Bloodstream form trypanosome plasma membrane proteins: antigenic variation and invariant antigens

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SUMMARY

Trypanosoma brucei is exposed to the adaptive immune system and complement in the blood of its mammalian hosts. The aim of this review is to analyse the role and regulation of the proteins present on the external face of the plasma membrane in the long-term persistence of an infection and transmission. In particular, the following are addressed: (1) antigenic variation of the variant surface glycoprotein (VSG), (2) the formation of an effective VSG barrier shielding invariant surface proteins, and (3) the rapid uptake of VSG antibody complexes combined with degradation of the immunoglobulin and recycling of the VSG.

Key words: Trypanosome, VSG, antigenic variation.

INTRODUCTION

Trypanosoma brucei lives in the blood and tissue fluids of mammalian hosts and an infection can last several years. This lifestyle requires invariant cell surface proteins for nutrient uptake, signaling and structural integrity. However, the invariant surface proteins need to be shielded from the immune system to establish a long-lasting infection.

African trypanosomes avoid clearance by the immune system in several ways. First, they modulate the innate and adaptive immune response by an incompletely understood mechanism dependent on trypanosome-derived factors (Mansfield and Paulnock, 2005; Leppert *et al*. 2007). Second, trypanosomes have a dense surface coat mainly composed of a single polypeptide, the variant surface glycoprotein (VSG), that shields the invariant surface proteins from effectors of the immune system and prevents complement activation (Ferrante and Allison, 1983). Third, antibody binding to VSG leads to rapid internalization of the antibody-VSG complex, subsequent proteolysis of the antibody and recycling of the VSG back to the surface (Engstler *et al*. 2007). This process prolongs the survival of a trypanosome at low antibody titres. Finally, the survival of the population is dependent on antigenic variation that allows the infection to stay ahead of the host immune system.

As an infection progresses, a high antibody titre against a particular VSG leads to the clearance of trypanosomes expressing that VSG, trypanosomes that have switched VSG expand as a new population until they are recognized in turn – these cycles can go on for years. The rate of VSG switching varies between isolates but models of long-term infections are consistent with estimated rates and temporal expression of *VSG* genes is dependent only on parasite-intrinsic factors and a density-dependent differentiation to the non-dividing trypanosomes (Lythgoe *et al*. 2007).

This review describes some components of the trypanosome plasma membrane, the endocytosis and recycling of VSGs back to the surface and finally summarizes what is known about antigenic variation.

THE TRYPANOSOME CELL SURFACE

An unusual feature of the external face of the plasma membrane of trypanosomes is the predominance of a glycosylphosphatidylinositol (GPI)-anchored protein, the VSG (Ferguson *et al*. 1988; Melhert *et al*. 2002). The numbers are staggering; the VSG is over 95% of externally disposed cell surface proteins and ~15% of the total cell protein of a trypanosome cell with $\sim 5 \times 10^6$ VSG dimers per cell (Jackson *et al*. 1985).

Why has there been selection for GPI-anchoring of the superabundant VSG? There are several possible advantages of a GPI-anchor over the single transmembrane helix present in type 1 membrane proteins that are probably only significant at high packing densities. First, a higher packing density on the cell surface could be achieved using GPI-anchors as an equivalent packing density of type 1 membrane proteins might congest the cytoplasmic face of the plasma membrane. This is important as it is probable

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that the VSG density on the surface approaches the maximum possible. Secondly, GPI-anchoring may play a role in trafficking and recycling of the VSG. In the absence of a GPI-anchor, VSG is mislocalised to the lysosome (Triggs and Bangs, 2003) or to non-lysosomal compartments close to the flagellar pocket (Bohme and Cross, 2002). In trypanosomes GPI-valence seem to play a role in the trafficking of GPI-anchored proteins. The dimeric VSG, with two GPI anchors, mainly localises to the cell surface whereas the transferrin receptor, with a single GPI-anchor, and reporter proteins, with a single GPI anchor, cycle through the flagellar pocket and endosome, and are delivered to the lysosome (Schwartz *et al*. 2005; Schwartz and Bangs, 2007). Thirdly, during differentiation from the bloodstream to the procyclic form the VSG coat is rapidly shed from the surface by the activities of the GPI-specific phospholipase C and the zinc metalloprotease TbMSP-B (Grandgenett *et al*. 2007).

