Life and times
Manna, Paul T.; Boehm, Cordula; Leung, Ka Fai; Natesan, Senthil Kumar; Field, Mark

Published in:
Trends in Parasitology

DOI:
10.1016/j.pt.2014.03.004

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Link to publication in Discovery Research Portal

Citation for published version (APA):
Manna, P. T., Boehm, C., Leung, K. F., Natesan, S. K., & Field, M. C. (2014). Life and times: synthesis, trafficking, and evolution of VSG. Trends in Parasitology, 30(5), 251-258. DOI: 10.1016/j.pt.2014.03.004

General rights
Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain.
• You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 23. Jul. 2018
Life and times: synthesis, trafficking, and evolution of VSG

Paul T. Manna¹, Cordula Boehm¹, Ka Fai Leung², Senthil Kumar Natesan²*, and Mark C. Field¹

¹Division of Biological Chemistry and Drug Discovery, University of Dundee, Dundee, DD1 5EH, UK
²Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QH, UK

Evasion of the acquired immune response in African trypanosomes is principally mediated by antigenic variation, the sequential expression of distinct variant surface glycoproteins (VSGs) at extremely high density on the cell surface. Sequence diversity between VSGs facilitates escape of a subpopulation of trypanosomes from antibody-mediated killing. Significant advances have increased understanding of the mechanisms underpinning synthesis and maintenance of the VSG coat. In this review, we discuss the biosynthesis, trafficking, and turnover of VSG, emphasising those unusual mechanisms that act to maintain coat integrity and to protect against immunological attack. We also highlight new findings that suggest the presence of unique or highly divergent proteins that may offer therapeutic opportunities, as well as considering aspects of VSG biology that remain to be fully explored.

The variant surface glycoprotein and a paradigm for antigenic variation

For African trypanosomes that must survive in the extracellular spaces of the mammalian host, a sophisticated immune evasion mechanism, antigenic variation, is essential. We have known for over 40 years that this process is mediated by the VSG, originally described by Cross in a pioneering piece of biochemistry, and that this protein corresponds to an electron-dense coat at the surface of mammalian infective forms, as observed by Vickerman [1–3]. Both aspects remain crucial components of the modern paradigm of antigenic variation in trypanosomes.

We now understand VSG to be a 58-kDa glycosphingolipid (GPI)-anchored glycoprotein, having predominantly an α-helical secondary structure, with highly variable sequences embedded at the N terminus and monomolecularly expressed from a repertoire of hundreds of genes [4,5]. We also know that VSG is not the sole mechanism by which trypanosomes manipulate the immune systems of their mammalian hosts [6]. However, the basics of this system remain a potent reminder of the importance of VSG, in contributing to understanding trypanosome biology, acting as a model for antigenic variation in general, for early dissection of GPI biosynthesis (e.g., conceptually similar, albeit nonidentical, mechanisms are present in Plasmodium [7]), and as a model for intracellular transport studies [8].

Early work demonstrated (i) rapid addition of the GPI anchor to the VSG protein [9]; (ii) fast export to the cell surface in a manner involving a complex dance in molecular-weight changes correlating with various post-translational modifications [10]; (iii) rapid endocytosis and recycling pathways serving to carry VSG through the endomembrane system [11,12], and (iv) the realisation of just how abundant the VSG polypeptide is in terms of overall protein, estimated at 10⁷ copies and approximately 90% of cell surface polypeptides [13,14]. Other early significant observations included the demonstration of rapid VSG antibody capping, with implications for immune evasion [15], a phenomenon that required nearly 30 years to explain mechanistically and whose true value to immune evasion remains to be rigorously tested in an in vivo setting.

The massive dominance of the trypanosome surface by VSG implied that mechanisms for protein transport, targeting, and sorting may be distinct from mammalian cells, where most surface molecules have a trans-membrane domain, but it is now clear that many other trypanosomats also rely heavily on a GPI anchor for surface molecule anchoring, suggesting broader significance for observations in Trypanosoma brucei [16]. In this review, we consider the molecular mechanisms that participate in the synthesis, targeting, and turnover of VSG, how these differ from canonical views of protein trafficking and modifications, and what such modifications may mean, both for VSG itself and for the trypanosome more generally, with some speculations on how the system arose. We explicitly do not deal with many recent advances in uncovering mechanisms of monoallelic VSG expression and switching; interested readers are referred to reviews in this area (e.g., [4]).

VSG biosynthesis and sorting through the secretory pathway

The bloodstream form of T. brucei is under stringent selective pressure from the host immune system to maintain the high-density VSG surface coat, which presumably
is of sufficient density to protect against antibody recognition of invariant antigens. However, responses to experimental manipulation of VSG synthesis demonstrate an importance for VSG coat fidelity beyond simply acting as a steric barrier against the humoral immune response. VSG depletion by RNAi triggers a precise cell cycle arrest and general protein translation block, suggesting that synthesis is monitored and tightly coupled to a cell cycle checkpoint, although, because these studies were performed in vitro, the impact on immune evasion has not been directly addressed [17,18]. However, this phenomenon may reflect a pressing need to balance precisely the various fluxes of VSG trafficking to and from the surface, and implies the presence of a counting mechanism. Thus, efficient VSG synthesis, processing, surface delivery, and turnover are essential to satisfy demands, both intrinsic and extrinsic to the parasite, that govern its proliferation and survival (Figure 1). Although the half-life of VSG is long compared with the cell cycle, of the order of 72 h [11], the parasite still synthesises approximately $10^7$ mature copies of VSG per cell during each 8-h cell cycle [13]. This equates to approximately 20 000 VSG polypeptides per minute, all of which must be correctly processed and targeted to the cell surface [19]. Given that additional evidence suggests that VSG is overexpressed by approximately threefold during synthesis [20], the true number may approach 80 000 VSG molecules synthesised per minute, likely a significant burden on the cell. The general features of the eukaryotic membrane protein synthetic and exocytic pathways are well conserved in T. brucei, albeit with some idiosyncrasies, notably in glycosylation pathways and spatial organisation (Figure 1). VSG biosynthesis is outlined below, with particular attention to these adaptations and how they may aid in meeting the extreme VSG demand.

