

PROTEIN TRAFFICKING IN AFRICAN TRYPANOSOMES: THE ROLE OF
LOCALIZATION OF THE SERUM RESISTANCE ASSOCIATED PROTEIN IN THE
INHIBITION OF TRYPANOSOME LYTIC FACTOR-1

by

NATALIE ANN STEPHENS

(Under the Direction of Stephen L. Hajduk)

ABSTRACT

Trypanosoma brucei or African trypanosomes are unicellular parasites that cause the veterinary wasting disease Nagana, and the human disease, Human African Trypanosomiasis. The veterinary pathogen, *T. b. brucei*, is unable to infect humans due to a trypanolytic subclass of high-density lipoprotein found in human serum called Trypanosome Lytic Factor (TLF-1). Conversely, the human infectious subspecies of African trypanosomes have evolved mechanisms of resistance that allow them to circumvent the innate immunity of their hosts. One example of these human infectious subspecies is *T. b. rhodesiense*, which circumvents TLF-1 killing and survives in the mammalian bloodstream through the expression of the Serum Resistance Associated protein (SRA).

In this study, I have defined the morphologies of TLF-1 mediated cell lysis and demonstrated that each of the lytic components of TLF-1 gives rise to a specific cell death morphology, that is likely to be due to different mechanisms of trypanosome killing. I have also identified the post-translational modifications of

SRA and defined its trafficking pathway from synthesis to degradation. SRA is GPI-anchored and has an unusual localization as a resident endosomal protein, before it is ultimately targeted to the lysosomal compartment and rapidly degraded. In addition, I have identified the initial point of colocalization of SRA and TLF-1 as the early endosomal compartment and demonstrated that TLF-1 appears to be less stable in the presence of SRA.

Previous work by our group proposed that TLF-1 interacts with the negatively charged lysosomal membrane in order to mediate lysis. This interaction occurs through membrane insertion of the TLF-1 protein, apolipoprotein L-I, which also binds SRA. The mechanism of resistance is proposed to involve the interaction of SRA and TLF-1 through the binding of ApoL-I, which blocks TLF-1 association with the lysosomal membrane. Failure of TLF-1 to associate with the membrane leaves the toxin exposed to proteolysis in the lysosomal lumen and subsequent accelerated degradation. Thus, the mechanism of *T. b. rhodesiense* resistance to TLF-1 killing is likely to be a two-step process that blocks the localization of the trypanosome toxin from its required site of action, followed by clearance through lysosomal degradation.

INDEX WORDS: Serum resistance, Trypanosome Lytic Factor, Lysis,
Trypanosoma brucei, Protein trafficking, GPI anchors

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DEDICATION

For my family.

My parents—Donald and Aileen Stephens

My sister—Kerene Stephens-Spencer

My grandmother—Violet Taylor

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I would like to thank my family— Donald and Aileen Stephens (Mommy and Daddy) and my big sister Kerene for their unending support over the years. My trips home to Jamaica are always filled with endless encouragement and love. My grandmother has also been one of my biggest cheerleaders and I am incredibly thankful for her prayers and continued support. Thanks guys for all the phonecalls, the advice and for all the long-distance love and care.

My time in graduate school has been wonderful and that is in large part due to my advisor, Steve Hajduk. Being away from my home and family in Jamaica has always been difficult and after twelve years, I still get homesick occasionally. Without the support of my excellent mentor, the strain of being away from home would have been far greater. As an advisor, Steve maintained the perfect balance between allowing me the space to develop as an independent researcher, but of also being there 100% when needed. Much more than just a PI, Steve has created an environment that feels like a second family. Whether it's talking science, playing bocce, or moving across country, Duk has always had a knack for making things seem easier. I feel extremely fortunate to have been a part of Steve Hajduk's research group and I'm extremely happy to be a Dukling. Thanks for everything Dukkie!

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benchmate was always there with “Justin” ideas and a truly unique perspective. Rudo Kieft, my birthday buddy, has been a brilliant source of advice and is undoubtedly the tryp guru. If you have a question, just ask Rudo. John Harrington has always been there to lend an ear and advice about experiments, or any other random notions that I may get. David Seidman and Steve Sykes, “the RNA guys”, have been great friends as well as labmates. I have also worked with several talented undergraduates and I am thankful for the opportunity to have been a teacher and mentor. Keri Jamison, Andrew Brunner and Carla Rutherford made significant contributions to my project and I’m happy to have met and worked with them. As labmates, we spend the majority of waking hours in the same space, and I feel extremely fortunate to have been a part of a group that made this an easy and enjoyable thing to do.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Trypanosomes are unicellular, protozoan parasites that infect a wide range of hosts from plants to humans [1, 2]. While many trypanosomes are limited to infectivity of non-human species, there are others that cause debilitating and fatal human disease. *Leishmania* subspecies like *L. major* and *L. tropica* cause the less severe cutaneous form of leishmaniasis, while *L. donovani* infection results in visceral leishmaniasis [3]. *Trypanosoma cruzi*, is able to invade host defense cells and heart muscle tissue, causing Chagas disease—a chronic and complex set of disorders involving both the heart and digestive systems [4]. *Trypanosoma brucei*, or the African trypanosome causes both acute and chronic forms of human African trypanosomiasis. Both *Leishmania* and *Trypanosoma cruzi* provide examples of obligate intracellular parasitism and survive in the mammalian host by invasion of host cells and manipulation of host cell biology. However, *Trypanosoma brucei* is an extracellular parasite that exists in the host bloodstream. The ability of this parasite to survive in the circulation, stems from the evolution of multiple lines of parasite defense against the innate and adaptive immune systems of the host. With these resistance mechanisms in place, it has proved difficult to develop desirable treatments that are not also highly toxic to patients.

The objective of this work is to investigate the mechanisms and associated morphologies of human serum lysis of *T. b. brucei*, as well as the resistance mechanism of *T.*

b. rhodesiense and the role of protein trafficking in serum resistance. In order to convey the complexities of these mechanisms, I have provided a comprehensive review of the organism *Trypanosoma brucei*, with emphasis on the mechanisms of human serum lysis and parasite resistance, outlined in the Introduction and Literature Review of this work. In Chapter 2, I have defined two morphologies associated with serum lysis and shown that each morphology is due to one of the two trypanolytic protein components of human serum. The characterization of the serum resistance associated protein, SRA, and the trafficking and localization of this protein, as well as the post-translational modifications involved in trafficking have been described in the third and fourth chapters of this dissertation, respectively. I have proposed a mechanism of SRA-mediated resistance in Chapter 5, which defines the critical steps and key factors of the resistance mechanism. In addition, the role of protein trafficking in serum resistance is also highlighted. Finally, the major conclusions of the research are also presented and discussed in the final chapter of this dissertation.

