrin-2 bilobe are normal parts of the trypanosome cell cycle, and the multiple structures present in TbPLK depleted cells therefore could represent an enlarged population of satellite Golgi-like organelles, implying a role for centrin-2 in determining Golgi size (de Graffenried et al., 2008; He et al., 2004). Certainly, studies of this process in trypanosomes should be especially informative due to the smaller and more compact nature of the Golgi complex compared to higher eukaryotes.

There is also evidence for developmental aspects of Golgi complex regulation, that is, that the organelle in procyclic forms may be regulated in a manner distinct from the BSF. For example, the PCF Golgi apparatus is

almost double in size and contains more cisternae compared to the BSF. This may reflect differential demands on the secretory system, and specifically an altered requirement for polypeptide-associated glycan elaboration (discussed in Field and Carrington, 2004). However, the presence of very large *N*-acetyllactosamine repeat N-glycans in a subset of BSF endosomal proteins and the presence of similar structures on the procyclin GPI-anchor may indicate a comparatively constant level of Golgi-mediated carbohydrate synthesis between life stages. However, developmental alterations are supported by a much increased expression of TbRab18 in the PCF and altered distributions of additional Golgi-resident Rab proteins (Dhir et al., 2004; Field et al., 2000; Jeffries et al., 2002). Moreover, knockdown of the ARL1 GTPase involved in maintenance of Golgi integrity only causes cell death in BSF and not PCF (Price et al., 2005). The functional consequences of these developmental differences between life stages and their implications for mechanisms of Golgi biogenesis remains unknown.

7.3. Cell-cycle and life-cycle coordination of Golgi complex replication and function

During the G2 phase of the cell cycle, duplicated BB and kinetoplasts separate in a microtubule-dependant manner (Robinson and Gull, 1991), and several lines of evidence now show that this is essential for Golgi complex segregation. Pharmacological inhibition of BB separation also inhibits Golgi segregation (He et al., 2004), and a pool of BB-associated centrin-2 may serve to connect duplication of these organelles in a manner similar to mammalian cells (He, 2007). Furthermore, centrin-1 is implicated in the spatial positioning of multiple organelles required for the initiation of cytokinesis in *T. brucei*. In addition, centrin-1 also localizes to the BB and a bilobed structure in close proximity to the Golgi complex. However, the two centrins do have distinct functions, as only centrin-1-depleted cells possess multiple BB and Golgi complexes suggesting that centrin-1 may function in organelle segregation rather than duplication *per se* (Selvapandiyan et al., 2007). Finally, a novel role for the class III PI-3-kinase Vsp34 in Golgi segregation has been demonstrated in trypanosomes. Attenuation of TbVsp34 levels severed the spatial connection between BB and Golgi complex replication and positioning; the two cisternae replicate but fail to segregate, resulting in considerably enlarged Golgi stacks with an increased number of cisternae (Hall et al., 2006). Employing interconnected duplication in this manner appears to facilitate coordinated biogenesis of multiple trypanosome organelles and hence their accurate partitioning at cytokinesis.

7.4. Retrograde transport

It is well established in yeast and mammalian cells that retrograde transport pathways are vital for correct functioning of the Golgi complex (Pavelka et al., 2008; Sannerud et al., 2003). However, the mechanism of delivery of material, and the extent to which this occurs, remains unresolved, and the role of retrograde transport pathways in organelle biogenesis remains an open question. As *T. brucei* contains orthologues of both retromer and COPI complex subunits, both of which participate in retrograde transport in higher eukaryotes, this is likely indicative of some functional conservation of these pathways (Berriman et al., 2005; J.B. Dacks, MCF, and V. Koumandou, unpublished data). Direct functional studies are clearly required to establish if such retrograde pathways are indeed operational in trypanosomes and what role, if any, they play in Golgi complex biogenesis and maintenance.