In addition to a GPI-anchor, VSGs are modified with N-linked oligosaccharides that are either high-mannose oligosaccharides or complex oligosaccharides (Bangs *et al*. 1988; Zamze *et al*. 1990; Zitzmann *et al*. 2000). It has been suggested that VSG glycosylation contributes to the volume of VSGs and thus its ability to form a protective surface barrier (Blum *et al*. 1993). The model of the N-terminal domain of the MITat1.5 glycoprotein shows that the N-linked oligosaccharides occupy a significant amount of the inter-VSG space (Mehlert *et al*. 2002).

There are three types of N-terminal VSG domains (A, B and C) and six types of C-terminal domains (type1–6) (Carrington *et al*. 1991; Berriman *et al*. 2005). The different VSG types are classified according to their pattern of conserved cysteine residues. All combinations of the N-terminal domains with the C-terminal domains appear possible (Hutchinson *et al*. 2003). The structures of the N-terminal domain of MITat1.2 and ILTat1.24, both type A, have been determined by X-ray crystallography (Freymann *et al*. 1990; Blum *et al*. 1993). Despite having only 16% sequence identity the proteins were folded in the same way (see Fig. 1). Disulphide mapping experiments showed that type B domains have a similar tertiary structure (Bussler *et al*. 1998). Type 2, 4 and 5 C-terminal domains are single domains, type 1, 3 and 6 di-domains. The structure of the type 2 domain of MITat1.2 (Chattopadhyay *et al*. 2005) and the type 1 di-domain of ILTat1.24 (Jones *et al*. 2008) has been solved. The C-terminal domain of MITat1.2 and the two parts of the type1 domain of ILTat1.24 adopt similar tertiary structures. The structural conservation of the N- and C-terminal VSG domains is probably due to a selection pressure to ensure a protective cell surface barrier, rapid VSG internalization and recycling despite the need for antigenic variation. The proper VSG fold seems to be required for high protein expression as mutating several structural features of VSGs resulted in reduced protein expression compared to wildtype VSGs (Wang *et al*. 2003). Several other cell surface proteins have a similar structure to the N-terminal domain of VSGs; amongst these are the invariant surface glycoproteins ISG65 and ISG75 (Carrington and Boothroyd, 1996), the transferrin receptor (Salmon *et al*. 1997) and the SRA protein which renders *T. brucei rhodesiense* resistant to lysis by human serum (Campillo and Carrington, 2003). The evolutionary relationship between VSGs and VSG-like proteins is unclear. They could have evolved in parallel from a common ancestor or the VSGs evolved from a VSG-like protein or the other way round. The VSG MITat1.5 of *T. brucei* shares extensive homology with the transferrin subunits ESAG6 and ESAG7. Interestingly, it was possible to make a chimeric transferrin receptor of VSG/ESAG6 and VSG/ESAG7 that was still able to heterodimerise and bind transferrin (Salmon *et al*. 1997). SRA looks like a truncated VSG which lacks the surface loops and again a VSG exists, WATat1.2, which has extensive homology to SRA (Campillo and Carrington, 2003). Why is the VSG fold conserved in non-VSG proteins? In the case of the transferrin receptor it is possible that the elongated VSG-like shape is needed to allow access to free transferrin in the flagellar pocket, but it is less clear why the ISGs have the VSG-like fold.

How does the VSG shield other cell surface proteins? The simplest explanation is that the VSG physically prevents immunoglobulin binding to other cell surface proteins. The VSG layer has a thickness of 12 to 15 nm (Vickerman, 1969; Blum *et al*. 1993) and there is evidence that antibodies are able to recognize VSG epitopes up to 6 to 8 nm into this layer (Hsia *et al*. 1996). Many proteins, such as the hexose transporters, have only small extracellular domains (Borst and Fairlamb, 1998) that could be physically protected from host antibodies by the larger VSG. However, ISG65 and 75 are type 1 transmembrane proteins and have an extracellular domain similar in the size to the VSGs and are distributed over the entire surface of the trypanosome (Ziegelbauer and Overath, 1993). The low abundance of ISG65 and ISG75 is only relative to the VSG, there are 7×10^4 copies of ISG65 and 5×10^4 copies of ISG75 per cell (Ziegelbauer *et al*. 1992), but in any other cell they would be moderately abundant cell surface proteins. Infection serum both from humans and mice contains antibodies that recognize invariant cell surface proteins but the trypanosome is not susceptible to a killing response mediated by these immunoglobulins (Ziegelbauer and Overath, 1993). The extracellular domains ISG65 and 75 are similar in size to VSGs, but it remains unclear if and how the VSG coat prevents or negates the