HUMAN AFRICAN TRYPANOSOMIASIS

Human African Trypanosomiasis (HAT) or African sleeping sickness is caused by *T. brucei* infection that is transmitted from the bite of the insect vector, the Tsetse fly. The chronic form of sleeping sickness results from infection by *T. b. gambiense*, while the acute form of the disease is due to infection by *T. b. rhodesiense* [5]. The first phase of the disease is called the hemolymphatic phase and is accompanied by fever, joint pain, itching and headache. The second phase occurs once the parasite infection crosses the blood brain

barrier and invades the central nervous system. This phase of the disease manifests as behavioral changes, decreased coordination, confusion and severe fatigue. With the chronic form of the disease, these signs and symptoms may not occur until years after the initial infection, while the acute disease manifests within weeks or months [5]. Regardless of the form of trypanosomiasis, without diagnosis and treatment of the disease, infection by *T. brucei* is fatal.

THE ULTRASTRUCTURE OF *TRYPANOSOMA BRUCEI*

The bloodstream form of *T. brucei* has an elongated, slender cell body that is approximately 20µm long and 2 µm wide, with a single attached flagellum that extends along the entire length of the cell. The cytoskeleton consists of a parallel network of subpellicular microtubules that is found along the entire plasma membrane, with the exception of the flagellar pocket region [6]. The cell body houses organelles customarily found in eukaryotic cells including the nucleus, endoplasmic reticulum (ER), Golgi, as well as endosomal and lysosomal compartments. Other organelles that distinguish *T. brucei* from mammalian cells are the kinetoplast and glycosomes, which house the mitochondrial DNA and glycolytic enzymes, respectively [7, 8]. In addition, *T. brucei* cells have a single, branched mitochondrion, as well as a single Golgi stack (Figure 1.1).

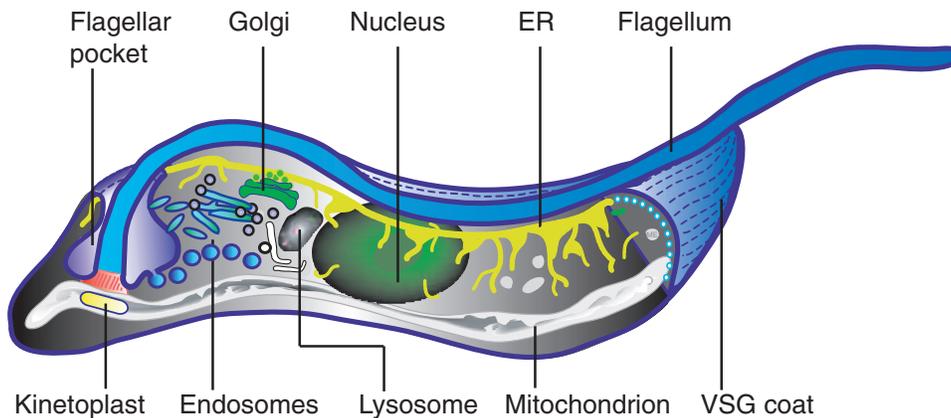


Figure 1.1. Schematic diagram of *T. brucei* ultrastructure. The organization of the cell is shown with the major organelles labeled. (Adapted from Overath and Engstler, *Molecular Microbiology*, 2004.)

THE LIFE CYCLE OF *TRYPANOSOMA BRUCEI*

Trypanosoma brucei undergoes a complex set of life cycle stages as it is transmitted between two widely different environments from the insect vector to the mammalian host (Figure 1.2). When the tsetse fly takes a blood meal, it ingests the bloodstream form of *T. brucei*, which rapidly differentiates into the procyclic form in the insect midgut. During differentiation, the cell undergoes both morphological and metabolic changes that facilitate its survival in the new environment. The cell becomes elongated, loses expression of the major surface coat antigen and has significantly decreased motility. Another major change that takes place is the development of an active mitochondrion. Mitochondrial expansion occurs in response to the change in available energy substrates, and the principal means of energy production becomes oxidative phosphorylation. After approximately 4 days, the

cells migrate to the salivary gland of the fly, and differentiate into epimastigotes, which become attached to the epithelium via the flagellum. As further differentiation into the non-proliferating mature metacyclic form proceeds, mitochondrial repression occurs and the cells regain a surface coat. Once the metacyclics become detached from the epithelium, they can be transmitted to the mammalian host when the tsetse fly takes a blood meal [9].

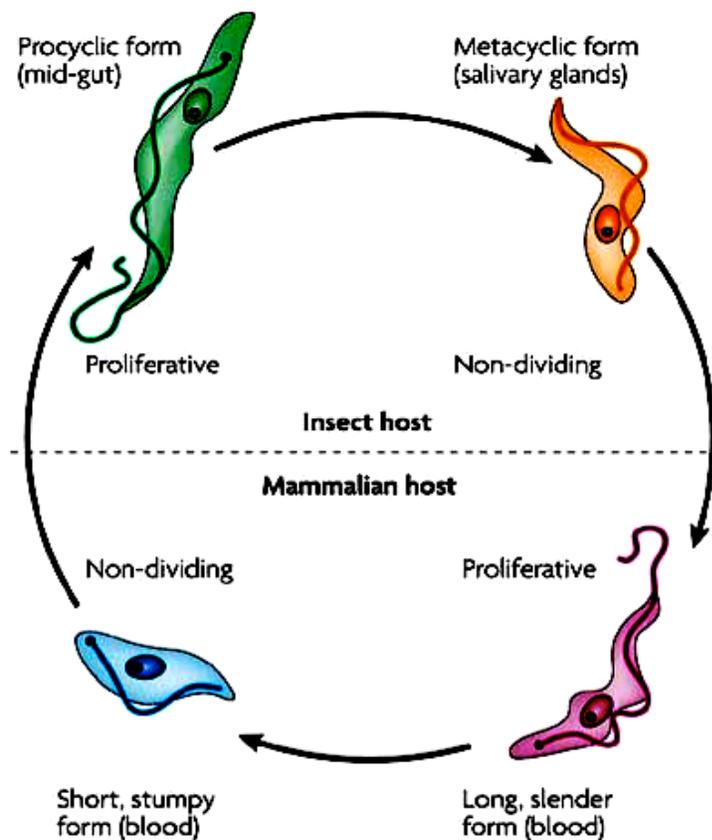


Figure 1.2. The Life Cycle of *T. brucei*. Schematic diagram showing the main life cycle stages of the cell. The upper half of the cycle shows the insect vector stages. The lower half of the cycle shows the host stages. (Adapted from Lee, Stephens and Englund. *Nature Reviews Microbiology*, 2007).

