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REVIEW ESSAY

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How are *Trypanosoma brucei* receptors protected from host antibody-mediated attack?

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Abstract

Trypanosoma brucei is the causal agent of African Trypanosomiasis in humans and other animals. It maintains a long-term infection through an antigenic variation based population survival strategy. To proliferate in a mammal, *T. brucei* acquires iron and haem through the receptor mediated uptake of host transferrin and haptoglobin-hemoglobin respectively. The receptors are exposed to host antibodies but this does not lead to clearance of the infection. Here we discuss how the trypanosome avoids this fate in the context of recent findings on the structure and cell biology of the receptors.

KEYWORDS

endocytosis, haem, haptoglobin-hemoglobin, iron, transferrin, trypanosomes

INTRODUCTION

Parasites have evolved to establish and maintain long term proliferative populations in their hosts, with infection causing disease of variable severity. Successful parasitism is dependent on acquisition of nutrients from the host, and the ability to survive both the innate and adaptive immune responses mounted by the host. In turn, these properties are dependent on molecular interactions between the parasite and host. The result is a molecular arms race involving the gain of new, or loss of old, interactions that favor either the host or the parasite. These random walks in evolution have tended to produce some species that are highly specialized for limited host ranges and others that are more generalists and can infect a wide range of diverse hosts. The difference between the two paths is probably reflected in the number of molecular interactions between host and parasite, with more specialized host specificity conferred by a larger number. The protozoan genus *Trypanosoma* contains both generalist parasites such as *T. brucei*, the causal agent of African trypanosomiasis[1] and others with limited host range such as *T. theileri* which is restricted to Bovids.[2–6]

The phylogenetic relationship between *Trypanosoma* species suggests that *T. theileri* and *T. brucei* arose from a common ancestor and diverged over 100 million years ago,[7,8] coincident with the emergence of mammals and their complex immune systems. Little is known at the molecular level about the interactions between *T. theileri* and its host but transcriptome evidence and characterization of the cell surface suggests that *T. theileri* expresses large numbers of different proteins on the cell surface, mostly *O*-glycosylated and attached to the external face of the plasma membrane byglycosylphosphatidylinositol (GPI)-anchor, as well as complex glycolipids.[9,10] This oligosaccharide-rich type of cell surface architecture probably acts as a more or less successful disguise or mask and the infecting population probably relies on manipulation of host immune response for survival which in turn may explain a narrow host range since most immune effectors and their receptors evolve rapidly.

The generalist parasite *T. brucei* expresses the single superabundant protein, the variant surface glycoprotein (VSG) that is anchored to the external face of the plasma membrane by a GPI-anchor.[11,12] The density of VSG on the cell surface is phenomenal and it acts as a barrier between the host immune system and the trypanosome plasma membrane.[13] The *T. brucei* genome contains a reservoir of thousands of VSG sequences but only one VSG gene is expressed in any one trypanosome at any one time. Long term survival of the infecting population in a host depends upon antigenic variation of the VSG. Within the population there is a low frequency of switching expression from one VSG to another that effectively samples the VSG reservoir.[14–17] If the new VSG is not recognized by the host adaptive immune system the trypanosome can proliferate. In contrast, the vast majority of trypanosomes that do not switch are killed by the antibody-mediated response, either through the classical pathway of complement or by phagocytosis after opsonization.[18] Any novel VSG will be recognized after a few days later but further switches allow the population to persist. The evolution of this system of antigenic variation has permitted *T. brucei* to infect a vast host range, in addition to nearly all mammals it has been reported in birds and reptiles.[19,20] Such a wide host range means that it is difficult to envisage how *T. brucei* would interact with effectors of the adaptive immune system without a huge array of receptors able to overcome the sequence diversity present in cytokines and the like. It is possible that the evolution of the VSG coat removed the requirement for specific interactions with the host immune response modulators and thus allowed a broadening of the host range. This is not to suggest that there is no manipulation of the host immune response. *T. brucei* secretes aromatic keto acids,[21] these metabolites potentially suppress the activation of host macrophages and dendritic cells, downregulate their capacity to produce pro-inflammatory cytokines and thereby altering the downstream host adaptive immunity [21–24]. Additionally, *T. brucei* has been identified to bypass the immunoglobulin M (IgM) mediated killing by preventing memory B cell development.[25,26]