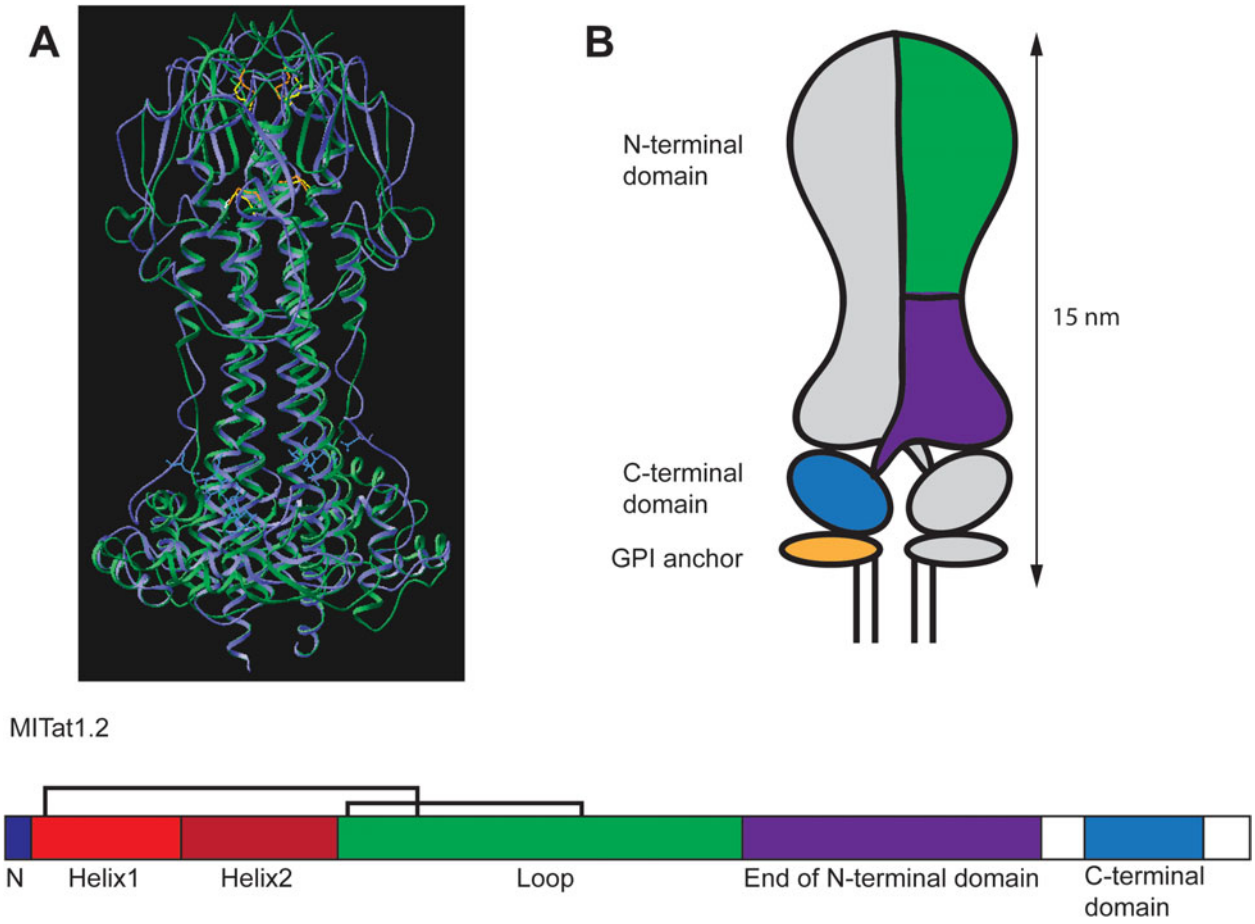

Fig. 1. VSG structure: A. The superimposed structures of the N-terminal domains of MITat1.2 and ILTat1.24 demonstrate the conservation of the tertiary structure of VSGs. The disulphide bridges are marked in yellow. B. Representation of a VSG dimer and linear representation of a VSG MITat1.2 monomer. In the structural cartoon, one monomer is shown in grey, the other is shown in different colours that correspond to regions indicated in the primary structure. The white boxes are regions for which the structure has not been solved. The vertical lines represent cysteines, the horizontal lines disulphide bonds.