When the metacyclics are deposited at the bite site, a sore called a chancre develops [5]. In the early stages of infection, cells migrate away from the chancre and into the bloodstream and lymphatic system, where they rapidly develop into the long and slender, dividing forms. This proliferating bloodstream form is highly motile with a robust endocytic system. The mitochondrion is also repressed, as glycolysis is the main form of energy production in this life stage. In the late stages of infection, long and slender trypanosomes develop into the non-dividing, short and stumpy form, which transitions back into the procyclic form when taken up by a fly during a blood meal [9].

VARIANT SURFACE GLYCOPROTEINS AND GPI ANCHORS

The variant surface glycoprotein (VSG) is the most abundant protein in *T. brucei*, making up 10% of total cellular protein. There are approximately 10^7 homodimers of VSG that are densely packed over the entire surface coat of the trypanosome and attached to the plasma membrane by a lipid anchor called the glycosylphosphatidylinositol (GPI) anchor [10]. Due to the abundance of VSGs, the biosynthesis of GPI anchors and the role of this lipid modification in protein trafficking have been extensively researched in *T. brucei*. In addition to VSGs, other bloodstream form trypanosome cell surface proteins have been shown to be, or proposed to be GPI-anchored proteins. The *T. brucei* transferrin receptor (TfR), which is also a VSG family protein is GPI-anchored [11], and the *T. brucei* haptoglobin-hemoglobin receptor (*TbHpHbR*) is also predicted to have this modification [12].

GPI anchors consist of an ethanolamine linked to the glycoprotein by an amide linkage. This ethanolamine moiety is attached to a glycan, which is linked to a glucosamine. Two myristate chains are attached to the glucosamine. The GPI anchor is synthesized in the endoplasmic reticulum (ER), where the glycoprotein is translated with both an N-terminal signal sequence and a C-terminal hydrophobic peptide [10, 13, 14]. The N-terminal signal sequence, which is a hallmark of cell surface or secretory proteins, is cleaved by the ER signal peptidase during translation. The C-terminal hydrophobic peptide is also cleaved in the ER immediately post-translation, and the GPI anchor is transferred *en bloc* to the attachment site of the glycoprotein within a minute of this cleavage [15-17]. Once glycoproteins are GPI-anchored and glycosylated within the ER, the proteins are trafficked to the Golgi for further processing and are subsequently targeted to the cell surface via vesicle-mediated secretion. The cleavage and release of *T. brucei* GPI-anchored proteins is carried out by the *T. brucei* glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) enzyme [18-20]. GPI-PLC cleavage of VSGs is proposed to have a role in the loss of VSGs during antigenic variation, as well as during differentiation to the procyclic form [21, 22]. Consistent with this, the enzyme has been localized to the exterior of the flagellar membrane [22, 23]. Additional intracellular localizations of GPI-PLC have also been reported [24-27].

The role of the GPI anchor in cell surface targeting and protein trafficking has been analyzed through mutational analysis. VSG deletion constructs were engineered with a stop codon immediately upstream of the GPI anchor attachment site to prevent GPI addition, and expressed in bloodstream form *T. b. brucei* in order to examine the effects of GPI anchor deletion [28]. The GPI-anchor deficient VSGs were delayed in ER exit and were

targeted to the lysosome for degradation, rather than secreted to the cell surface. These studies suggest that the GPI anchor may act as a forward trafficking signal through the ER [28]. It is possible that cell surface targeting may occur in a manner similar to that observed for targeting to the apical membrane of polarized mammalian cells. These lipid-modified proteins associate with lipid rafts via the GPI anchor and selectively traffic to the plasma membrane for secretion to the cell surface through a specialized region of the plasma membrane called the flagellar pocket.

THE FLAGELLAR POCKET, ENDOCYTOSIS AND PROTEIN TRAFFICKING

All secretion and endocytosis in bloodstream form *T. brucei* occurs through a specialized region of the plasma membrane called the flagellar pocket. This specialized area is formed from the invagination of the plasma membrane at the point of the cell through which the flagellum emerges from the trypanosome cell body. One end of the flagellar pocket is delineated by the basal body where the flagellum originates, while the other end of the pocket borders at the flagellar pocket collar, at the neck of the flagellum [29]. Although extracellular, the flagellar pocket has a luminal quality due to the enclosed nature of the pocket. This is caused by the close arrangement of the flagellar and plasma membranes at the point of invagination of the plasma membrane (Figure 1.3). The lumen-like environment contains an undefined carbohydrate-rich matrix [30], as well as the cell surface receptors that are shielded from the adaptive immune response in this sequestered region [12, 31-33].

The specialization of the flagellar pocket for endocytosis and secretion is evident from several key elements of the architecture and organization of the posterior end of cell. First, the invagination of the plasma membrane at this region is possible because this is the only area of the plasma membrane that does not have a subpellicular network of microtubules. This region is therefore less rigid and more accessible for vesicle budding and fusion, which are critical to trafficking. Second, all components of the endocytic machinery are localized to the posterior end of the cell between the nucleus and the kinetoplast, which is the region of the cell most closely localized to the flagellar pocket. This arrangement undoubtedly facilitates the efficiency of endocytosis. Finally, the main organelles involved in secretion are also found in close proximity to the flagellar pocket (Figure 1.3). Electron microscopy revealed that both the endoplasmic reticulum and the Golgi are found proximal to the flagellar pocket membrane in order to facilitate the trafficking of cell surface proteins. Thus, the polarization of the trypanosome is critical to the functioning of the flagellar pocket as the endocytic and secretory organelle.