**Endoplasmic reticulum translocation**

The nascent VSG polypeptide encodes an N-terminal hydrophobic signal sequence, targeting the protein to the endoplasmic reticulum (ER) lumen via the Sec61 translocon pore [21,22]. Early work suggested that this signal sequence peptide was cleaved co-translationally [21], supporting a model of classical signal recognition particle (SRP)-mediated, co-translational ER translocation [23]. More recent analyses have suggested that SRP-dependent co-translational translocation is more important for polytopic membrane proteins in T. brucei, with proteins destined as becoming GPI anchored by using an alternative post-translational pathway [24–26]. Given that involvement of these two pathways in VSG translocation has not been addressed directly, the exact mechanism of VSG ER import remains an open question.

---

**Figure 1.** Routes responsible for trafficking of variant surface glycoproteins (VSG). (A) The major intracellular compartments that are known to be associated with VSG trafficking, with the flagellar pocket shown at top left. Rab GTPase proteins, which are convenient markers for many subcellular compartments, are shown as green ovals, whereas arrows indicate the major known transport routes and red indicates several molecular complexes within the endocytic system that are of significance. (B) The overall structure of a VSG dimer, colour coded with α-helical elements in red, β-sheets in blue, comprising the X-ray and NMR structures for the N- and C-terminal domains [83,84] (note that the arrangement of these domains with each other is arbitrary because this is not experimentally known). (C) A hypothetical array of VSGs with the tips of the C-terminal domains just touching. (D) A simplified version of (A) but rekeyed to illustrate the significant concentration gradient of VSG as it exits the ER and is then concentrated at the plasma membrane. Arrows indicate major routes, with blue for biosynthesis, red for degradation and grey for recycling. The approximate number of VSG molecules that are in flux through each route is also indicated. Abbreviation: CAPS, clathrin-associated proteins; ERES, endoplasmic reticulum exit sites; ESCRT, endosomal-sorting complex required for transport.
Signal sequence cleavage and glycosylation
Concomitant with translocation, nascent VSG polypeptide is exposed to ER-resident enzymes and maturation of the protein begins. The N-terminal signal sequence is cleaved [21,22], presumably by the signal peptidase complex (for a comprehensive review, see [27]). Although this complex is yet to be formally characterised in T. brucei, a catalytic Sec11 subunit homologue is readily identifiable by homology searches (P.T. Manna and M.C. Field, unpublished). Additionally, Asn-X-Ser/Thr consensus sites are N-glycosylated by oligosaccharyltransferase (OST), an eight-subunit complex in higher eukaryotes, predicted by homology searches to be represented in T. brucei by only three paralogous catalytic stauroporins and temperature-sensitivity protein 3 (STT3) subunits in trypanosomes. This represents a potentially extreme level of minimisation, but the precise composition of the trypanosome OST remains to be elucidated and may be more complex. N-linked glycosylation in T. brucei is central to VSG expression levels [28], with all known VSGs being N-glycosylated at up to three sites [29,30].

N-glycosylation has some unusual features in trypanosomes. Whereas most eukaryotes transfer a triantennary triglucosylated oligomannose structure, Glc3MαMan9GlcNAc2, from the dolichol-linked donor, trypanosomes are unable to produce glucosylated lipid precursors and, hence, transfer nonglucosylated glycans [29]. Additionally, whereas the STT3B transfers a triantennary oligomannose structure, Manα2GlcNAc2, the STT3A subunit has preference for an unusual biantennary pucimannose glycan, Manβ2GlcNAc2. Direct transfer of pucimannose glycans may provide a route to pucimannose or complex glycan structures, comprising approximately half of VSG N-glycans, sparing lipid-linked mannosyl precursors required for oligomannose and, importantly, also GPI-anchor biosynthesis, a potential adaptation to the enormous VSG biosynthetic load [30-32]. Although N-glycosylation in T. brucei is perhaps the clearest departure from the classical eukaryotic pathway, a range of seemingly complementary modifications has arisen in more distal processes, such as ER quality control and further processing in the Golgi complex.

GPI anchoring
The VSG polypeptide contains a C-terminal cleavable signal for GPI-anchor addition [22,33]. Upon translocation, the GPI signal sequence is cleaved and replaced with a preformed GPI anchor by the GPI trans-amidase complex comprising conserved GAA1, GPI8, and PIG-T subunits, together with trypanosomatid-specific TTA1 and TTA2 [34]. The VSG GPI anchor is unique in having only myristate (C14:0) fatty acids and a poly-α-galactose side chain, at least one galactose likely being transferred in the ER [35,36]. Only correctly GPI-anchored VSG is able to reach the cell surface, subject to proper folding and dimerisation, with GPI-deficient VSGs being retained in the ER before degradation [37,38].