effect of antibody binding. The problem is perhaps exemplified by comparison with the transferrin receptor which is a similar size to the ISGs but is localised to the flagellar pocket, an invagination of the plasma membrane where the flagellum exits the cell, which renders them inaccessible to effectors of the immune system. Whether the transferrin receptor is restricted to the flagellar pocket or not is unknown. Overexpression of the transferrin receptor by serum starvation or by introduction of extra copies of the transferrin receptor subunit genes resulted in localisation over the entire cell surface (Mussmann *et al.* 2003; Schwartz *et al.* 2005). Several explanations are possible: (1) The transferrin receptor is localised to the entire cell surface but due to the limit of detection it is only detected in the flagellar pocket. (2) There is an easily saturable transferrin receptor retention mechanism in the flagellar pocket (Mussmann *et al.* 2003). (3) As overexpressed transferrin receptor on the surface was not able to bind transferrin and was not shed into the medium at a higher rate, the single GPI-anchor on the transferrin receptor does not seem sufficient for passive plasma membrane retention, and the detected cell surface transferrin receptor could be in reality a homodimer of the GPI-anchored ESAG6 (Schwartz *et al.* 2005). The biological significance of the finding that serum starvation leads to overexpression of the transferrin receptor also remains questionable. Trypanosomes encode in their bloodstream form expression sites different transferrin receptors that have different affinities for the diverse mammalian transferrins. It was suggested that overexpression of the transferrin receptor takes place at the beginning of an infection when a low-affinity receptor is expressed until the trypanosomes have switched to a bloodstream form expression site encoding for a high-affinity receptor. Calculations based on the K_d values show that trypanosomes expressing a low-affinity receptor should nevertheless be able to grow in any mammalian host without the need to switch to another bloodstream form expression site (Steverding, 2003).

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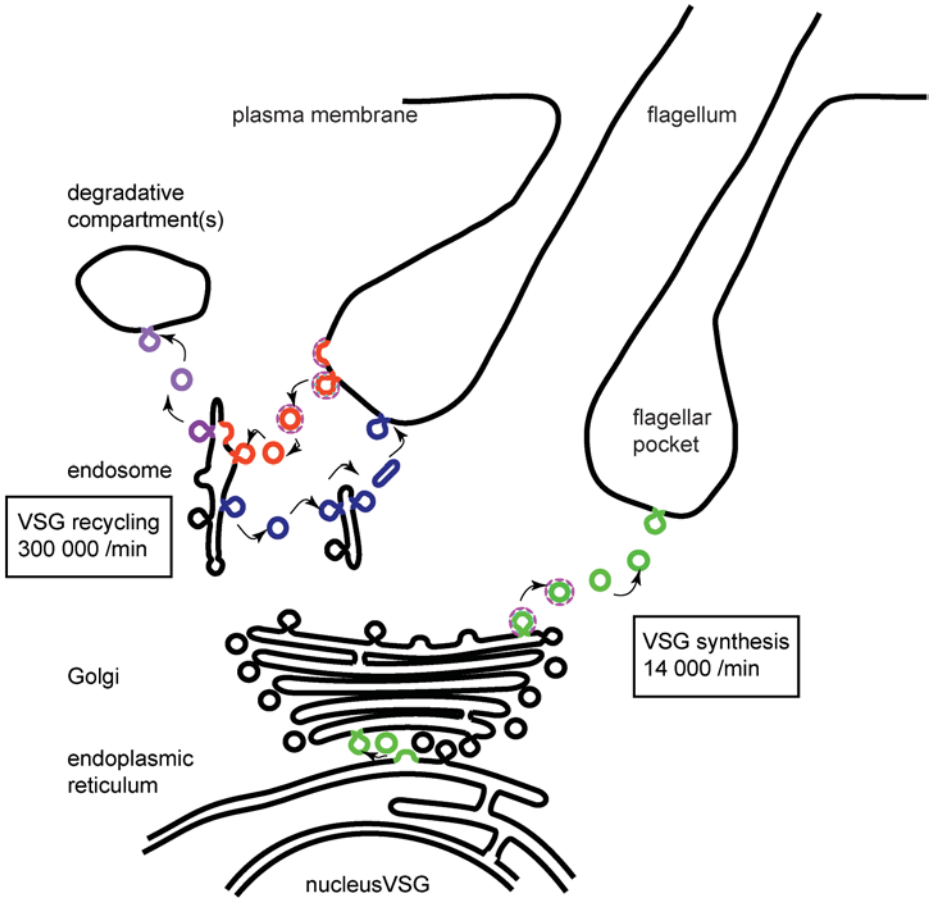

Fig. 2. Diagram of a simplified model of VSG transport in the trypanosome. VSG is synthesised on the rough endoplasmatic reticulum and is exported to the flagellar pocket plasma membrane in vesicles (green) via the Golgi apparatus. VSG is internalised from the plasma membrane by the removal of vesicles (red) from the flagellar pocket. The VSG and receptors are separated from bound immunoglobulin and cargo in the endosome and the VSG is returned to the cell surface in elongated vesicles (blue). Immunoglobulin and possibly ligands are transported to a degradative compartment(s) via vesicles (purple). The rate of VSG recycling far exceeds that of new synthesis. Clathrin-mediated steps are indicated by the purple shell around some vesicles, although a complete description of clathrin-dependent transport remains an aspiration. The values for rates of VSG movement are estimates based on published measurements (Grunfelder et al. 2003) and a cell doubling time of 6 hours.