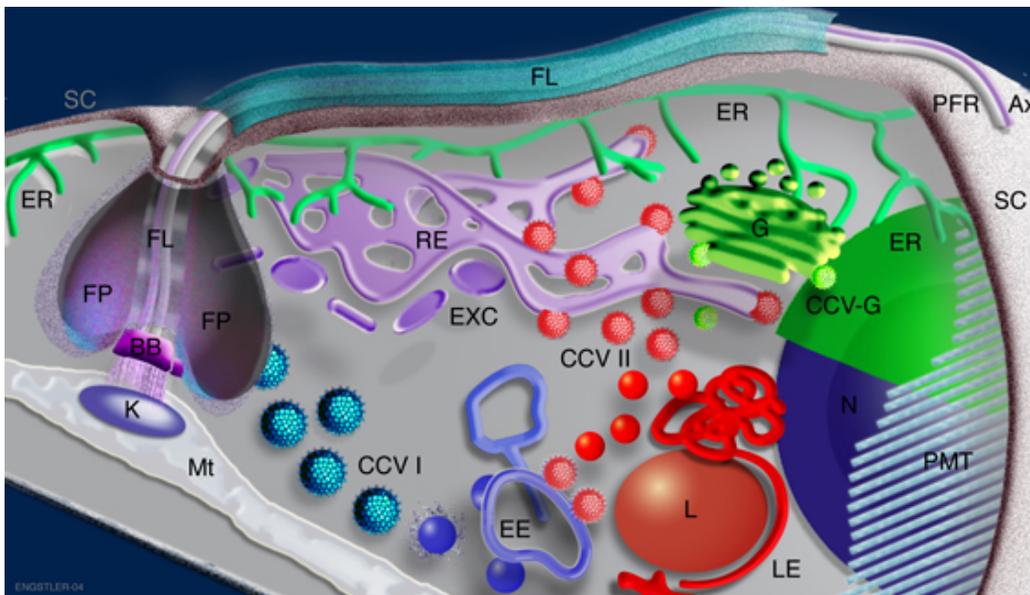


Figure 1.3. The flagellar pocket and endocytic machinery. An illustration depicting the organelles and trafficking vesicles involved in endocytosis and secretion. FP – flagellar pocket; N – nucleus; Mt – mitochondrion; ER – endoplasmic reticulum; G – golgi; K – kinetoplast; FL – flagellum; BB – basal body; L – lysosome; EE – early endosome; RE – recycling endosome; LE – late endosome; CCV – clathrin coated vesicle (Adapted from Overath and Engstler, *Molecular Microbiology*, 2004).

The organization of the flagellar pocket and the endocytic/secretory machinery in *T. brucei* has led to the development of a highly efficient protein trafficking process. In fact, the rate of endocytosis measured for bloodstream form *T. brucei* is one of the highest rates of endocytosis recorded to date [34], especially considering that the flagellar pocket occupies < 5% of the entire plasma membrane [35]. Studies on the kinetics of endocytosis have shown that the flagellar pocket volume is turned over within 1-2 minutes, while the entire surface coat of VSGs is endocytosed within 12 minutes and the intracellular volume of VSG turned over within a minute [36]. The high efficiency of VSG clearance from the cell surface is thought to occur as a result of the hydrodynamic forces that drive the movement of VSGs along the cell surface towards the posterior end of the cell into the flagellar pocket [37]. Thus, the positioning of the flagellar pocket is significant to efficient uptake of endocytic cargo, whether by bulk phase endocytosis, as with VSGs, or by receptor-mediated endocytosis, as with high-density lipoproteins (HDLs).

Once cargo is endocytosed, sorting into different classes of endosomes occurs. The various classes of endosomes have been well characterized and are each associated with

specific Rab GTPases, that allow for localization and resolution of the trafficking pathways of different cargo. Ultimately, endocytic cargo is either recycled or targeted to the terminal compartment of the endocytic pathway—the lysosome. The trafficking pathways of both VSGs and the transferrin (Tf)-TfR complex have been well-documented using the Rab GTPases as markers. Upon endocytosis, VSGs and Tf are internalized by bulk phase membrane flow and are immediately sorted into the early endosomes designated by the presence of the Rab5 GTPase [36, 38-42]. Subsequent sorting into microtubular vesicular bodies lead to the distillation of various cargo to several different potential destinations. Both VSGs and TfR are typically sorted into the recycling endosomes characterized by the Rab11 GTPase, and rerouted back to the flagellar pocket for secretion to the cell surface [36, 43]. The endocytic cargo that is sorted for lysosomal targeting, rather than recycling, for example, Tf, is routed into late endosomes that are denoted by the presence of the Rab 7 GTPase [39, 44, 45]. These late endosomes are the last stop before cargo is ultimately transported to the lysosome for degradation. This terminal organelle is localized by the presence of the highly glycosylated, LAMP-like protein p67 [30, 46], as well as by a luminal *T. brucei* Cathepsin L-like protease (*TbbCatL*) [47, 48].

While the endocytic routes of various cargo have been well characterized and documented, the trafficking of secretory proteins is less well known. The key organelles involved are the ER and the Golgi. As seen in higher eukaryotes, secretory and cell surface proteins are translated at the ER with N-terminal signal sequences that allow for translocation into the ER for post-translational modification. Subsequently, proteins undergo further processing while trafficking through the Golgi before being packaged into vesicles that fuse with the cell flagellar pocket membrane.

THE TRYPANOSOME LYTIC FACTOR-1

The trypanolytic activity of normal human serum (NHS) was first discovered in 1902 by Laveran. The selectivity of serum toxicity was later discovered when it was found that neither *T. b. gambiense* nor *T. b. rhodesiense*, the human infectious subspecies, were susceptible to serum lysis [49]. It was not until 1978, however, that the trypanocidal component of human serum was isolated [50]. Through a series of density ultracentrifugation flotations, Rifkin found that the trypanocidal activity was associated with the high-density lipoprotein (HDL) fraction of serum. Apolipoprotein A-I (apoA-I), the characteristic protein found in all HDLs was also identified and confirmed the HDL classification of the lytic serum fraction.