Folding and quality control
Intuitively, the high abundance of VSG suggests selection for efficient folding and maturation. However, considerable overproduction of VSG and subsequent proteosomal degradation of a large proportion of presumably misfolded protein does occur, suggesting that VSG is not particularly efficient in its ability to attain the native fold [20]. Although relatively little detailed mechanistic information is available, a functioning ER quality control system likely operates in VSG synthesis. The trypanosome genome encodes orthologues of many key genes for ER oligosaccharide processing and quality control, including calreticulin, the deglucosylating α-glucosidase GII enzyme and the reglucosylating UDP-Glc-glycoprotein glucosyltransferase (UGGT) [39]. Although VSG does not receive glucosylated glycans via OST, UGGT selectively binds to unglucosylated unfolded proteins and glucosylates the N-glycans, promoting binding to calreticulin and associated chaperones, such as BiP. VSG with glucosylated glycans is in turn a substrate for α-glucosidase GII and is further deglucosylated [39]. Once the VSG molecule has reached its native conformation, it is no longer a substrate for disorder-sensing UGGT and remains deglucosylated. Thus, freed from calreticulin, properly folded VSG is competent for ER export.

In other eukaryotes, a timing mechanism operates to free terminally misfolded proteins from this cycle and target them instead for proteosomal degradation. This involves the removal of mannosyl residues from terminally misfolded proteins to reveal a glycan structure recognised by the ER lectin Yos9, which subsequently targets the protein for proteosomal degradation [27]. Trypanosoma brucei encodes a cluster of ER degradation-associated mannosidase-related (EDM) genes and a Yos9 orthologue [20,40]. Experimental support for this pathway comes from RNAi depletion of T. brucei EDMs, leading to VSG accumulation [20,40]. In addition to these conserved components, two trypanosomatid-specific genes, ERAP18 and ERAP32, with positive effects on VSG copy number, were recently identified in a screen for ER residents [41]. These ER proteins have clear, but mechanistically opaque, effects upon the copy number of VSG expressed at the surface, with a potential role in quality control or ER export [41]. Together, these studies argue against VSG having superior folding competence and also demonstrate a conserved ER quality control and ER-associated degradation (ERAD) system functioning in VSG biosynthesis.

ER export
Newly synthesised, glycosylated, and dimerised VSG is exported from the ER at specific exit sites (ERES) that, in T. brucei, are closely apposed to the Golgi apparatus, suggesting that they are a streamlining adaptation for high VSG flux [42]. As in other eukaryotes, transport from the ERES is via coat protein complex II (COPII) vesicles, formed following Sar1 activation and recruitment of a prebudding complex of Sec23 and Sec24, which in turn recruits the outer coat of Sec13 and Sec31 [43]. Trypanosoma brucei has two isoforms each of Sec23 and Sec24, with VSG export depending upon TbsSec23.2 and TbsSec24.1 [42]. How VSG is selectively loaded into COPII vesicles by this prebudding complex, in the absence of cytosolic sorting signals, is yet to be defined.

Processing in the Golgi
ER-derived COPII vesicles fuse with the cis face of the Golgi, and cargoes undergo further processing and migrates
towards the trans-Golgi network (TGN) for sorting to their correct cellular location. In a further example of the streamlining of the trypanosome secretory system, the Golgi is present as a single copy and occupies a defined position within the cell, adjacent to the ERES and polarised towards the flagellar pocket (FP) [44]. Within the Golgi, the α-galactose side chain of the VSG GPI anchor is extended [45,46], and N-glycans are further modified. It appears that only the unusual biantennary Man3GlcNAc2 glycans are processed to complex oligosaccharides, suggested to result from the lack of a Golgi α-mannosidase and an unusual preference of trypanosome N-acetylgluco-
saminyltransferase I (GnTI) for Man4GlcNAc2 and Man3GlcNAc2 structures [47].

**Cell surface delivery**

Compared with the earlier stages of VSG biosynthesis, little is known of the mechanisms of post-Golgi VSG exocytosis. Newly synthesised VSG does not enter the system for Rab11-dependent surface delivery alongside endocytosed VSG [48,49]. Golgi export of newly synthesised VSG is also actin independent [50]. In other organisms, the export of GPI-anchored proteins from the Golgi has been suggested to occur through accumulation in specific TGN microdomains, potentially involving localised lipid interactions [51], but VSG-enriched microdomains have not been observed in the *T. brucei* endomembrane system [14]. The amount of newly synthesised VSG being delivered to the cell surface at any one time is dwarfed by the constantly recycling cell surface pool, significantly complicating experimental dissection of this aspect.