The tsetse fly is the definitive host for *T. brucei*, so the evolutionary pressure on trypanosomes is to infect and then be transmitted by the insect vector. In addition to the population survival strategy based on antigenic variation, there are processes that have evolved to extend the life of individual cells, increasing chances of transmission, based on rapid endocytosis of immunoglobulins bound to the VSG. The site of endocytosis is imposed on the trypanosome by the subpellicular array of microtubules that underlie the plasma membrane and are too closely spaced to allow passage of endocytic vesicles. The one region of the plasma membrane excluded from this constraint is the flagellar pocket, an invagination at the base of the flagellum at the posterior end of the cell, and endocytosis only occurs here.[27,28]

The endocytosis of immunoglobulins bound to VSG is remarkably rapid and occurs within seconds [29] (Figure 1A). First, the VSG layer results in a smooth external surface and immunoglobulins bound to VSGs protrude above this surface and are subject to hydrodynamic forces imposed by the surrounding fluid as the trypanosome swims. The result is selective movement of VSG-immunoglobulin complexes towards the posterior end of the cell and the flagellar pocket. Second, the rate of plasma membrane endocytosis and recycling is exceptionally fast, an area equivalent to the entire cell surface is internalized and recycled in ~7 min.[30] Thus, the effect of the hydrodynamic forces on the VSG-immunoglobulin complex is to concentrate it near a well-draining plug hole.

The compartments associated with handling endocytosed cargo are arrayed between the flagellar pocket and nucleus, with the lysosome normally located distal to the flagellar pocket and proximal to the nucleus.[31] The endocytosis of VSGs and plasma membrane through the flagellar pocket occurs via class I clathrin coated vesicles (CCV) which migrate to the Rab5 positive early endosome. The next step is either trafficking to Rab11 marked recycling endosomes or to Rab7 positive late compartments[29–31] (Figure 1B). Estimates for the VSG half-life, up to 30 h equivalent to five cell doublings, indicate it is recycled many times.

The problem with receptors for host proteins

T. brucei proliferates in the extracellular milieu of tissue fluids and blood within the mammalian host. The problem for trypanosomes with receptors for host macromolecular nutrients is that availability to bind ligand is co-incident with accessibility to host antibodies. Host anti-receptor immunoglobulins could potentially lead to trypanosome lysis via a membrane attack complex or phagocytosis if enough opsonins remain bound to the cell surface for long enough, although it is difficult to put numbers on how much and how long is enough. In this context, we discuss below how trypanosomes adjust the fine tuning of nutrient uptake to acquire transferrin for Fe^{3+} and haptoglobin hemoglobin for haem, while avoiding receptor-directed antibody-mediated killing.

Iron acquisition and transferrin

Iron plays a key role in different biological reactions as it can readily cycle between two oxidation states, Fe^{2+} and Fe^{3+}. Thus, it can either accept or donate electrons working in redox reactions and it is essential for all life forms being an integral part of cellular processes like oxygen and electron transport where it acts as a cofactor.[32] However, free Fe^{2+} can potentially generate toxic superoxide radicals via the Fenton reaction [33] and organisms have evolved mechanisms to minimize this risk. In mammals, iron is bound to the transporter protein transferrin (Tf) in the bloodstream and extracellular spaces, presumably minimizing its toxic effect.[34,35] Transferrin is a glycoprotein of ~80 KDa synthesized mostly in the liver. It has a bi-lobed structure containing two structurally similar domains with an interconnecting loop.[36,37] Each lobe contains a single iron binding site with a high affinity for Fe^{3+} around neutral pH.[38,39] Each lobe switches between two conformational states, either an iron-free form in apo-transferrin or the iron-bound form in holo-transferrin. In the bloodstream of mammals, the concentration of transferrin (~30 μM) normally results in an excess and with some binding sites are not occupied by Fe^{3+}.[40] Once one or both binding sites contain Fe^{3+}, holo-transferrin is captured by the mammalian transferrin receptor (TfR) and the receptor ligand complex is endocytosed.[41] The affinity of transferrin towards iron decreases with the lowering of pH, so as the pH drops during the maturation of the endosomal compartment a conformational change of holo-transferrin to apo-transferrin occurs which in turn releases the iron.[42–45] Subsequently, the transferrin-TfR complex recycles back to the cell surface