8. Ubiquitylation and Endocytosis of *Trans*-Membrane Domain Proteins

A fundamental issue in protein sorting is defining the mechanism by which the endomembrane system machinery is able to discriminate between different cargo. A comparatively limited set of polypeptide signals, together with diverse physicochemical properties dependent on the mode of membrane attachment, appears to account for this process. In trypanosomes, and especially the bloodstream stages, the sheer bulk of VSG at the surface possesses a considerable challenge for faithful sorting of any non-VSG polypeptides.

8.1. The VSG sorting problem

VSG is present at $\sim 10^7$ copies per cell, overwhelmingly as dimeric forms, representing $\sim 90\%$ of cell-surface glycoprotein. The turnover of VSG is extremely slow; loss of VSG from the cell occurs with a half-life in excess of 36 h, and detailed analysis of the fate of VSG suggests that the vast majority of this loss is via slow shedding, such that if all degradation were by internalization and proteolysis, the half-life of VSG would be in excess of 250 h (Seyfang et al., 1990), more than 30 cell generation times. Therefore, VSG is recycled with extreme efficiency, and any degradation of the polypeptide is a minor pathway. Knockdown analysis indicates that maintenance of the expression level of VSG is essential for viability and continued normal morphology (Sheader et al., 2005), suggesting

that even in the absence of antigenic variation there is a considerable burden to the trypanosome cell to maintain the surface VSG density at correct levels.

In sharp contrast, the major families of type I *trans*-membrane domain proteins, the ISGs, are represented at more modest copy number; both ISG65 and ISG75 are at $\sim 10^4$ copies per cell (Ziegelbauer and Overath, 1992), that is, a 1000-fold lower than VSG. Moreover, these ISG polypeptides are actively degraded with half-lives of ~ 4 h (ISG65) and ~ 2 h (ISG75) (Chung et al., 2004, K.F. Leung and MCF, manuscript in preparation). Therefore, for the ISGs, degradation represents the major fate of the protein, and recycling makes a comparatively modest contribution to the itinerary. Given that all endocytosis is mediated by a single clathrin-mediated mechanism, this is independent of AP-2 (Morgan et al., 2002), and both VSG and ISG are dependent on TbEpsinR as discussed earlier, how is such selectivity achieved? At least part of the process appears to be due to posttranslational ubiquitylation of the ISG proteins.

8.2. Ubiquitylation and endocytosis

In higher eukaryotes, the contribution of ubiquitylation to endocytosis has been well characterized (Williams and Urbé, 2007). Several surface receptors, including the growth hormone and epidermal growth factor receptors are modified by ubiquitin at the cell surface; this process requires the action of highly specific plasma membrane E3 ubiquitin ligases that transfer the ubiquitin to the target molecule (Katzmann et al., 2002, 2004); at least two, c-*cbl* and Rsp5, have been described that carry out this function in metazoa and yeast. Both monoubiquitylation and oligoubiquitylation appear to take place, which contrasts with the polyubiquitylation that occurs on soluble cytoplasmic proteins destined for degradation by the proteasome, and presumably confers specificity in recognition. Ubiquitylated endocytic cargo molecules are recognized by a number of factors, most of which utilize a conserved UIM to facilitate the interaction; UIM–ubiquitin interactions are low affinity (Babst, 2005), but the large number of the UIMs present in the system likely facilitate sequential handing off of ubiquitylated molecule as it progresses through the endosomal pathway. Further, several UIM-containing proteins, for example epsin, also interact with both the lipid bilayer and clathrin, providing a mechanism for recognition and incorporation of ubiquitylated cargo into clathrin-coated pits. Ubiquitylated cargo is ultimately handled by the ESCRT system, a supercomplex that in higher eukaryotes, at least, contains ~ 20 distinct polypeptides. Final disposal of the ubiquitylated protein requires the action of a deubiquitylation peptidase, Doa4p, that removes the ubiquitin and allows the cargo molecule to be further degraded by the lysosome (Clague and Urbé, 2006).