**Concentration and sorting along the exocytic pathway**

Newly synthesised VSG reaches the cell surface with a t1/2 of approximately 15 min [10], with the concentration of VSG at the cell surface approximately 50-fold greater than in the ER, and the Golgi having an intermediate density of approximately 2.7-fold that of the ER [14]. Rapid transit of VSG through the exocytic apparatus, together with a large concentration gradient, suggests efficient sorting of VSG at multiple levels [14]. The first sorting and concentration step for which we have evidence is selective export of VSG from the ER in Sec23.2/Sec24.1 COPII vesicles, and away from a population of COPII vesicles bearing the alternate paralogues Sec23.1/Sec24.2 [42]. It is calculated that a single COPII vesicle should be capable of accommodating approximately 600 VSG dimers, at a density of approximately 30 000 dimers/μm², the observed density of VSG at the cell surface [13,14,19]. The observed density of VSG at the Golgi, together with the rate of synthesis, suggests one fully loaded COPII vesicle equivalent docking at the Golgi every 3.5 s. There is no apparent gradient in VSG density across the Golgi, although it is likely that this has not been measured with sufficient sensitivity to detect a moderate gradient [14], but this does suggest similar kinetics for transport between cisternae. However, substantial discrepancies exist between the observed transit rate and the theoretical rate as calculated, based on the required flux of VSG required to build the surface, indicating that there are fundamental details remaining to be uncovered [19]. Therefore, any consideration of the kinetics of VSG transit through specific exocytic organelles currently remains speculative. A second major sorting step likely occurs on exit from the Golgi, where VSG is again presumably concentrated; unfortunately, the mechanism of this transport step also remains elusive.

**Adaptations and specialisations of the endocytic machinery**

The plasma membrane recycling machinery has a major role in VSG coat maintenance. All surface membrane traffic in *T. brucei* is routed via the flagellar pocket (FP), a flask-shaped invagination of the plasma membrane surrounding the base of the flagellum, where exocytosis, endocytosis and/or recycling intersect. The FP represents 2% of total surface membrane and is delineated by an electron-dense flagellar pocket collar acting as a tight cytoskeletal barrier (discussed in [8]). Uptake from the FP depends exclusively on clathrin-mediated endocytosis (CME), with inhibition of CME by depletion of clathrin resulting in severe FP enlargement, likely a result of imbalance in trafficking of material to and from the surface [52]. Endocytosis is developmentally regulated and upregulated approximately tenfold in mammalian- compared with insect-form trypanosomes, sufficient so as to internalise an area equivalent to the entire plasma membrane every 12 min [12,53,54]. Developmental regulation is mainly restricted to early endocytic gene expression as detected by transcriptome analysis, with little evidence for changes elsewhere, that is, anterograde transport or the terminal steps in endocytosis [55].

**Endocytic activity and removal from the FP**

Compared with most other studied eukaryotes, the CME machinery in trypanosomes appears simplified: notably one of the CME key players, adaptor protein 2 (AP-2), a heterotetrameric complex responsible for cargo receptor binding and clathrin recruitment to the plasma membrane, is absent from all trypanosomatids that have VSG, likely a critical adaptation for rapid endocytosis and apparently unique to this lineage [56]. The absence of AP-2 also suggests that the initial endocytic step in trypanosomes, budding and internalisation of membrane from the FP, is nonconcentrative. Indeed, further concentration of VSG into endocytic vesicles may be precluded by the density of the molecule at the cell surface, with sorting of other surface molecules delegated to later steps to maximise the speed of VSG removal. It is also significant that the level of conservation of proteins interacting with clathrin appears to be low, with evidence for significantly distinct cohorts of polypeptides operating [57,58]; however, a full description of this process is lacking and, hence, the real level of divergence is unclear.

**Sorting, recycling, and return to the surface**

The high rate of endocytosis necessitates a mechanism to return VSG to the surface. This process is rapid, with estimates of between approximately 1 and 10 min, and the itinerary well characterised, with dependence on Rab5 and Rab11 being clear from both morphological and kinetic studies [6,8,12,48,59]. Much of this machinery is also likely
conserved; for example, recycling depends on the trypanosome orthologue of RME-8, which is essential [60], and Rab11, which interacts with the exocyst subunit Sec15, itself a well-characterised player in exocytic transport in other eukaryotes [61,62]. However, Rab11 also interacts with at least one protein restricted to trypanosomes, RBP74, and a protein restricted to taxa with motile cilia and/or flagella, AZ11, which may link Rab11 to flagellar function [62]. Rab11 is likely a major organiser of the endomembrane system, and may receive input allowing coordination of transport to balance VSG synthesis, recycling, and the requirement for protein turnover. Significantly, Rab11 and RME-8 are upregulated in the mammalian stage [54,60,63]. The mechanisms of sorting, and specifically how and where VSG is sorted away from surface, receptors, and other surface molecules remains to be explored (discussed below); recent evidence indicates considerable complexity within the endocytic apparatus, with a new compartment defined by Rab21 being added to the itinerary [64].

One fundamental question that remains is how the highly abundant VSG is sorted efficiently to a recycling pathway, while other molecules are delivered to the lysosomal pathway for degradation. The current model, supported by immunoelectron microscopy experiments, suggests a default sorting of VSG into the recycling pathway, analogous to the known route for membrane lipids and rapidly recycling cargoes, such as the transferrin receptor in mammalian systems [48]. Under this scheme, the question better asked is perhaps how other surface molecules are sorted to the retrograde pathway. Recent evidence supports a role for ubiquitylation in this process. Invariant surface glycoprotein (ISG) 65 and 75 family proteins are among the most abundant surface molecules after VSG in mammalian stages, with approximately 50 000–70 000 molecules per cell. ISGs have a trans-membrane domain and conserved cytoplasmic lysines that are substrates for ubiquitin modification, which act as endocytic and sorting signals [65–68]. This modification is likely recognised by the conserved endosomal-sorting complex required for transport (ESCRT) machinery [69], although the role of ESCRTs in ISG degradation has been recently challenged [70].