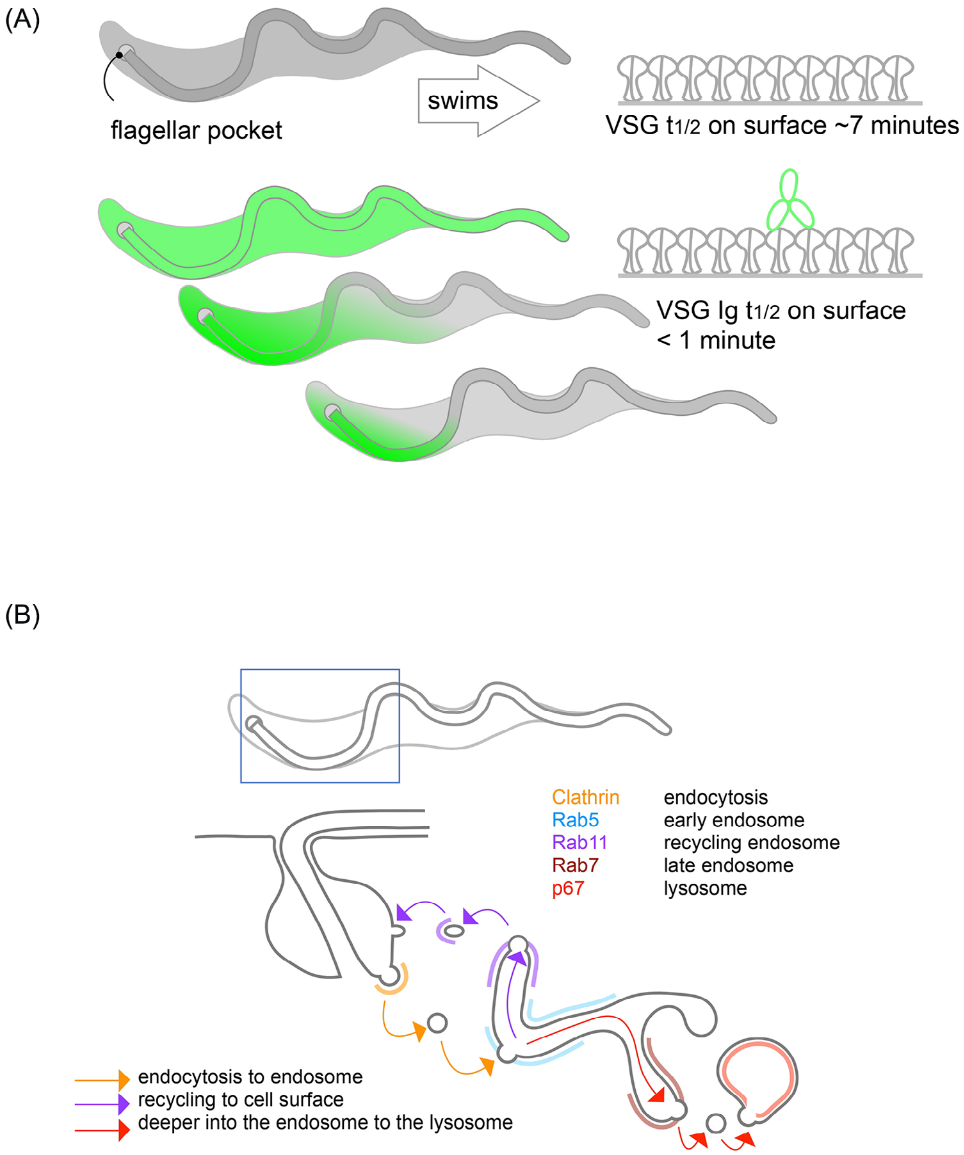

FIGURE 1 A model of endocytosis in trypanosome. (A) A cartoon of a trypanosome showing the location of the flagellar pocket at the posterior end of the cell and the swimming direction. The hydrodynamic force generated as a result of anterior end directed motion results in the backward movement of antibody-cell surface protein complexes (green) protruding above the plane of the VSG coat. This effectively concentrates the complexes near the flagellar pocket, the sole site of endocytosis. This movement combined with the continuous rapid recycling of VSG (grey) coated plasma membrane through flagellar pocket and return to the cell surface is sufficient to clear the bound antibody in seconds. (B) Endocytosis starts via clathrin coated vesicles that are transported to the endosome, a continuous membrane network compartmentalized into distinct functional subdomains with the presence of early, late, or recycling endosomal markers.[31] The ligand delivered to Rab5 marked early endosome is carried to Rab7 positive late endosome from where a vesicle may bud out to deliver the cargo to lysosome, otherwise, Rab11 marked endosome can recycle the ligand back to the flagellar pocket to rejoin the plasma membrane.

where increased pH promotes release of apo-transferrin into the extracellular environment and the TfR is free for another uptake cycle.[42]