THE ENDOCYTOSIS OF SURFACE PROTEINS AND REMOVAL OF BOUND ANTIBODIES TO VSG

The endocytic system in bloodstream form trypanosomes is exceptionally active with the equivalent of the entire cell surface being recycled every 12 minutes (Engstler *et al*. 2004). For comparison, cell surface turnover in macrophages is 2.6 times slower and in fibroblasts 5–9 times slower than in *Trypanosoma brucei* (Thilo, 1985; Engstler *et al*. 2004; Overath and Engstler, 2004). There is evidence that this high rate of endocytosis is restricted to mammal-infective forms with much lower rates in insect forms (Morgan *et al*. 2001; Natesan *et al*. 2007). It has been suggested that the up-regulation in bloodstream form cells contributes to the evasion of the immune system (Morgan *et al*. 2002). The VSG half-life is 32 hours, representing ~5 cell doublings, with VSG being shed to the medium rather than degraded by internal turnover (Bulow *et al*. 1989; Seyfang *et al*. 1990). Hence, VSGs are able to remain functional through more than 100 cycles of traversing acidic endosomal compartments.

All endocytosis and exocytosis occurs via the flagellar pocket and VSG is taken up from the plasma membrane by large clathrin-coated vesicles and transported to the early endosome, which is marked by TbRAB5 (Fig. 2). Small clathrin-coated vesicles not containing VSG bud from the early endosomes resulting in an indirect concentration of the VSG. These vesicles presumably contain cargo for late endosomes and lysosomes. The mechanism of the VSG exclusion from these clathrin-coated vesicles is not known. From the early endosomes the VSGs move to recycling endosomes which are TbRAB11-positive structures and, from there, to the flagellar pocket via TbRAB11-positive exocytic carriers (Jeffries *et al*. 2001; Grunfelder *et al*. 2003). The

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volume of vesicular transport involved in VSG recycling far exceeds the volume of vesicular transport from the Golgi apparatus delivering newly synthesized VSG to the flagellar pocket (Fig. 2).

VSGs diffuse freely in the surface coat but movement is under some restriction due to the dense VSG packing in the plasma membrane. The diffusion coefficient for a VSG in the plane of a membrane is 1×10^{-10} cm^2/s, similar to other plasma membrane glycoproteins (Bulow *et al*. 1988). VSG molecules form a complex with anti-VSG immunoglobulins and these complexes move more rapidly in the plane of the membrane than free VSG. The movement of the complexes is directional, towards the posterior pole, the location of the flagellar pocket. This directional movement is dependent on trypanosome cell motility and is explained by hydrodynamic flow forces dragging the antibody VSG complex as the cell swims forward (Engstler *et al*. 2007). Endocytosed immunoglobulins are present in TbRAB5A-positive sorting endosomes (early endosomes) and in the recycling endosome. The antibodies are degraded to short peptides and then secreted showing that a proteoloytic system is operating within the endosome (Pal *et al*. 2003).

ANTIGENIC VARIATION

African trypanosomes express a surface coat composed of a single VSG. There are two interconnected processes important for antigenic variation; first, the genetic or epigenetic changes that lead to a change in the identity of the expressed VSG, and second the mechanisms that ensure monoallelic expression of the active VSG gene so that any one cell presents only a single VSG.

A change in VSG being expressed occurs stochastically and independently of any host factors, the trypanosome population generates cells expressing different VSGs and each variant will expand unless or until killed by the host immune system. The rate of switching varies from once per 1×10^2 to 1×10^6 cell doublings depending on the origin and history of the isolate investigated (Turner and Barry, 1989; Turner, 1997). Up to 20% of the protein coding capacity of the genome is dedicated to VSGs, a huge repertoire of at least 1700 potential *VSGs* per diploid genome (Horn and Barry, 2005). Nearly all VSG sequences are located in tandem arrays in subtelomeric regions, a location that favours mitotic recombination (Linardopoulou *et al*. 2005). The central 'housekeeping' parts of homologous chromosomes have synteny but the *VSG* arrays do not (Melville *et al*. 1998; Berriman *et al*. 2005), the effect is to increase the *VSG* repertoire but it also implies that the arrays are excluded from meiotic recombination (Hutchinson *et al*. 2007).