**Contribution to immune evasion**

African trypanosomes evolved at least three VSG-dependent strategies for immune evasion. Long-term persistence is accomplished foremost by antigenic variation [71–74]. Additionally, the host immune system is weakened by general immune suppression via as yet unknown mechanisms, but which appear to compromise the ability of the antibody response to mature fully; there is evidence that fragments of VSG contribute to this [75–77]. A third mechanism involves rapid clearance of antibodies, aided by hydrodynamic flow generated by the motility of the parasite [15,78].

Given that VSG monomers are packed at high density, only the external N terminus of the homodimer is exposed to the host immune system [79,80]. Therefore, antibody-bound VSG molecules protrude from the surface coat and accumulate at the posterior pole of the cell due to increased drag, before internalisation and rapid degradation by a process requiring both the endocytic system and recycling [81]. Accumulation of VSG–immunoglobulin G (IgG) complexes at the posterior pole is independent of endocytosis, because downregulating clathrin by RNAi blocks endocytic trafficking, whereas VSG–IgG accumulation at the posterior pole is unaffected [80]. Motility is required for this phenomenon, and downregulation of the dynein arm intermediate chain DNA11, which reverses the swimming direction of the parasite, results in accumulation of IgG at the anterior pole of the cell [80]. Immunoglobulin degradation is sensitive to E46d and K11777 (cathepsin and cysteine protease inhibitors, respectively), supporting a role for lysosomes in degradation (Figure 2), whereas VSG is efficiently recycled back to the surface [12,48,81].

Although the precise role of surface antibody clearance in the mammalian host is yet to be fully elucidated, its effectiveness is evident at low to moderate antibody concentrations, but clearly insufficient to mediate protection at high antibody titres [82]. Thus, this represents a mechanism that protects an individual cell during emergence of a specific humoral immune response, and may also be of importance during early infection, when antibody titres are low or after differentiation to the cell cycle-arrested short stumpy form.

**Evolution of the VSG surface**

How then did VSG arise, and with it some of the remarkable aspects of trypanosome cell biology? We can presume that *T. brucei* initially arose from an organism more similar to *Leishmania* and *Trypanosoma cruzi*, which implies a more heterogenous surface proteome than African trypanosomes currently have. Massive increases in expression level, expansion into a large paralogous gene family, and the advent of monoallelic expression were all required to achieve the VSG system, but the order of events is unclear (Figure 3). Comparisons of the gene complements and genome structures between the African and American
trypanosomes, and *Leishmania* spp. indicate that the major coding variance resides within those genes encoding surface determinants, with a remarkable level of conservation for most other gene functional classes, even extending to the level of retaining synteny and polycistronic transcription unit composition [85]. This insight agrees well with much of the earlier biochemical analysis demonstrating clear diversification of the surface composition of the African and American trypanosomes and *Leishmania* spp, and is presumably a reflection of the major emphasis of selective pressure for parasites with radically different life cycles and styles [86].

Examination of more closely related genomes also suggests how VSG itself has evolved, with clear evidence for ongoing and lineage-specific diversification of this family. For example, the VSG repertoire of *Trypanosoma vivax* is greater than *T. brucei* in terms of sequence diversification, with evidence for differential recombination events between taxa and little overlap between the repertoires. A deep division into two distinct subfamilies, a-VSG and b-VSG, appears to have been maintained through the evolution of the African lineage, suggesting that isolation between these two subfamilies occurred early [87,88]. Furthermore, VSGs also appear to have given rise to nonvariable antigens, such as the transferrin receptor and serum resistance associated (SRA), and overall surface diversity may also be connected with the expression site (ES) architecture. For example, it has been suggested that ISGs are related to ESAG11, providing additional possible mechanisms for surface phylum diversification and separation of the evolutionary trajectories of surface proteins encoded by core chromosomal regions and those that have migrated to subtelomeric sites [88]. *Trypanosoma grayi*, also a tssetse-transmitted trypanosome of African origin and closely relate to *T. cruzi* [89], lacks VSG, which suggests that emergence of the VSG system was rapid, because no intermediate forms between *T. grayi* and the *T. brucei African clade* are known [56].
Concluding remarks
VSG has played the role of a prototypical model protein for understanding the cell biology of the GPI anchor and the molecular genetics and immunology of antigenic variation, making it an exceptionally generous molecule, if at the same time sitting at the heart of a deadly immune evasion machine. Moreover, interest in just these topics propelled the emergence of African trypanosomes as an important model organism, so that understanding of the cell biology of VSG is now at an advanced stage, albeit with many questions that still remain. Without the initial spur provided by VSG, it is doubtful that the interest or research activity into African trypanosomes would be as strong or as vibrant as it is today, and many of us have benefited from the generosity of VSG. VSG continues to challenge and fascinate; however, a full appreciation of this remarkable example of evolution and immune evasion machinery remains to be achieved.

Acknowledgements
Work in our laboratory is supported by a Wellcome Trust program grant (082813 to M.C.F.). M.C.F. would like to thank many members of the laboratory during our time in Cambridge, especially for their insights, contributions, and efforts that form part of the work that is described here.