8.3. ISGs are ubiquitylated

Until recently, this entire system was uncharacterized in trypanosomes. While a clear role for ubiquitylation in protein turnover, and hence cell-cycle progression, has been demonstrated by knockdown of individual components of the trypanosome proteasome, as well as via inhibition with proteasome blocking compounds (Li and Wang, 2002; Mutomba et al., 1997), a role in intracellular trafficking has not been defined. Interestingly, the cytoplasmic domains of the ISGs are rather short, between 28 and 35 amino acids (Fig. 1.3). The absence of dileucine or tyrosine-based canonical endocytosis signals, which are represented on the lysosomal protein p67, for example (Allen et al., 2007; Tazeh and Bangs, 2007), but the conservation of a high proportion of lysines within these sequences (11–15%) is suggestive of ubiquitylation. Further, in contrast to earlier reports, it is apparent that the ISGs have a substantial endosomal presence in addition to their expression at the cell surface (Chung et al., 2004).

The *trans*-membrane and cytoplasmic domains of ISG65 or ISG75 are sufficient and necessary for targeting to endosomes and the plasma membrane (Chung et al., 2004, 2008; K.F. Leung and MCF, unpublished data), and precludes a major role for the ISG ectodomain in targeting. Further, deletion mutants demonstrate a role for the cytoplasmic domain of ISG65 in anterograde transport as removal of the 16 very C-terminal residues traps the protein within the ER. Mutagenesis demonstrates a role for the cytoplasmic lysine residues in endocytosis, turnover, and ubiquitylation of both ISG65 and ISG75 (Chung et al., 2008; K.F. Leung and MCF, unpublished data). Moreover, the reporter protein is oligoubiquitylated, with up to five ubiquitin chains. Remarkably, this is identical to the situation in higher eukaryotes, indicating a very ancient evolutionary origin and retention of this process (Williams and Urbé, 2007).

Significantly, these observations indicate a hierarchy in the importance of cytoplasmic lysine residues, with those at the C-terminus bearing a predominant role as ubiquitin acceptors (Chung et al., 2008). However, there is also sequence context at work, and this hierarchy is not just the result of proximity to the C-terminus and/or distance from the cell membrane. C-terminal fusion of the ubiquitin-like polypeptide, NEDD8, does not support additional ubiquitylation, despite presenting a number of new sites for modification. However, at variance with the system in higher eukaryotes is the finding that the trypanosome lacks clear homologues of Rsp5 or c-Cbl (Chung et al., 2008), and at present the ubiquitin ligases remain unknown.

Internalization of ISGs is presumed nonselective (Field and Carrington, 2009; Grünfelder et al., 2003), but there is a clear compartment where such a sorting could take place, post the early endosome, and which also contains clathrin coats on the cytoplasmic membrane face (Grünfelder et al., 2003).

In this structure, VSG clearly segregates into the central regions of cisternae and away from clathrin, which is localized to peripheral budding profiles. It is possible that ubiquitylated cargo is separated via preferential incorporation into clathrin-rich membrane domains.

8.4. Late endocytosis and the multivesicular body

The final steps in turnover of ubiquitylated molecules are also broadly homologous to higher eukaryotes, with at least one important distinction. *In silico* analysis demonstrates that the ESCRT system is highly conserved, and in trypanosomes the majority of the ESCRT subunits are represented in the genome (Leung et al., 2008 and table 1). Representatives of ESCRT I-complex factors can be localized to the endosomal system and are juxtaposed to Rab5 early endosomes and the lysosome, a position closely paralleling higher eukaryote MVBs. Knockdown of TbVps23, one of the ESCRT I factors, leads to partial inhibition of ISG65 degradation, while treatment with weak bases suggests that degradation requires the function of low pH compartments, strongly indicative of a role for ESCRTs and MVBs in this process (Chung et al., 2008; Leung et al., 2008). The major difference between higher eukaryotes and trypanosomes, which is in fact shared with all non-Opistokhont taxa, is the absence of the ESCRT 0 complex (Fig. 1.6). ESCRT 0 is a heterodimer that has been implicated in several processes, including recognition of ubiquitylated cargo via a UIM motif in Vps23/HRS, membrane attachment through a PIP3-binding FYVE domain in Hse1/STAM, and also in recruitment of the ESCRT I complex (Babst, 2005). The latter is present in higher eukaryotes as both soluble and membrane-bound populations, a property shared with the trypanosomes. These data suggest that a distinct mechanism must exist in trypanosomes for both initiation of ESCRT assembly at the endosomal membrane and also for recognition and delivery of ubiquitylated cargo into that compartment. It is formally possible that a divergent ESCRT 0 complex, that has not been detected *in silico,* is present but restriction to animals and yeasts strongly suggest this is a lineage-specific innovation. Rather, the possibilities that either ESCRT I directly binds endosomes or that a completely distinct mechanism is acting are attractive and aspects that require further analysis.