The *VSG* genes are located in four different types of loci (see Fig. 3A). The first is the bloodstream expression site (BES). There are at least 10 BESs in the diploid genome and only one is active per time (Becker *et al*. 2004; Hertz-Fowler *et al*. 2008; Young *et al*. 2008). Each BES has a *VSG* promoter and contains several expression site-associated genes (ESAGs). The gene content is variable but, in addition to the VSG, most contain *ESAG6* and 7 which encode the two subunits of the transferrin receptor (Hertz-Fowler *et al*. 2008). The *VSG* gene is always located at the very end of each BES that is closest to the telomere. In *T. brucei rhodesiense* one or two BESs contain the *SRA* gene. When *T. brucei rhodesiense* infects a human, this specific BES needs to be active to prevent lysis by human serum (Xong *et al*. 1998). Later in the infection, switching is only possible by recombination of the VSG to a BES containing a *SRA* gene. Transcriptional switching, activation of another BES not containing *SRA*, presumably leads to cell death. The BESs are polycistronically transcribed (Johnson *et al*. 1987; Kooter *et al*. 1987) by RNA polymerase I (Gunzl *et al*. 2003). Single mRNAs are produced by *trans*-splicing and simultaneous polyadenylation. In the 'inactive' BESs transcription is initiated but transcriptional elongation and processing is inhibited so that no *VSG* mRNA is made from these sites (Vanhamme *et al*. 2000). Only the active BES is located in an expression site body (ESB) that is an extranucleolar structure in the nucleus where transcription of the BES takes place (Navarro and Gull, 2001). VSG double expressors utilising two BES are unstable, thus it seems that the ESB favours the expression of a single BES (Chaves *et al*. 1999). A BES can be driven by an rDNA promoter (Rudenko *et al*. 1995) and such a BES is also located in an extranucleolar structure, presumably in an ESB. This suggests that sequences other than the promoter sequence are responsible for ESB recruitment of a BES (Chaves *et al*. 1998). Early in the differentiation to procyclic cells the active BES is repositioned from the ESB to the nuclear envelope. In this location, the VSG promoter is inactivated and undergoes chromatin condensation (Landeira and Navarro, 2007).

VSG genes are also located in less than 25 metacyclic expression sites (MES). MESs are only active in metacyclic trypanosomes, the life stage that differentiates in the tsetse fly salivary gland and infects a mammalian host when the fly feeds. These expression sites have also a promoter but are monocistronic and are not expressed once differentiation to bloodstream forms has occurred (Graham *et al*. 1999).

The silent repertoire of VSG sequences is present in subtelomeric arrays and minichromosomes. At subtelomeres there are around 1500 silent *VSG* genes located in arrays ranging from 5 to more than 150 genes. From these genes, only around 4% are intact genes, 65% are pseudogenes, 21% gene fragments and 9% encode for not-functional