T. brucei transferrin receptor

Trypanosomes are unable to survive and proliferate in the absence of iron [46] and need to acquire iron from their host.[47,48] *T. brucei* expresses a TfR that is unrelated to the mammalian receptor and it is a heterodimer encoded by the *ESAG6* and *ESAG7* genes. The two subunits have between 80% and 90% amino acid sequence identity.[49,50] In addition, both are encoded by multigene families with ~15 copies of each.[11] The most divergent variants of each gene have around 90% amino acid sequence identity.[50,51] The expression of ESAG 6 and 7 is linked to monoallelic VSG expression and only one TfR variant is expressed at any one time. The TfR is attached to the external face of the plasma membrane by a GPI-anchor on the ESAG6 subunit.[52,53] It has a similar structure to dimeric VSGs and has roughly the same dimensions,[50] this size is probably necessary to allow it to access transferrin in the context of the VSG coat (Figure 2A and B).

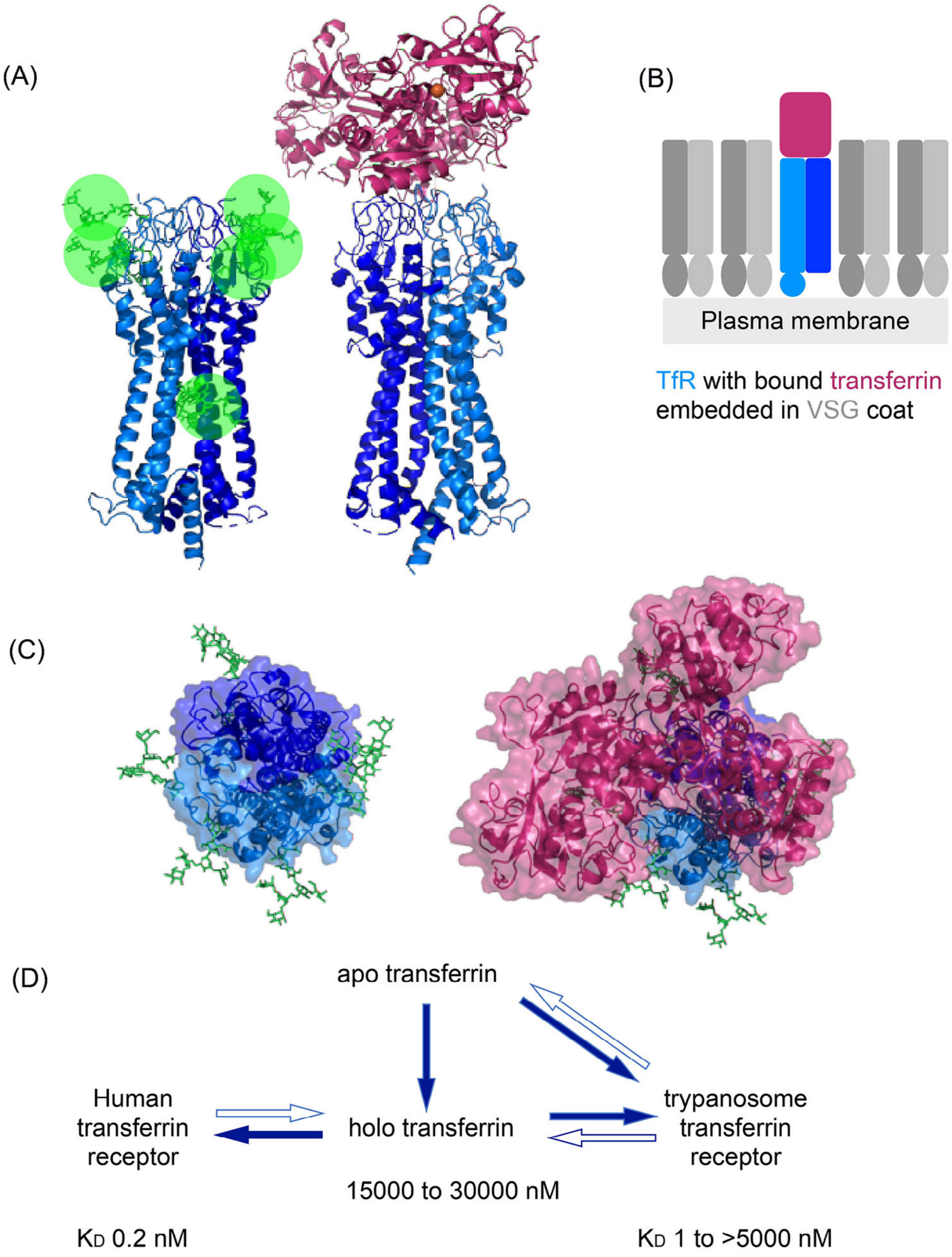

FIGURE 2 Dynamics of trypanosome transferrin receptor. (A) A side view of *T. brucei* transferrin receptor (TfR) composed of ESAG6 (light blue) and ESAG7 (dark blue). BES17 TfR (left) contains seven N-linked oligosaccharides (green circles approximating to their volumes). A side view of the human transferrin complexed with TfR is depicted on the right. At the membrane distal region, divergence of ESAG6 and ESAG7 generates N- terminal loops that results in an asymmetric binding site for human Tf (red). (B) The TfR- Tf complex remains embedded in the VSG coat as depicted in the cartoon where the C-terminal GPI- anchor present in ESAG6 (light blue) attaches the receptor with plasma membrane. (C) Extracellular top view of the TfR (blue) from the outside of the cell illustrating the arrangement of glycans (green), once TfR binds transferrin (red) most of the receptor is occluded and the remaining exposed receptor contains N-linked oligosaccharides. (D) Kinetics of the competition between host and trypanosome TfRs for transferrin binding. The filled arrows are indicative of the favored forward reaction under in vitro condition.

After binding the receptor, transferrin enters the trypanosome by endocytosis.

Let's now look at how the TfR avoids being an easy target for host antibodies. First, the binding site is at the membrane distal end of TfR where the heterodimer forms an asymmetric surface that binds a single transferrin. As the TfR is embedded in a sea of VSGs, if the binding site is occupied by transferrin most of the accessibility to antibodies has been occluded (Figure 2A and C). Is it likely that the TfR is almost always occupied? The concentration of transferrin is ~30 μM in blood and ~15 μM in tissue fluids, the affinities (described by the dissociation constant, K_D) of two TfR variants for transferrins from a range of different mammals were determined and ranged from 1.4 to 500 nM for one and 80 to > 5000 nM for second.