References
1 Cross, G.A. (1975) Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of Trypanosoma brucei. Parasitology 71, 393–417
2 Bridgen, P.J. et al. (1976) N-terminal amino acid sequences of variant-specific surface antigens from Trypanosoma brucei. Nature 263, 613–614
3 Vickerman, K. (1969) On the surface coat and flagellar adhesion in trypanosomes. J Cell Sci 5, 163–193
4 Morrison, L.J. et al. (2009) Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complexity. Cell. Microbiol. 11, 1724–1734
5 Blum, M.L. et al. (1993) A structural motif in the variant surface glycoproteins of Trypanosoma brucei. Nature 362, 603–609
6 Field, M.C. et al. (2009) Macromolecular trafficking and immune evasion in African trypanosomes. Int. Rev. Cell Mol. Biol. 278, 1–67
7 Thomas, J.R. et al. (1990) Structure, biosynthesis, and function of glycosphingolipid glycosyltransferases. Biochemistry 29, 5413–5422
8 Field, M.C. and Carrington, M. (2009) The trypanosome flagellar pocket. Nat. Rev. Microbiol. 7, 775–786
9 Ferguson, M.A. and Cross, G.A. (1984) Myristylation of the membrane form of a Trypanosoma brucei variant surface glycoprotein. J. Biol. Chem. 259, 3011–3015
10 Bangs, J.D. et al. (1986) Post-translational modification and intracellular transport of a trypanosome variant surface glycoprotein. J. Cell Biol. 103, 255–263
11 Seyfang, A. et al. (1990) Degradation, recycling, and shedding of Trypanosoma brucei variant surface glycoprotein. J. Protozool. 37, 546–552
12 Engstler, M. et al. (2004) Kinetics of endocytosis and recycling of the GPI-anchored variant surface glycoprotein in Trypanosoma brucei. J. Cell Sci. 117, 1105–1115
13 Jackson, D.G. et al. (1985) A new method for the rapid purification of both the membrane-bound and released forms of the variant surface glycoprotein from Trypanosoma brucei. Biochem. J. 230, 195–202
14 Grünfelder, C.G. et al. (2002) Accumulation of a GPI-anchored protein at the cell surface requires sorting at multiple intracellular levels. Traffic 3, 547–559
15 Barry, J.D. (1979) Capping of variable antigen on Trypanosoma brucei, and its immunological and biological significance. J. Cell Sci. 37, 287–302
16 Field, M.C. et al. (2007) Intracellular trafficking in the trypanosomatids. Traffic 8, 629–639
17 ShSeeder, K. et al. (2005) Variant surface glycoprotein RNA interference triggers a precytokinesis cell cycle arrest in African trypanosomes. Proc. Natl. Acad. Sci. U.S.A. 102, 8716–8721
18 Smith, T.K. et al. (2009) Blocking variant surface glycoprotein synthesis in Trypanosoma brucei triggers a general arrest in translation initiation. PLoS ONE 4, e7532
19 Warren, G. (2013) Transport through the Golgi in Trypanosoma brucei. Histochem. Cell Biol. 140, 235–238
20 Field, M.C. et al. (2010) Chaperone requirements for biosynthesis of the trypanosome variant surface glycoprotein. PLoS ONE 5, e8468
21 McConville, J. et al. (1981) Biosynthesis of Trypanosoma brucei variant surface glycoprotein. I. Synthesis, size, and processing of an N-terminal signal peptide. Mol. Biochem. Parasitol. 4, 225–242
22 Boothroyd, J.C. et al. (1981) Variant surface glycoproteins of Trypanosoma brucei are synthesised with cleavable hydrophobic sequences at the carboxy and amino termini. Nucleic Acids Res. 9, 4735–4743
23 Wickner, W. and Schekman, R. (2005) Protein translocation across biological membranes. Science 310, 1452–1456
24 Liu, L. et al. (2000) RNA interference of signal peptide-binding protein SRP54 elicits deleterious effects and protein sorting defects in trypanosomes. J. Biol. Chem. 277, 47348–47357
25 Lustig, Y. et al. (2007) Down-regulation of the trypanosomatid signal recognition particle affects the biogenesis of polytopic membrane proteins but not of signal peptide-containing proteins. Eukaryot. Cell 6, 1865–1875
26 Goldshmidt, H. et al. (2008) Role of protein translocation pathways across the endoplasmic reticulum in Trypanosoma brucei. J. Biol. Chem. 283, 32083–32098
27 Barlowe, C.K. and Miller, E.A. (2013) Secretory protein biogenesis and traffic in the early secretory pathway. Genetics 193, 383–410
28 Wang, J. et al. (2003) Structural features affecting variant surface glycoprotein expression in Trypanosoma brucei. Mol. Biochem. Parasit. 128, 135–145
29 Parodi, A.J. (1993) N-glycosylation in trypanosomatid protozoa. Glycobiology 3, 193–199
30 Mehert, A. et al. (1998) The glycosylation of the variant surface glycoproteins and procyelic acidic repetitive proteins of Trypanosoma brucei. Mol. Biochem. Parasit. 91, 145–152
31 Izquierdo, L. et al. (2009) Distinct donor and acceptor specificities of Trypanosoma brucei oligosaccharidyltransferases. EMBO J. 28, 2650–2661
32 Smith, T.K. and Büttikofer, P. (2010) Lipid metabolism in Trypanosoma brucei. Mol. Biochem. Parasit. 172, 66–79
33 Udenfried, S. and Kodukula, K. (1995) How glycosylphosphatidylinositol-anchored membrane proteins are made. Ann. Rev. Biochem. 64, 563–591
34 Nagamune, K. et al. (2003) GPI transamidase of Trypanosoma brucei has two previously uncharacterized (trypanosomatid transamidase 1 and 2) and three common subunits. Proc. Natl. Acad. Sci. U.S.A. 100, 10682–10687
35 Mayor, S. et al. (1992) Galactose-containing glycosylphosphatidylinositol in Trypanosoma brucei. J. Biol. Chem. 267, 754–761
36 Ferguson, M.A.J. (1999) The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosomes research. J. Cell Sci. 112, 2799–2809
37 Böhme, U. and Cross, G.A.M. (2002) Mutational analysis of the variant surface glycoprotein GPI-anchor signal sequence in Trypanosoma brucei. J. Cell Sci. 115, 805–816
38 Triggs, V.P. and Bangs, J.D. (2003) Glycosylphosphatidylinositol-dependent protein trafficking in bloodstream stage Trypanosoma brucei. Eukaryot. Cell 2, 76–83
39 Izquierdo, L. et al. (2009) Trypanosoma brucei UDP-glucose:glycoprotein glucosyltransferase has unusual substrate specificity and protects the parasite from stress. Eukaryot. Cell 8, 230–240
40 Banerjee, S. et al. (2007) The evolution of N-glycan-dependent endoplasmic reticulum quality control factors for glycoprotein folding and degradation. Proc. Natl. Acad. Sci. U.S.A. 104, 11676–11681
41 Wang, Y.N. et al. (2010) Trypanosoma brucei: trypanosome-specific endoplasmic reticulum proteins involved in variant surface glycoprotein expression. Exp. Parasitol. 125, 208–221
Review