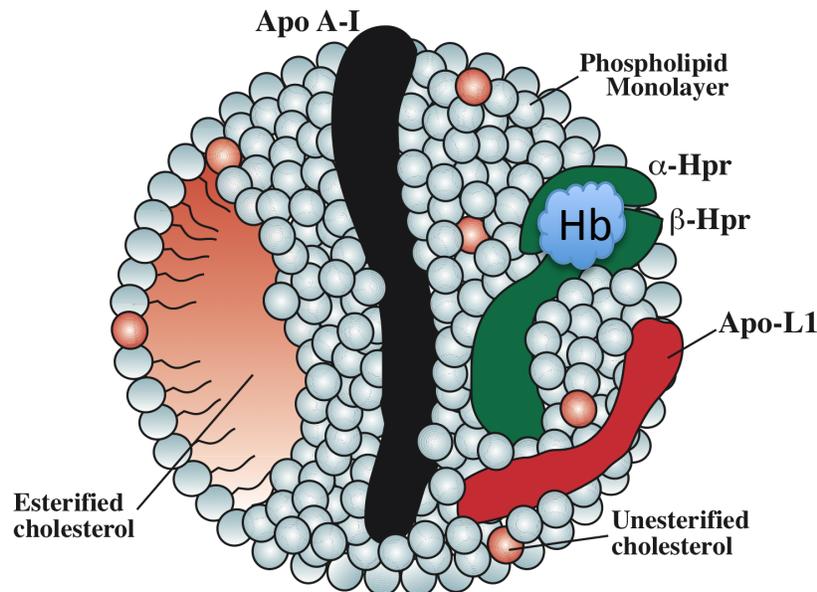


Figure 1.4. The trypanosome lytic factor-1. An illustration depicting the components of the TLF-1 molecule. There are three apolipoproteins embedded in a lipid core (clear circles, pink circles). Apolipoprotein A-I (ApoA-I) is the requisite apolipoprotein of HDLs and is

shown in black. Apolipoprotein L-I (ApoL-I) and haptoglobin related protein (Hpr) are the trypanocidal proteins of TLF-1 and are shown in red and green, respectively. Both the α - and β - subunits of Hpr are depicted. Hemoglobin (Hb) (blue) is shown bound to Hpr.

In both *in vitro* and *in vivo* assays with *T. brucei*-infected mice, the HDL fraction of serum was found to cause trypanosome lysis with the same lysis phenotype observed with unfractionated serum. Consistent with the lysis resistance that was observed with serum treatment of *T. b. gambiense* and *T. b. rhodesiense*, these human infectious subspecies were not killed by the HDL fraction of human serum [51]. Further evidence of the HDL fraction as the trypanosome toxin came from examination of serum trypanolytic activity from Tangier's patients, who have HDL-deficient serum. NHS isolated from Tangier's patients was found to have significantly less trypanolytic activity, likely due to the lack of HDLs in this serum, thus supporting the HDL fraction of serum as being the toxic component of serum [50]. Following Rifkin's discovery, further characterization of the HDL fraction of serum reported that not all HDLs were toxic to *T. brucei*, but rather that a specific minor subclass of HDLs (1.21 – 1.24 g/ml) caused trypanosome lysis [52]. This minor subclass was called the Trypanosome Lytic Factor-1 (TLF-1) (Figure 1.4) [52]. With an estimated diameter of approximately 18-22 nm and molecular weight of 500 kDa, TLF-1 is unusually large for an HDL particle. In addition to apoA-I, TLF-1 was also found to contain two primate-specific apolipoproteins that are specific to the trypanolytic subset of HDLs—haptoglobin related protein (Hpr) and apolipoprotein L-I (ApoL-I).

Haptoglobin related protein is a hemoglobin (Hb) binding protein [53] that shares >90% amino acid sequence identity to the serum protein, haptoglobin (Hp) [54]. Haptoglobin is an acute phase protein that scavenges free hemoglobin found in serum resulting from infection or injury-induced hemolysis [55]. Haptoglobin related protein may exist in either a dimeric form or a tetrameric form, consisting of the α -subunit and β -subunit of the protein. One unusual feature of Hpr is its secretion from liver cells without the cleavage of the N-terminal signal sequence [56]. This retained signal sequence is thought to be involved in Hpr assembly into HDLs. Apolipoprotein L-I is a colicin-like protein with pore-forming activity. Bioinformatic modeling of the protein revealed a helical region similar to those of bacterial colicins, and capable of similar function [57]. Other domains of apoL-I include a membrane addressing domain that is required for targeting to lipid bilayers, as well as a second helical region proposed to form a coiled-coil protein-protein interaction with SRA, a trypanosome protein involved in resistance to TLF-1 lysis [57]. Both Hpr and apoL-I have trypanocidal activity [56-59]. The individual properties of the proteins as an Hb-binding partner and a pore-forming protein, are proposed to be critical for their individual roles in lysis.

THE MECHANISM OF TLF-1 MEDIATED LYSIS

Lysis of trypanosomes by TLF-1 has been the focus of extensive investigation. The fundamentals of *T. brucei* killing by TLF-1 include receptor-mediated uptake of the particle and vesicle trafficking to the terminal lysosomal compartment for initiation of cell lysis [12, 60-62]. The series of events that lead to cell death begin with the high affinity binding of

the TLF-1 particle to the *T. brucei* haptoglobin hemoglobin receptor (*TbHpHbR*), which localizes to the flagellar pocket [12]. This interaction occurs via the Hpr-Hb complex, which is the ligand for receptor-mediated uptake. The Hb-bound TLF-1 particle is then endocytosed into endosomal vesicles and is trafficked to the acidic lysosomal compartment where cell lysis is initiated [12, 60-62]. TLF-1 lysis assays carried out at low temperatures at which endocytosis (3-5°C), and endosomal trafficking are halted (15-17°C), result in inhibition of trypanosome lysis [60]. In addition, lysis assays carried out in the presence of pH raising substances such as ammonium chloride (NH₄Cl) and chloroquine, also inhibit lysis [60]. Thus, internalization, lysosomal localization and acidification of TLF-1 have been shown to be critical for cell lysis to occur.