[50] Thus, most TfRs should ideally be occupied by ligand most of the time (Figure 2D). Second, the concentration of transferrin means that binding will probably out compete antibody binding on many TfR variants. In addition to masking by the ligand, the TfR has a set of N-linked glycans around the membrane distal end of the receptor,[50] positioned to avoid interference with transferrin binding but probably providing steric hindrance to antibody access to epitopes nearer the plasma membrane (Figure 2C and A). The high concentration of transferrin also means that the competition between trypanosome and host TfRs will not limit availability, indeed expression of a TfR with high affinity is not necessary for infection, at least in rodents.[50,54] In some combinations of a particular TfR variant and host species, the transferrin ligand binding site may be available for antibody binding, especially in a long-term infection as antibody maturation will increase the affinity. The diversity in TfRs may counter this,[54–56] when the location of variation was mapped onto the receptor structure, most was present around the area of the ligand binding site but did not affect residues involved in ligand binding.[50] This suggested that the variation might be more involved in mitigating an antibody response, rather than modulating affinity for different transferrins, by allowing selection of cells that had switched to a different receptor. Both *ESAG6* and *ESAG7*, the transferrin receptor genes, along with the *VSG*, are found in ∼15 BESs without exception. While a library of over thousand *VSG* genes is readily available for recombination into an expression site, the parasite only has access to the ∼15 available TfR sequences. It is important to emphasize that in this model any changes in TfR expression would be on a much longer time scale than VSG switching.

There are mitigation strategies at the cellular level as well as the molecular adaptations described above. There is no evidence of a link between ligand binding and endocytosis. Instead, it is probable that the TfR, and other GPI-anchored receptors, are swept along with the VSG in the constitutive endocytosis of the VSG coated plasma membrane. This means that any TfR with antibody bound will be subject to hydrodynamic forces causing it to migrate to the posterior end of the cells where it is rapidly endocytosed via the flagellar pocket.[57] This process takes seconds from ligand binding to internalization.[58,59] Although Rab5 and Rab11 positive endosomes are involved in trafficking of transferrin,[60] the fate of transferrin bound TfR still remains unclear [59,60]. The half-life of TfR has been measured in four labs and the estimates ranged from ∼ 40 min to 7 h.[61–64] Even the lower end of these values suggests a degree of TfR recycling back to the cell surface, consistent with the one estimate of a recycling time of 11 min,[63] and a system to deliver transferrin to the lysosome but rescue TfR.[30] It is likely that any antibodies bound to TfR follow the same route to the lysosome, as with VSG-bound antibodies.