Trends in Parasitology  May 2014, Vol. 30, No. 5

42 Sevova, E.S. and Bangs, J.D. (2009) Streamlined architecture and glycosylphosphatidylinositol-dependent trafficking in the early secretory pathway of African trypanosomes. Mol. Biol. Cell 20, 4739–4750

43 Lord, C. et al. (2013) The highly conserved COPIII coat complex sorts cargo from the endoplasmic reticulum and targets it to the Golgi. CSH Perspect. Biol. 5, a013367

44 Silverman, J.S. and Bangs, J.D. (2012) Form and function in the trypanosomal secretory pathway. Curr. Opin. Microbiol. 15, 463–468

45 Bangs, J.D. et al. (1988) Biosynthesis of a variant surface glycoprotein of Trypanosoma brucei. Processing of the glycolipid membrane anchor and N-linked oligosaccharides. J. Biol. Chem. 263, 17697–17705

46 McConville, M.J. and Ferguson, M.A. (1993) The structure, biosynthesis and function of glycosylated phosphatidylinositol in the parasitic protozoa and higher eukaryotes. Biochem. J. 294, 305–324

47 Manthri, S. et al. (2008) Deletion of the ThaLG3 gene demonstrates site-specific N-glycosylation and N-glycan processing in Trypanosoma brucei. Glycobiology 18, 367–383

48 Grünfelder, C.G. et al. (2005) Endocytosis of a glycosylphosphatidylinositol-anchored protein via clathrin-coated vesicles, sorting by default in endosomes, and exocytosis via RAB11-positive carriers. Mol. Biol. Cell 14, 2029–2040

49 Hall, B.S. et al. (2005) Developmental variation in Rab11-dependent trafficking in Trypanosoma brucei. Eukaryot. Cell 4, 971–980

50 Nolan, D.P. and García-Salcedo, J.A. (2008) Loss of actin does not affect export of newly synthesized proteins to the surface of Trypanosoma brucei. Mol. Biochem. Parasitol. 157, 233–235

51 Aneja, M. and Hoffack, B. (2011) Exit from the trans-Golgi network: from molecules to mechanisms. Curr. Opin. Cell Biol. 23, 443–451

52 Allen, C.L. et al. (2003) Clathrin-mediated endocytosis is essential in Trypanosoma brucei. EMBO J. 22, 4991–5002

53 Morgan, G.W. et al. (2002) The endocytic apparatus of the kinetoplastida. Part II: machinery and components of the system. Trends Parasitol. 18, 540–546

54 Natesan, S.K. et al. (2007) Activation of endocytosis as an adaptation to the mammalian host by trypanosomes. Eukaryot. Cell 8, 2029–2037

55 Koumandou, V.L. et al. (2008) The trypanosome transcriptome is remodelled during differentiation but displays limited responsiveness within life stages. BMC Genomics 9, 298

56 Manna, P.T. et al. (2013) Adaptin evolution in kinetoplastids and emergence of the variant surface glycoprotein coat in African trypanosomatids. Mol. Phylogenet. Evol. 67, 123–128

57 Gabernet-Castello, C. et al. (2009) The single ENTH-domain protein of trypanosomes; endocytic functions and evolutionary relationship with epsins. Traffic 10, 894–911

58 Adung’a, V.O. et al. (2013) Proteomic analysis of clathrin interactions in trypanosomes reveals dynamic evolution of endocytosis. Traffic 14, 440–457