Overall, the identification of ubiquitylation as a mechanism for internalization of ISGs explains many facts. Firstly, absence of canonical endocytosis motifs from these proteins, but their efficient internalization is now explained. Secondly, these insights also show how endocytosis can function in the absence of AP-2, and further that sorting probably does not take place at the plasma membrane. Further, the involvement of the ESCRT complexes also explains the presence of MVB profiles as seen by ultrastructure, and suggests a mechanism for segregation of GPI-anchored and *trans*-membrane domain proteins. It is currently unknown if other *trans*-membrane domain

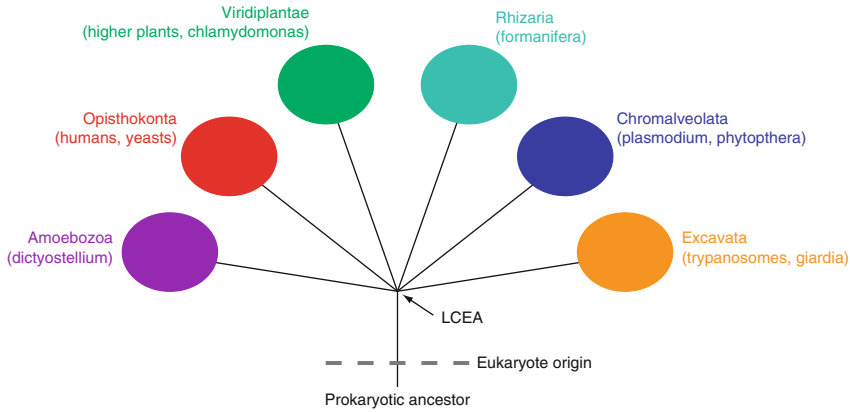

Figure 1.6 Simplified eukaryotic phylogeny. Each supergroup is represented by a colored lollipop, and representative species are indicated by each. An unresolved root, corresponding in the last common eukaryotic ancestor (or LCEA) is shown. The detailed implications of this view of eukaryotic lineages are discussed elsewhere, but the relevant point for this discussion is the absence of a primitive versus modern lineage. However, trypanosomes are highly derived organisms due to their parasitic lifestyle and coevolution with vertebrate hosts for tens of millions of years.

proteins enter this pathway. Finally, the possibility that ubiquitylation could be exploited for therapeutic gain is raised. Significant differences in the E3 ligases appear to be present, with the exciting possibility that if the factors responsible for modification of ISGs can be identified, then specific inhibitors may be designed.