The localization of the TfR to the flagellar pocket has been proposed to protect the trypanosome from downstream consequences of antibody binding to receptors. The size limit for access to the flagellar pocket is greater than 1 MDa, so, localization to the flagellar pocket is likely to be protective against phagocytosis but would not prevent the assembly of a membrane attack complex. The localization of proteins within the VSG layer is technically challenging and some experiments support complete segregation to the pocket (and endosome) whereas others detect TfR on the surface of the cell body.[61,57,64–66]

Haem acquisition and haptoglobin-hemoglobin

Unlike most aerobic organisms, trypanosomes do not have the complete haem biosynthetic pathway and instead acquire it from their hosts.[67] In the case of *T. brucei* in a tsetse fly gut, this is achieved via a haem transporter (HRG) as presumably the free haem sloshing about during digestion of the blood meal by the tsetse makes this a feasible strategy.[68,69] In a mammalian host, there is very little free haem as hemoglobin released from damaged erythrocytes is rapidly bound by haptoglobin.[70,71] The haptoglobin-hemoglobin is then taken up and detoxified by macrophages after binding to the CD163 scavenger receptor and endocytosis.

T. brucei haptoglobin-hemoglobin receptor

To acquire haem in mammalian hosts, *T. brucei* has evolved a receptor to specifically bind haptoglobin-hemoglobin, it has far less affinity for haptoglobin alone or hemoglobin alone.[72] *T. brucei* haptoglobin hemoglobin receptor (HpHbR) is in competition with the CD163 scavenger receptor expressed on host macrophages for haptoglobin-hemoglobin [73,74] and, unlike transferrin, the concentration of the ligand is variable depending on the degree of hemolysis in circulation.

The structure of haptoglobin is complex and variable, in humans there are two gene variants, Hp1 has a single CCP domain [75] and can only form dimers whereas Hp2 has a duplication of the CCP domain and can form multimers with a minimum of three monomers.[76,77] In cattle, only the equivalent of Hp2 is present and the haptoglobin is multimeric whereas in pigs it is solely dimeric.[77–79] The monomeric dissociation constant ($K_D \sim 2 \times 10^{-9}$ M)[80] for human CD163 and human haptoglobin-hemoglobin is greater than that for HpHbR ($K_D \sim 1 \times 10^{-6}$ M).[81] Human CD163 has evolved to bind human haptoglobin-hemoglobin whereas the *T. brucei* HpHbR binds it from a diverse range of mammals and this will come at a cost in affinity. However, multimeric haptoglobin hemoglobin from humans and other mammals is probably bound by two or more HpHbRs which results in an avidity [73,81–83] that reduces the effective dissociation constant, so most of the exposed receptors are probably occupied. The binding of two or more receptors to a single ligand is possible because of the free diffusion of the GPI-anchored HpHbR in the plasma membrane and a ∼50° kink in the three helical bundle of HpHbR [84] (Figure 3A–C), the resultant avidity allowing it to compete with CD163 (Figure 3D).

How does the trypanosome avoid antibody-mediated attack via the exposed HpHbR? Like the TfR, the binding of ligand will occlude most of the receptor from antibodies and there are N-linked oligosaccharides in other locations (Figure 3A and C). A combination of these masking strategies along with a low copy number of 300–400 per cell and the rapid endocytosis of HpHbR with ligand or antibody bound is