While these elements of TLF-mediated lysis are widely agreed upon and accepted by the TLF-1 research community, there are currently two differing hypotheses on the trypanosome cell death mechanism. The Pays research group proposes that apoL-I is the sole trypanolytic component of TLF-1. Apolipoprotein L-I forms a pore in the lysosomal membrane, resulting in an influx of Cl⁻ ions that leads to extensive swelling of the lysosome [57, 59]. This swelling leads to the formation of a large vacuole that ultimately occupies the entire cell body, causing distortion of the trypanosome morphology from a long and slender form, to a balloon-like appearance. The vacuole is proposed to expand until the entire cell ruptures, resulting in cell lysis [57, 59].

The mode of lysis proposed by the Hajduk group postulates that Hpr and apoL-I are both trypanocidal and act synergistically to cause cell lysis, albeit by different mechanisms

[56, 58]. Consistent with Pays' reports, apoL-I-mediated cell lysis leads to the formation of the swollen vacuole and is therefore likely to be acting as a pore-forming protein.

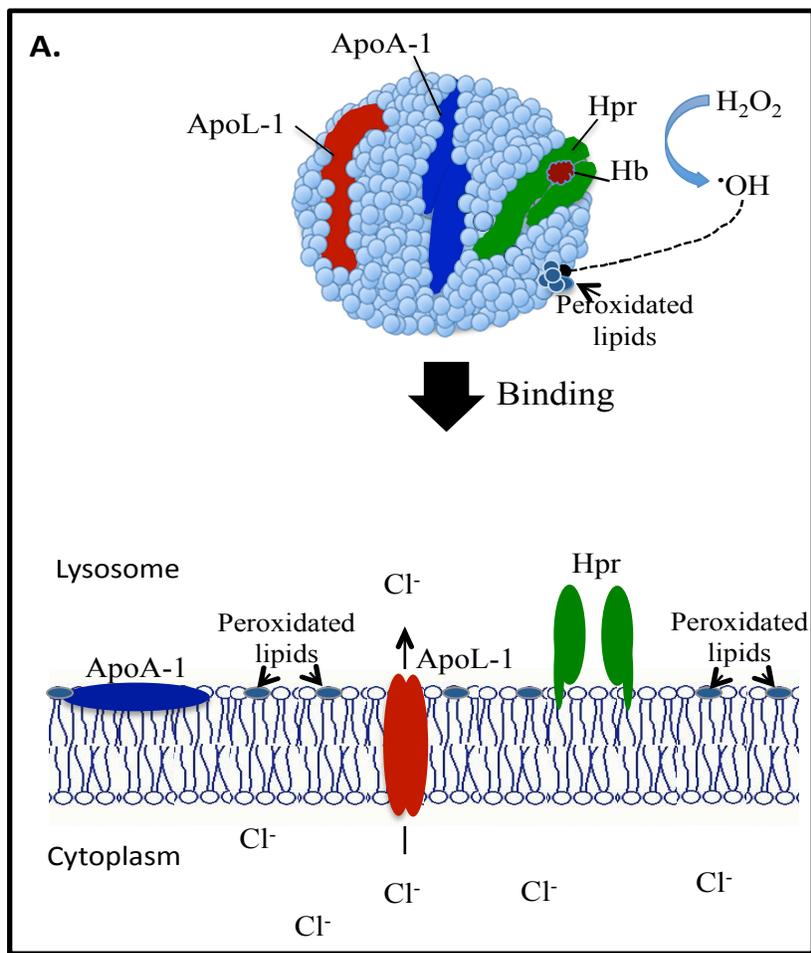


Figure 1.5. A proposed model of the mechanism of TLF-1-mediated lysis. TLF-1 binds Hb via Hpr (green) and is targeted to the lysosomal membrane. This low pH and peroxide-rich environment facilitate Fenton chemistry, initiating with the lipids of the TLF-1 molecule. ApoL-I (red) inserts into the lysosomal membrane, forming a pore and in so doing, targets TLF-1 to the membrane, where the Fenton chemical reaction is propagated. Hpr and apoA-I (blue) also associate with the membrane. (Adapted from Harrington *et. al.*, *J. Biol. Chem.* 2009).

Hpr binds Hb and is thought to facilitate delivery of heme to the acidic lysosome, where low pH, free peroxide radicals, and Fe^{2+} ions lead to the initiation of Fenton chemistry [53, 56]. This peroxidation reaction is proposed to initiate with the lipids of the TLF-1 molecule and propagate via the lipids of the lysosomal membrane, resulting in membrane disruption and subsequent cell lysis (Figure 1.5). Furthermore, cell lysis is inhibited by the presence of iron chelators and free radical scavengers [63], which remove the reactants of Fenton chemistry. In addition, a model liposome *in vitro* system was used to demonstrate that TLF-1 selectively permeabilizes unilamellar liposomes at low pH. TLF-1 also selectively binds negatively-charged liposomes [64]. This specificity of the membrane disrupting activity of TLF-1 is consistent with early reports by the Hajduk group that lysosomal localization is critical for cell lysis since this compartment provides the acidic and anionic membranes required for TLF-1 activity.

PARASITE RESISTANCE MECHANISMS

The intriguing interplay between parasitic organisms and their hosts is both a complex and meticulously modulated one. Unlike most pathogenic microbes, parasites must find a delicate balance that allows for non-detrimental existence within their hosts in order to survive themselves. This dependence means the parasite must navigate and/or manipulate host biology by developing resistance mechanisms in order to survive the host immune response. Resistance is typically due to evasion of the host immune response through the neutralization of host toxins and the manipulation of host cell biology, as well as regulation of parasite biology. One example of modulation of parasite biology is the

process of antigenic variation. The major surface coat proteins of pathogens are typically highly antigenic and elicit an antibody-mediated immune response. Through regulation of expression of the genes encoding these surface proteins, parasites are able to switch expression of the major coat protein and therefore evade the adaptive immune response. This particular type of resistance to host immunity is probably best exemplified by *T. brucei*, which expresses a single variant surface glycoprotein at a time from a pool of several hundred [65].

Evasion of the host adaptive immune response may also take place via invasion of host cells, which is the strategy taken by intracellular parasites like *Leishmania* and *Trypanosoma cruzi*. These microbes are able to survive by sequestering themselves within host cells in order to escape the macromolecules of adaptive immunity. Though invasion is a seemingly, passive strategy of “hiding-out”, once ensconced within the host cell, these parasites rapidly execute more aggressive survival tactics that alter host cell signaling pathways to promote parasite resistance.