9. Evolution of the Trypanosome Endomembrane System

9.1. Changing views of the eukaryotic tree of life

Our views of the evolution of the eukaryotes, and hence the position of trypanosomes within this history, have been revolutionized in the last 5 years (Adl et al., 2005). A reexamination of taxon relationships, facilitated by major advances in DNA sequencing technology and hence the quantity of molecular data, together with recognition of technical issues with earlier topologies based on rRNA data (Dacks et al., 2008b) has led to a redrawing of the tree of life (Fig. 1.6). Principal implications of this new topology are that a rapid explosion of eukaryotic lineages occurred early in evolutionary history, and that this radiation was so fast as to preclude robust resolution of branching

order at the deepest levels (Embley and Martin, 2006). The result is a view of eukaryotes as comprised of at least six supergroups, all originating at approximately equivalent times. The major consequences are that protistan lineages can no longer be considered as ancient (or metazoa and yeast as modern), nor is there evidence for long periods of common evolutionary history between the members of different supergroups. Experimentally, this means that trypanosomes are no closer to the last common eukaryotic ancestor (LCEA) than yeasts, plants, or amebae. A further consequence is that one cannot extrapolate, with any degree of accuracy, across supergroups, unless there is additional good evidence to do so. Trypanosomes and *Plasmodium*, for example, do not share an early origin, and hence are not especially related. It is, therefore, essential that model organisms representative of individual supergroups are carefully selected, so that a broader understanding of the diversity of the eukaryota can be obtained (Dacks et al., 2008a,b). Fortunately, the African trypanosome is well placed in this regard, being very experimentally tractable, and as a result has attracted considerable attention as a model system for evolutionary cell biology.

9.2. Comparative genomics

The application of comparative genomics to trypanosomes has uncovered a significant level of divergence between these organisms and the higher eukaryotes. Many of these are examples of lineage-specific features that appear restricted to the Opisthokont, and therefore are not unique features of trypanosomes. The list of absences includes ESCRT0, caveolin (suggesting the absence of large stable lipid rafts), the GGA and stonin sorting adaptors, some GRASPs, UIM-containing ENTH-domain proteins (true epsins), and numerous factors that are part of the clathrin interactome (Field et al., 2007a,b; Gabernet-Castello et al., 2008; MCF and J.B. Dacks, unpublished data). Some uncertainty remains concerning the absence of specific factors, that is, if these are true absences or just undetectable, but overall it is clear that trypanosomes lack a considerable number of trafficking factors. These observations do suggest, however, that while trypanosomes cannot be considered as ancient, the absence of many Opisthokont-specific factors indicates that they may be more representative of the vast majority of lineages, and, in this context at least, that they are perhaps more similar to the LCEA. However, there have been few successful attempts to identify trypanosome trafficking factors that do not directly depend on a knowledge of Opisthokont systems, and hence any truly novel factors likely remain to be described, the "asymmetry" problem (Dacks and Field, 2007).

9.3. G-protein signaling complexity and evolution

Exploration of the potential signaling complexity that subtends and integrates the trypanosome trafficking system is also relatively uncharted, and our lack of information concerning molecular pathways remains a major challenge. The kinome is of relative high complexity, but the functions of the vast majority of these factors are unexplored (Parsons et al., 2005). Significantly several kinases are implicated in control of endocytosis, which may suggest some similarity with higher eukaryotes (Koumandou et al., 2008; Pelkmans et al., 2005) and there is clear evidence for a role for phosphoinositide-based signal transduction (Hall et al., 2005a,b; Rodgers et al., 2007). The ratio of the numbers of Rab to Rab GAPs genes is comparable to that found in many unicellular systems distributed across the eukaryota (Field and O'Reilly, 2008) and the level of connectivity within the Rab and GAP interactome is also remarkably similar between trypanosomes and *S. cerevisiae* (C. Gabernet-Castello and MCF, unpublished data); while very preliminary, these observations are suggestive of a conserved level of integration between trafficking in trypanosomes and other unicellular organisms.