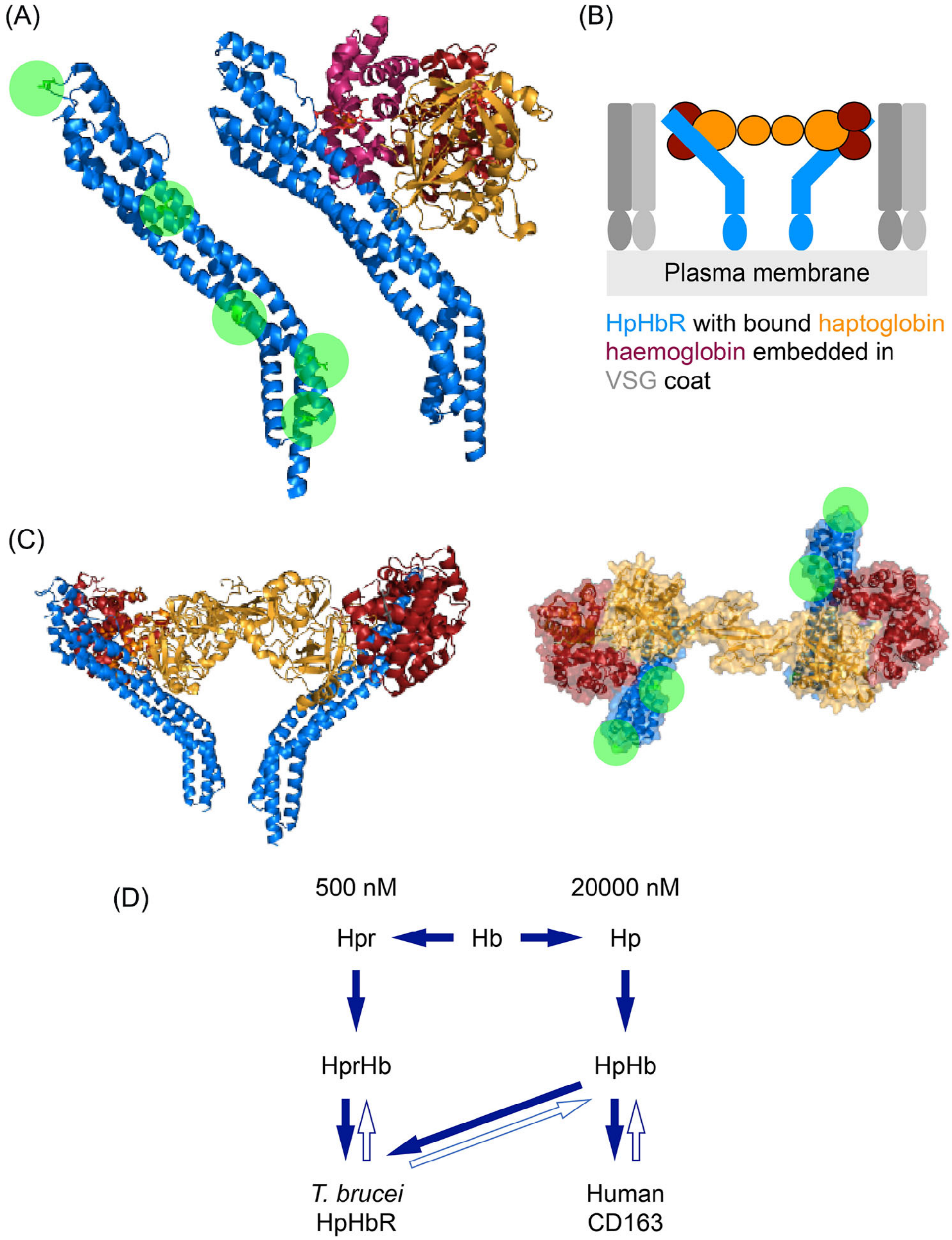

FIGURE 3 Structure of *T. brucei* HpHbR and binding of its ligand Hp-Hb. (A) A side view of *T. brucei* haptoglobin-hemoglobin receptor (HpHbR) (left) showing the location of N-linked oligosaccharides (green circles approximating to their volumes) and the ligand binding (right). (B) A cartoon diagram on the arrangement of HpHbR within VSG layer. Human Hp1-1 can form a dimer which is captured by two monomeric HpHbR attached to the plasma membrane via their C- terminal GPI anchor. (C) A side view (left) of the two monomeric HpHbRs bound to the dimeric HpHb. Extracellular top view (right) of the same complex showing the presence of glycosylation sites (green circles) within the exposed parts of the receptors potentially protecting them from host immunoglobulins. (D) Kinetics of the competition between human and trypanosome receptors for HpHb and TLF binding. The filled arrows are indicative of the favored forward reaction under in vitro condition.

probably sufficient to minimize the effect of antibody binding to the receptor.[72,85] However, the combined strategies are not sufficient to prevent antibody binding as HpHbR monoclonal antibodies readily gained access to the *T. brucei* endosomal system both in vitro and in the mouse infection model.[86]