In a *Leishmania* infection, the promastigote form of the parasite is injected into human skin through the bite of a sandfly and is phagocytosed by macrophages present at the bite site [66, 67]. The promastigotes localize to the phagosome, resulting in the delayed maturation of the vacuole, demonstrated by a lack of lysosomal and late endosomal markers [67, 68]. The selective occupation and deactivation of macrophages, which function as part of the host’s immune defense, is an ingenious part of the *Leishmania* resistance mechanism. The parasite is able to disable macrophage response by manipulation of specific signaling pathways including those involving protein kinase C,

JAK/STAT, mitogen-activated protein (MAP) kinase, as well as cytokine signaling such as IL-10 and IL-12 [69]. At least two of the surface coat proteins of *Leishmania*, lipophosphoglycan (LPG) and GP63, have been implicated in the altering of host cell signaling pathways. One effect of this manipulation is the suppression of nitric oxide (NO) burst, and other proinflammatory responses that would typically aid in the elimination of the parasite [70].

During a *T. cruzi* infection, these cells begin the manipulation of host cell biology from the point of invasion. *T. cruzi* is able to invade different cell types including macrophages, fibroblasts as well as smooth and striated muscle cells. The parasite binds the mammalian cell surface and activates a calcium-dependent signaling pathway in the host cell, which leads to the recruitment of host cell lysosomes to the plasma membrane. These lysosomes fuse with the plasma membrane and provide membrane for the formation of parasitophorous vacuoles at the sites of parasite binding [71-74]. *T. cruzi* is then able to enter these vacuoles, using them as portals for invasion of the cell. In addition, *T. cruzi* has also been shown to bind receptors on neuronal cells and once internalized, alter MAP kinase signaling pathways to promote host cell survival, thereby ensuring its own persistence [75]. Furthermore, *T. cruzi* invasion leads to the activation of signaling pathways involving extracellular-regulated-signal kinase (ERK), activating transcription factor-1 (AP-1) and NF- κ B signaling. Cell proliferation is also affected, as evidenced by increased cyclin D levels [76]. These changes in cell signaling are involved in the cardiac remodeling that is a hallmark of *T. cruzi*-induced, chronic cardiomyopathy. Thus, both the *Leishmania* and *T. cruzi* trypanosomes are able to commandeer host cell biology, alter cell

signaling and architecture and ultimately manipulate host defensive mechanisms to prolong survival within the host.

The human infectious subspecies of *T. brucei*, *T. b. gambiense* and *T. b. rhodesiense*, have also developed resistance mechanisms that enable survival in humans. Since *T. brucei* is an extracellular parasite, this organism does not employ host cell invasion as part of its resistance mechanism but instead has developed highly effective mechanisms of neutralization of host cell immune mediators. Since TLF-1 uptake and lysosomal targeting are required for cell lysis of *T. brucei*, *T. b. gambiense* has developed a resistance mechanism involving a two-dimensional regulation of the *TbHpHbR*, which is also the TLF-1 receptor [12, 77]. Firstly, quantitative studies on the abundance of the *T. b. gambiense* HpHb receptor (*TbgHpHbR*) transcript revealed a 5-fold decrease in transcript levels when compared to mRNA levels of the receptor in TLF-sensitive *T. b. brucei*. Furthermore, when compared to serum-sensitive *T. b. brucei*, *T. b. gambiense* *TbgHpHbR* was found to have several loss-of-function mutations [77]. Therefore, both downregulation and mutation of *TbgHpHbR* gene led to a loss of functional *TbgHpHbR* receptor in *T. b. gambiense*. Consistent with the lack of expression of a functional receptor, binding and uptake studies showed that *T. b. gambiense* failed to bind or internalize TLF-1 and as a result, the cell circumvents lysis [77]. Thus *T. b. gambiense* has developed a dual-layered resistance mechanism that enables survival in the host by evasion of the TLF-1 molecule, by avoiding endocytosis of the toxin.

T. b. rhodesiense has also developed a highly effective resistance mechanism against lysis by NHS. This resistance is unstable and is due to the expression of the VSG-family

protein, the serum resistance associated (SRA) protein [78, 79]. A comparative analysis of mRNA transcripts in human infectious and non-human infectious *T. b. rhodesiense* revealed that the serum-resistant forms of *T. b. rhodesiense* expressed the *SRA* gene, while serum-sensitive forms did not [78, 80]. Serum-resistant *T. b. rhodesiense* lost *SRA* expression when grown in culture in the absence of NHS and became sensitive to lysis. When these sensitive cells were re-exposed to NHS, a revertant population that had regained *SRA* expression and serum resistance emerged [78]. Unlike *T. b. gambiense*, *T. b. rhodesiense* expresses a functional *TbHpHbR* that allows for TLF-1 binding and endocytosis [12]. The resistance mechanism of SRA therefore involves the neutralization of TLF-1 molecules that has been taken up by the trypanosome. To date, there have been no other proteins identified as mediators of serum resistance in *T. b. rhodesiense*. The expression of this single protein, SRA, is therefore sufficient to confer resistance to TLF-1 mediated lysis.

SRA evolved from a VSG due to an internal truncation event [81], and therefore has several VSG-like properties. Amino acid sequence analysis of SRA predicted post-translational modifications that are similar to those of the VSGs, as well as transferrin receptor, another VSG family protein [81]. Protein modeling also indicated that the tertiary structure of SRA would be similar to VSGs and that it is likely to be a dimer [81]. Despite these predictions, the dimerization of SRA has not been reported, nor has a binding partner been identified. The cellular localization of SRA was also shown to be very different from that of VSG. While, *T. brucei* VSGs and transferrin receptor localize to the cell surface and flagellar pocket respectively, localization studies of SRA revealed an intracellularly localized protein [59, 82, 83]. In addition to having an unexpected intracellular

localization, there have been inconsistencies in reports on the identification of the specific subcellular compartment to which SRA localizes [59, 82, 83].