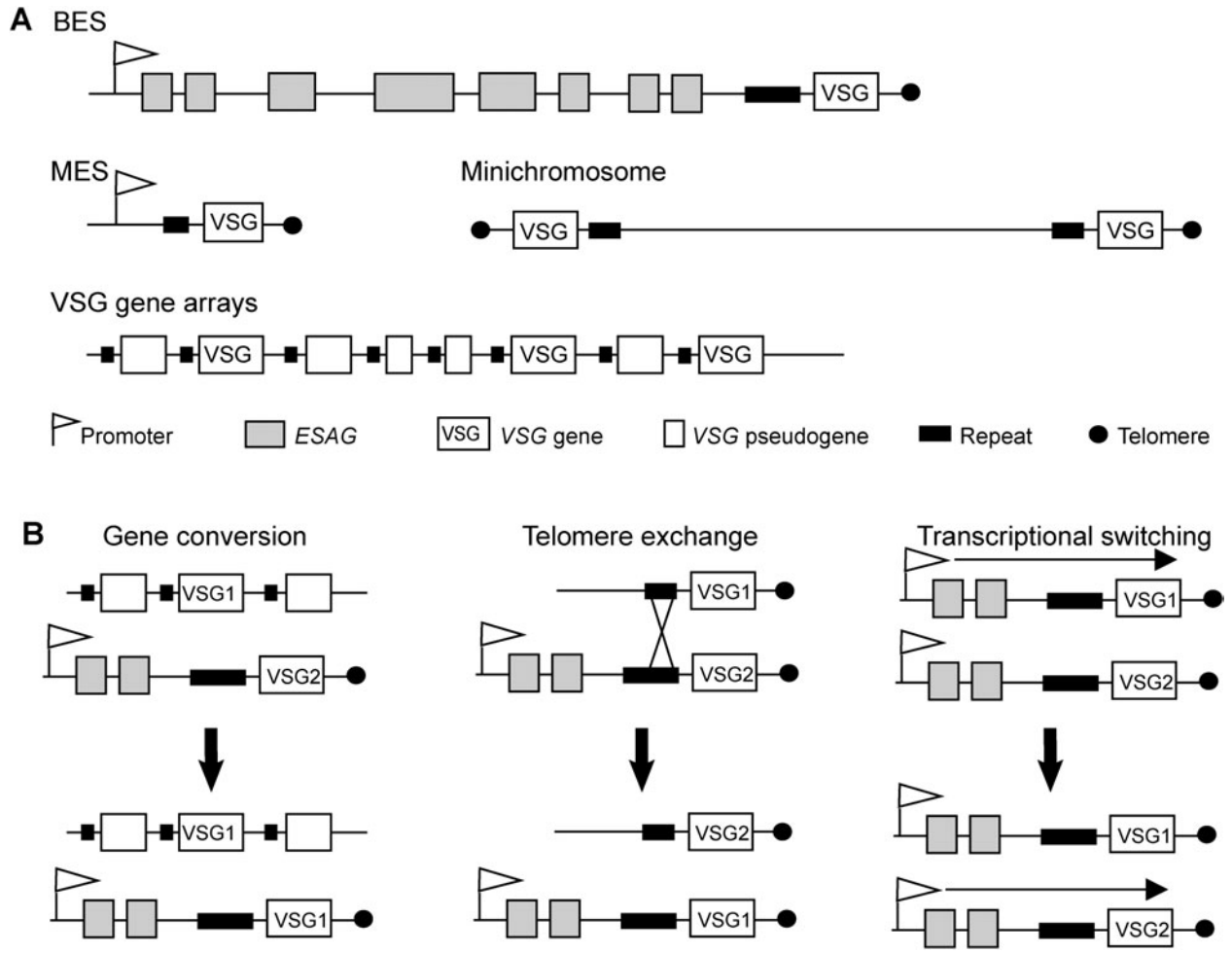

Fig. 3. A. VSG loci: There are around 10 or more VSG genes located in bloodstream form expression sites, 25 in metacyclic expression sites, around 1500 in subtelomeric arrays and around 200 on minichromosomes in the diploid genome. B. VSG switching mechanisms: When gene conversion occurs, the VSG gene in the bloodstream form expression site is replaced by a copy of another VSG gene, often located in subtelomeric arrays. Telomere exchange involves the crossover of the active bloodstream form expression site telomere with another telomere from minichromosomes. The active VSG gene is transferred to a 'silent' location on the other telomere and the formerly silent VSG gene is transferred into the active bloodstream form expression site. Transcriptional switching involves no recombination event unlike gene conversion and telomere exchange. Here the active bloodstream form expression site is down-regulated by an epigenetic mechanism and another bloodstream form expression site is activated.

whole-length *VSG*s which lack the required modification signals. In addition, there are around 200 *VSG* genes located on minichromosomes within 5 kbp of the telomere tract. These are less well characterized but it is believed that there is only one and sometimes no *VSG* gene per subtelomere (Weiden *et al*. 1991; Wickstead *et al*. 2004).

Antigenic variation occurs when the identity of the active VSG gene switches. There are three different switching mechanisms called duplicative gene conversion, telomere exchange and transcriptional switching (see Fig. 3B). Gene conversion is the predominant switching mechanism in pleomorphic bloodstream form cells (Robinson *et al*. 1999). Monomorphic bloodstream form cells that have been cultivated for a long time in the laboratory without selective pressure to undergo antigenic variation have a 10,000 fold reduced switching rate compared to pleomorphic bloodstream form cells (Lamont *et al*. 1986) and more often use transcriptional switching rather than gene conversion (Liu *et al*. 1985).