However, several lineage-specific factors have been uncovered. For example, three of the trypanosome Rab proteins, X1, X2, and X3, appear to be restricted to the Excavata. At the time of writing, it is unclear what the functions of these three proteins may be, although double gene knockout has shown that X1 and X2 are nonessential *in vitro* or *in vivo,* and a specific role in trafficking has so far eluded characterization, despite a clear location for RabX1 and RabX2 on exocytic compartments (Field et al., 1999, 2000; S. Natesan et al., submitted for publication). Comparisons of the Rab gene repertoire between *T. brucei* and *Leishmania* and *T. cruzi* has demonstrated the presence of likely secondary losses in *T. brucei* as both of the other kinetoplastida genomes encode additional Rabs, some of which are shared across the eukaryota, suggestive of a presence in the LCEA (Ackers et al., 2005; Berriman et al., 2005). The selective pressure that has molded the Rab repertoire is unclear, but the trivial explanation that the simpler system in *T. brucei* is due to loss of cell-invasive forms can be confidently rejected as the ancestral kinetoplastid was almost certainly free-living, and the intracellular stages of *Leishmania* and *T. cruzi* are the later adaptation. A final insight from the Rab gene family is that the division of endocytic pathways is also lineage specific. In particular, Rab5 plays an intimate role in control of the early stages of endocytosis, and this is common between trypanosomes and higher eukaryotes (Pal et al., 2002a,b; Zerial and McBride, 2001). Further, most species possess more than one Rab5 (*T. brucei* possesses two, 5A and 5B) and there is good evidence that this represents division of early endocytosis pathways. Unexpectedly, phylogenetic analysis indicates that the Rab5 subfamilies arose following the radiation of the supergroups

(Dacks et al., 2008a,b); significantly this suggests independent evolution of multiple Rab5 isoforms, and hence the selective pressure for such differentiation has been separately exerted on the individual supergroups. But, this also indicates that the precise manner in which division of labor between the Rab5 subfamily members is likely distinct to each lineage. However, the Rab5 subclasses were established prior to separation of *T. brucei* and *Leishmania* and hence the functions within the kinetoplastida are likely conserved (Dacks et al., 2008a,b).

9.4. Convergent evolution

Two potent examples of convergent evolution are provided by the TfR and the lysosomal protein p67. The former is a heterodimer consisting of the ESAG6 and ESAG7 gene products, and binds mammalian apo-transferrin with high affinity (Steverding et al., 1994). Most significantly ESAG6/7 is restricted to the flagellar pocket, anchored to the plasma membrane by a GPI-anchor and serves to facilitate efficient endocytosis of transferrin via the common clathrin pathway. However, both subunits are clearly related to VSG and hence have a completely distinct evolutionary origin from the host TfR. ESAG6 and 7 are the only ESAGs that are present in all expression sites, underscoring an absolute requirement for transferrin accumulation in the bloodstream stage (Berriman et al., 2002). It has been suggested that the presence of multiple and different copies of ESAG6/7 may facilitate an increased host range (van Luenen et al., 2005) but other data suggest that any one heterodimer may be sufficiently efficient in transferrin accumulation to fulfil the parasite's iron requirements (Salmon et al., 2005).

9.5. Evolutionary exploitation of the flagellar pocket

The flagellar pocket represents an evolutionary adaptation that many workers have proposed is exploited for the sequestration of surface receptors. For example, the ESAG6/7 complex is restricted to this membrane subdomain, which likely has the dual advantage of increased endocytic efficiency and also removal from the bulk surface and hence recognition by the host. However, it needs to be recognized that these observations remain correlative or ''just-so'' stories, and that a flagellar pocket-like invagination predates antigenic variation or parasitism; a similar structure is present in many free-living kinetoplastids and even in the more distant photosynthetic *Euglena*. This does not, however, prevent the exploitation of a preexisting structure for new functions, and potentially facilitating the acquisition of new ecological niches.