59 Pal, A. et al. (2002) Differential endocytic functions of Trypanosoma brucei Rab5 isoforms reveal a glycosylphosphatidylinositol-specific endosomal pathway. J. Biol. Chem. 277, 9529–9539

60 Koumandou, V.L. et al. (2013) Evidence for recycling of invariant surface transmembrane domain proteins in African trypanosomes. Eukaryot. Cell 12, 339–342

61 Gabernet-Castello, C. et al. (2011) Rab11 function in Trypanosoma brucei: identification of conserved and novel interaction partners. Eukaryot. Cell 10, 1082–1094

62 Heider, M.R. and Munson, M. (2012) Exercising the exocyst complex. Traffic 13, 898–907

63 Jeffries, T.R. et al. (2001) A developmentally regulated rab11 homologue in Trypanosoma brucei is involved in recycling processes. J. Cell Sci. 114, 2617–2626

64 Ali, M. et al. (2014) The ancient small GTPase Rab21 functions in intermediate endocytic steps in trypanosomes. Eukaryot. Cell 13, 304–319

65 Ziegelbauer, K. et al. (1992) Molecular characterization of two invariant surface glycoproteins specific for the bloodstream stage of Trypanosoma brucei. J. Biol. Chem. 267, 10797–10803

66 Chung, W.L. et al. (2004) Cytoplasmic targeting signals in transmembrane invariant surface glycoproteins of trypanosomes. J. Biol. Chem. 279, 5487–5490

67 Chung, W.L. et al. (2008) Ubiquitylation is required for degradation of transmembrane surface proteins in trypanosomes. Traffic 9, 1681–1697

68 Leung, K.F. et al. (2011) Ubiquitylation and developmental regulation of invariant surface protein expression in trypanosomes. Eukaryot. Cell 10, 916–931

69 Leung, K.F. et al. (2008) Evolution of the multivesicular body ESCRT machinery; retention across the eukaryotic lineage. Traffic 9, 1698–1716

70 Silverman, J.S. et al. (2013) Characterization of the late endosomal ESCRT machinery in Trypanosoma brucei. Traffic 14, 1078–1090

71 Berst, P. and Cross, G.A. (1982) Molecular basis for trypanosome antigenic variation. Cell 29, 291–303

72 Berst, P. et al. (1980) The genes for variant antigens in trypanosomes. Annu. Trop. Med. Hyg. 29, 1033–1036

73 Cross, G.A. (1996) Antigenic variation in trypanosomes: secrets surface slowly. Bioessays 18, 283–291

74 Lythgoe, K.A. et al. (2007) Parasite-intrinsic factors can explain ordered progression of trypanosome antigenic variation. Proc. Natl. Acad. Sci. U.S.A. 104, 8095–8100

75 Magee, S. et al. (1998) The glycosyl-inositol-phosphate and dimyristoylglycerol moieties of the glycosylphosphatidylinositol anchor of the trypanosome variant-specific surface glycoprotein are distinct macropheage-activating factors. J. Immunol. 160, 1949–1956

76 Donelson, J.E. et al. (1998) Multiple mechanisms of immune evasion by African trypanosomes. Mol. Biochem. Parasitol. 91, 51–66

77 Radwanska, M. et al. (2000) Comparative analysis of antibody responses against HSP60, invariant surface glycoprotein 70, and variant surface glycoprotein reveals a complex antigen-specific pattern of immunoglobulin isotype switching during infection by Trypanosoma brucei. Infect. Immun. 68, 848–860

78 Engstler, M. et al. (2007) Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. Cell 131, 505–515

79 Overath, P. and Engstler, M. (2004) Endocytosis, membrane recycling and sorting of GPI-anchored proteins: Trypanosoma brucei as a model system. Mol. Microbiol. 53, 735–744

80 Schwede, A. et al. (2011) The VSG C-terminal domain is inaccessible to antibodies on live trypanosomes. Mol. Biochem. Parasitol. 175, 201–204

81 Pal, A. et al. (2003) Rab5 and Rab11 mediate transferrin and anti- variant surface glycoprotein antibody recycling in Trypanosoma brucei. Biochem. J. 374, 443–451

82 McIntock, L.M. et al. (1993) Comparison of the effects of immune killing mechanisms on Trypanosoma brucei parasites of slender and stumpy morphology. Parasite Immunol. 15, 475–480

83 Freymann, D. et al. (1990) 2.A resolution structure of the N-terminal domain of a variant surface glycoprotein from Trypanosoma brucei. J. Biol. Chem. 216, 141–160

84 Chattopadhyay, A. et al. (2005) Structure of the C-terminal domain from Trypanosoma brucei variant surface glycoprotein M1 tet. J. Biol. Chem. 280, 7226–7235

85 El-Sayed, N.M. et al. (2005) Comparative genomics of trypanosomatid parasitic protozoa. Science 309, 404–409

86 Field, M.C. et al. (2007) Intracellular trafficking in trypanosomes. Traffic 8, 629–639

87 Jackson, A.P. et al. (2012) Antigenic diversity is generated by distinct evolutionary mechanisms in African trypanosomes. Proc. Natl. Acad. Sci. U.S.A. 109, 3416–3421

88 Jackson, A.P. et al. (2013) A cell-surface phylome for African trypanosomes. PLoS NTD 7, e2121

89 Mann, P. et al. (2013) Co-evolution of antigenic variation and adaptins in trypanosomatids. Mol. Protein Evol. 67, 123–128