VSG switching is significantly more frequent than the rates of background implying that it is initiated in a locus-specific manner, possibly by an endonuclease. The rate of switching can be increased by more than two orders of magnitude by engineering an endonucleolytic cleavage (Boothroyd *et al*. 2009). The 5′ endpoint of gene conversion lies in the tandem array 70 bp repeats upstream of the VSG (Boothroyd *et al*. 2009), and the 3′ endpoint occurs within a region spanning the 3′ end of the VSG coding sequence to the subtelomeric repeats (Bernards *et al*. 1981; Campbell *et al*. 1984; Matthews *et al*. 1990). There are some unusual features to the recombination mechanisms involved in antigenic variation. RAD51 is one of the key proteins

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for homologous recombination and enables a DNA single strand to pair with a homologous region in a DNA double strand. The switching rate was reduced in *RAD51* knock-out cells but nevertheless the cells were able to use recombination for switching to some degree (McCulloch and Barry, 1999). Mutating RAD51-3, a RAD51-related protein, (Proudfoot and McCulloch, 2005) and BRCA2, one of the regulators of RAD51 function, (Hartley and McCulloch, 2008) also impaired antigenic variation. There are two homologous recombination pathways in trypanosomes (Conway *et al*. 2002), one is RAD51-dependent pathway and the other is RAD51-independent that uses DNA substrates with very short lengths (minimal substrate length 24 bp). It has been proposed that the latter is perhaps responsible for the microhomology-based recombination that is observed in segmental gene conversion events. In addition to gene conversion, VSG switching can result from telomere exchange, the crossover of the chromosome end carrying the active BES with the chromosome end of another chromosome. This leads to the location of the former inactive *VSG* gene into the active BES and the formerly expressed gene is inactivated by recombination into the silent chromosome end (Pays *et al*. 1985).

When the epigenetic change that leads to VSG switching occurs, the active BES is inactivated and a different BES is activated. The process must involve two linked events: (1) the loss of the ESB from the silenced BES and the appearance of a new ESB on the activated BES, and (2) the appearance of silencing modifications on the chromatin of the old BES and the removal of such modification on the newly activated BES. The difficulty is to separate cause and effect. Transcriptional switching is slowed in cells deleted of the histone methyltransferase DOT1B so that switching intermediates, cells expressing two VSGs, are detectable for weeks and silent BESs become partially derepressed in DOT1B deleted cells (Figueiredo *et al*. 2008). *Tb*ISWI, a SWI2/SNF2-related chromatin-remodeling protein, is also required for the maintenance of BES down-regulation (Hughes *et al*. 2007). The DNA of silent BESs and telomeres contains base J (β-glucosylhydroxymethyluracil) but it is not known if this base has an active role in BES silencing or if base J modification is just a consequence of BES silencing (van Leeuwen *et al*. 1997). Recently, the accuracy of the inheritance of the ESB during the cell cycle has been investigated. An experimentally induced decrease in the amount of cohesin was used to cause a degree of premature separation of sister chromatids. The effect was to disrupt the inheritance of the ESB, probably due to one daughter cell inheriting the ESB and thus maintaining expression of the same VSG, the other daughter cell activated a BES *de novo*. The implications from these results are that the ESB replicates late in mitosis and that a new ESB can form spontaneously (Landeira *et al*. 2009).

The genomic structure of the VSG repertoire provides an explanation for the stochastic nature of antigenic variation. Intact *VSG* genes, which tend to be telomeric and have greater numbers of 5′ repeats, are activated early during infection. Later in infection, mosaic *VSG* genes assembled from pseudogenes from subtelomeric arrays are expressed (Morrison *et al*. 2005; Marcello and Barry, 2007). Thus, expression of VSGs is constrained by the likelihood of recombination to an active BES.

FUTURE CHALLENGES

The study of the trypanosome surface and antigenic variation has led to the discoveries ranging from the first description of the GPI-anchor in eukaryotes, protein-sorting by hydrodynamic flow forces, polycistronic transcription of protein-coding genes by RNA polymerase I in an expression site body, the extreme efficient rate of VSG uptake and recycling outstanding in eukaryotes, accurate mathematical modeling of an infection and so on. Nevertheless, there is still much work to do. It is not understood how the VSG proteins are able to protect the invariant surface proteins from the adaptive immune system. Nothing is known about the surface coat of trypanosomes like *Trypanosoma theileri* which also live in the mammalian bloodstream but do not use classical antigenic variation for protection. Why is the VSG genetic repertoire used for antigenic variation so large? An infection with *Anaplasma marginale*, causative agent of bovine anaplasmosis, can persist for years although this parasite uses only 5–7 functional pseudogenes and a single expression site for antigenic variation (Brayton *et al*. 2005; Palmer *et al*. 2006). Is *T. brucei* really able to use the entire *VSG* repertoire? Is the enormous amount of *VSG* genes required to allow superinfection of an animal by a second strain (Hutchinson *et al*. 2007) or is there another reason for the huge number of *VSG* genes?

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