HpHbR and human innate immunity

Some primates including humans exploit the expression of HpHbR by *T. brucei*. There are primate specific innate immune complexes, Trypanolytic Factors (TLF1 and TLF2),[87,88] that can act as a second ligand for the HpHbR.[81] The TLFs are remarkably potent and adding 0.1% (v/v) human serum to a culture of *T. brucei* is sufficient to kill all of them by 24 h.[89] Both TLFs contain two primate specific proteins, haptoglobin-related protein (Hpr) and apolipoprotein L1 (ApoL1). Hpr arose following duplication of the haptoglobin gene and binds hemoglobin comprising part of TLF. ApoL1 acts as a toxin and a recombinant form is sufficient to kill *T. brucei* alone if added directly to culture media.[90–94] Simplistically, Hpr-hemoglobin results in receptor mediated endocytosis of TLFs and then ApoL1 kills.[72,90–92] The mechanism of action of ApoL1 is contentious, but all agree that a loss of membrane selective permeability is an integral part. One subtlety of the gene duplication producing Hpr is that it has lost the CD163 binding site and so the TLFs are not cleared from circulation by macrophages.

In addition to Hpr and ApoL1, TLF1, and TLF2 both contain apolipoprotein A1, and this reflects the biosynthesis of TLF1 and a part of the high-density lipoprotein (HDL)–3 particle.[95,96] In addition, TLF2 contains a germline IgM.[97] The biosynthetic pathway of TLFs is currently unknown. Amongst others, outstanding questions are: what is the mechanism to ensure that Hpr and ApoL1 are included in the same HDL particle but individually excluded from the vast majority of others? How does the IgM associate to form TLF2?

There are two subspecies of *T. brucei* able to infect humans. *T. brucei rhodesiense* has evolved to express the SRA protein, a classical antitoxin that binds ApoL1 and prevents its action.[58,98] SRA expression is sufficient to confer human infectivity on *T. brucei*.[99] The second subspecies, *T. brucei gambiense*, has evolved a more complex mechanism, first the HpHbR has a point polymorphism that results in a ∼20-fold reduction in affinity for ligand.[81] This results in a K_D for TLFs that is larger than the TLF concentration and will thus reduce occupancy and uptake. It also expresses the *T. b. gambiense* specific glycoprotein (TgsGP) that has been reported to be necessary for survival in human serum but not sufficient to confer resistance to *T. brucei*.[100] Deletion of the HpHbR gene in either *T. b. brucei* or *T. b. gambiense* is not sufficient to confer resistance to TLF2.[100–102] The kinetics of killing suggest that TLF2 can access the cell more rapidly than would occur by fluid phase endocytosis and this may be the function of the IgM; any weak affinity for the VSG would be amplified by multivalent binding increasing avidity and enabling efficient uptake by receptor mediated endocytosis.

A genome predisposed for the emergence and evolution of novel receptors

The two receptors and the two proteins necessary for human infectivity described above provide examples of the evolutionary steps that occur in host pathogen interactions. The *T. brucei* genome contains more than 1000 sequences encoding VSGs that act as a substrate for evolution of new function.[103] TfR, SRA, and TgsGP are all related to VSGs in structure and sequence.[104–107] HpHbR has the same core structure as VSGs consisting of a three helical bundle.[81] This raises the chicken and egg question of which came first, VSGs or receptors? The presence of proteins with a predicted three helical bundle structure in *T. theileri*, distantly related without a VSG-based system of antigenic variation, suggests that this structure evolved before the VSG. In contrast, SRA and TgsGP are recently evolved and clearly VSG derivatives. TfR probably falls into the same category but arose some time ago as it is also present in *T. congolense*, another African trypanosome. However, how *T. theileri* acquires transferrin is not clear. The HpHbR is an example of this rapid evolution of receptors. In *T. brucei* HpHbR makes contacts with both Hp and Hb and has little affinity for either Hp or Hb individually. In *T. congolense* the homologous receptor has a very strong preference for hemoglobin binding, with a K_D ∼1000-fold lower than HpHb.[84]

The evolution of a VSG-based antigenic variation in African trypanosomes probably allowed an expansion in host range by effectively simplifying the interactions with the mammalian adaptive immune system. The densely packed coat and an increased diversity of hosts required co-evolution of receptors able to bind ligands in the context of the coat and, second, able to interact with a greater variation in ligand sequence. In *T. brucei*, HpHbR evolved from an ancient three helical bundle structure and this included the appearance of a kink to facilitate avidity. TfR, TgsGP and SRA evolved from VSGs taking advantage of the sequence reservoir in the genome that facilitated antigenic variation. As new nutrient receptors are identified and characterized, there will be further examples of how to grab your ligand without giving yourself away to antibodies.

AUTHOR CONTRIBUTION

Sourav Banerjee, Nicola Minshall, Helena Webb, and Mark Carrington contributed in preparing the article.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

This is not applicable as no new data is added in this article.

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