A recent study demonstrated elegantly how the flagellar pocket is now indispensable for trypanosomes. Ablation of expression of Bilbo-1, a component of the hemi-desmosomal flagellar pocket collar complex mediating

flagellum and flagellar pocket membrane adhesion results in loss of the pocket, but also major failures to vesicular transport, resulting in secretory cargo becoming trapped within accumulating cytoplasmic transport vesicles (Bonhivers et al., 2008). The resulting cells also exhibit major cytokinesis defects. It is tempting to speculate that this trafficking defect results from dilution of factors that mark the flagellar pocket membrane and that mis-sorting then results in defective cell division. However, the polarity of the trypanosome cell and hence faithful cytokinesis, is strongly dependent on flagellum positioning, so it is likely that this aspect of Bilbo-1 function also contributes. Regardless of the mechanism that leads to lethality, this work is an important reminder that whatever advantages the flagellar pocket may bring to the African trypanosome, there are additional and essential aspects that dictate retention beyond any role in protein sorting or immune evasion.

10. Conclusions and Future Perspectives

The last decade or so has seen a revolution in the depth of analysis that is possible for trypanosomes. In multiple areas, including cell-cycle control, differentiation, cytoskeletal function, and membrane transport, advances have been considerable (e.g., Broadhead et al., 2006; Fenn and Matthews, 2007; Hammarton et al., 2007). Completion of the genome sequence of *T. b. brucei*, partial sequences for additional African trypanosomes and complete sequences for *Leishmania* and *T. cruzi* have facilitated rapid analysis, comparative approaches, and much else. The recent reworking of the eukaryotic tree of life (Adl et al., 2005) may have displaced trypanosomes as putative ancient systems, but the new topology more than underscores their importance for understanding the range of eukaryotic diversity. In short, trypanosomes have emerged as an important model for cell and evolutionary biology, while retaining their preeminence for understanding of antigenic variation, immune evasion, virulence, and for design of antikinetoplastid therapeutics (Barrett et al., 2007). This optimism in laboratory or molecular-based science is somewhat tempered by limited progress in disease control, but there is also a basis for a positive outlook even here, with new funding approaches and postgenomics programs (Barrett et al., 2007). Drug resistance, ongoing difficulties with bridging the gap between bench and bedside and current political, agricultural and economic turmoil in Africa, however, caution against misplaced optimism (The Economist, 2008).

What are the priorities for ongoing work, in terms of macromolecular transport? There are at least three areas which require addressing at this time; systematic exploration of the cell biology, more thorough investigations of

therapeutic intervention and direct assessment of the contributions to immune evasion. All of these are clearly aspects of the same overall question.

Much of the progress in study of macromolecular transport has relied on mapping the functions of homologues of higher eukaryote factors into trypanosomes. This has served well, and demonstrated deep evolutionary connections for many pathways. Further, lineage-specific features have also been documented (Field et al., 2007a,b). However, an absence of a direct analysis by forward genetics or other approaches leads to an asymmetry; specifically we are ignorant of the level that trypanosome- or excavate-specific factors play in intracellular transport. Given that $\sim 40\%$ of trypanosome open reading frames remain unassigned, the likelihood of a contribution of trypanosome-specific factors is high. Clearly, identification of such factors and pathways to which they contribute would provide deep insights into kinetoplastid-specific processes, parasitism, and also evolution of the endomembrane system.

Such a deeper exploration of the protein networks participating in trypanosome macromolecular transport are of direct practical application. Specifically, it is clear that endocytosis is essential as multiple knockdown experiments attest. However, the factors investigated so far are both homologous to the host system, and also nondruggable at present; small GTPases and structural elements like clathrin are unappealing targets. But, identification of potential differences in the endocytic ubiquitylation enzymology, importance of the PI-3-kinase TbVps34 and likely participation of additional kinases and phosphatases all suggest that factors with enzymatic roles are present, and potentially much more accessible.

Finally, the role that the endomembrane system plays in immune evasion remains incompletely understood. Copious *in vitro* evidence has been accumulated; essentiality of endocytosis and VSG expression, correlation of high endocytic activity with mammalian infectivity, active antibody capping mechanisms coupled to extensive proteolysis within the endocytic/recycling system, and roles for various transport factor in these processes have all been reported. However, it remains that a detailed understanding of how the host immune system interfaces with trypanosome macromolecular transport in an *in vivo* context has not been forthcoming, and remains a challenge for the future.

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