Previously, one set of localization studies described SRA as a resident lysosomal protein [59], while another observed that SRA was a non-lysosomal protein found in undefined cytosolic vesicles localized between the nucleus and kinetoplast [83]. In addition to uncertainty of SRA localization, there were also discrepant reports on the compartment of the cell in which SRA bound TLF-1. Both a cytosolic colocalization of SRA and TLF-1 [83], as well as a lysosomal colocalization of SRA and apoL-I have been observed [59]. Given the discrepancies in these reports, it was not surprising that different SRA-mediated resistance mechanisms have been proposed. One proposed mechanism of SRA-mediated inhibition of TLF-1 involves SRA/apoL-1 binding and the inhibition of apoL-1 insertion into the lysosomal membrane [59]. This inhibition of apoL-1 membrane insertion subsequently prevents pore formation, lysosomal swelling and cell lysis. A second mechanism predicts that SRA inhibits TLF-1 is by preventing the toxin from trafficking to the lysosome. Since lysosomal localization of TLF-1 is critical for cell lysis, blocking TLF-1 from being targeted to its site of action would prevent trypanosome lysis [83].

Despite identification of SRA as a critical resistance protein in *T. b. rhodesiense* over two decades ago, and the evolution of multiple hypotheses regarding the method of TLF-1 detoxification, the mechanism of SRA-mediated resistance remains undetermined. This dissertation sought to 1) characterize and define the trafficking and localization of SRA, and 2) determine the mechanism of SRA-mediated resistance. These studies have defined the

trafficking pathway of SRA and have shown that this resistance protein is a glycosylated, GPI-anchored protein that has an unusual steady-state localization in the early endosomal compartment. Contrary to previous findings by Oli *et. al.*, SRA does not prevent TLF-1 from trafficking to the lysosome as both SRA and TLF-1 colocalized in the lysosome when proteolysis was inhibited. Thus the final stop for SRA and TLF-1 is the lysosome where both infectivity factors are degraded. While recent developments have elucidated the trafficking pathways of SRA and TLF-1, the specific events that occur *en route* to the lysosome that bring about neutralization of the trypanosome toxin remain unclear.

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CHAPTER 2

THE LYSIS MORPHOLOGIES OF HUMAN SERUM-MEDIATED KILLING OF AFRICAN
TRYPANOSOMES¹

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ABSTRACT

Normal human serum (NHS) is toxic to the protozoan parasite, *Trypanosoma brucei brucei*, due to the presence of an innate immune subclass of high-density lipoproteins (HDL) called Trypanosome Lytic Factor (TLF-1). The trypanocidal activity of human serum exhibits species selectivity, as well as varying degrees of lytic activity. This variation in cytotoxicity of NHS is thought to be associated with two distinct lysis morphologies that have been previously described. Consistent with this, two lytic mechanisms have also been proposed. Here, we characterize the cellular changes that accompany human serum-mediated killing and the conditions under which these changes to cell structure and appearance are observed. These results indicate that there is a rapid lysis accompanied by general cell swelling that is attributed to high specific lytic activity. This activity is likely due to the synergistic activity of two toxins, haptoglobin related protein (Hpr)/hemoglobin (Hb) and apolipoprotein L-I (apoL-I). In addition, we also report a course of lysis in which extensive lysosomal swelling occurs that is associated with low specific activity of TLF-1, as well as with apoL-1 mediated killing.

INTRODUCTION

Trypanosoma brucei brucei is a highly infectious parasite that causes the veterinary wasting disease, Nagana, in sub-Saharan Africa. The host range of *T. b. brucei* is restricted to non-primates due to the presence of an innate immune, anti-parasitic component found in primate sera. The trypanolytic activity of human serum was initially documented by Laveran as early as 1902, and has subsequently been the focus of extensive investigation [1]. This serum cytotoxicity was found to be specific only for *T. b. brucei*, while *T. b. rhodesiense* and *T. b. gambiense*, the human disease-causing subspecies of *T. brucei*, were highly resistant to serum killing [2]. It was not until several decades after Laveran's initial finding, that the lytic activity was shown to be associated with the high density lipoprotein (HDL) fraction of serum [3]. The specific subclass of trypanocidal HDL was later identified as a very minor fraction of HDLs and was called trypanosome lytic factor (TLF) [4]. This trypanocidal toxin contains two lytic apolipoproteins—apolipoprotein L-I (apoL-I) and haptoglobin related protein (Hpr), which are independently toxic to trypanosomes [5-9]. ApoL-I was found to have pore-forming properties and inserts into membranes to form ion channels [6]. Haptoglobin related protein is a hemoglobin (Hb) binding protein, which forms an Hpr-Hb complex that acts both as a ligand for the *T. brucei* haptoglobin-hemoglobin receptor (*TbHpHbR*), as well as a trypanolytic complex [5, 8-11].

In addition to discovering the trypanocidal factor of human serum, Rifkin also examined the biochemical and morphological changes that occurred during cell lysis [12]. These reports were consistent with the earliest observations of cell lysis by both Hawking

and Yorke *et. al.*, as well as more recent reports by Hajduk [13-15]. The progression of serum-associated lysis is typically described as occurring with an initial pre-lysis phase in the first 20 – 30 minutes of exposure to serum, in which there is no cell lysis. During the pre-lytic phase, the trypanosomes lose their slender, elongated appearance and take on the characteristic swollen, rounded form. The flagellum remains attached, but appears to extend away from the cell body, with the entire cell taking on the appearance of a kite on a string. The cells retain this morphology and remain motile until lysis occurs, usually within 2 hours, leaving behind a cytoskeletal ghost. Another distinguishing feature of lysis is the disruption of the plasma membrane, observed in electron micrographs of human serum-treated cells [12, 13].

The Pays research group has presented an alternate set of cellular changes associated with human serum lysis of *T. b. brucei*. The distinguishing feature being the formation and progressive swelling of a cytosolic vacuole in treated cells, which was identified as the lysosome [6]. During the time course of lysis, there is initially no swelling or overall change in cell shape. The flagellum remains attached and the cell continues to be motile while the cytosolic vacuole progressively increases in size until much of the cell body volume is occupied by the expanded lysosome. Another distinguishing feature is that lysis also occurred more slowly with the majority of cells dying 6 – 12 hrs after addition of human serum [6, 16]. Furthermore, the plasma membrane was intact during expansion of the lysosome until the point of cell rupture [17].

The differences in reported morphologies may reflect different mechanisms of human serum-mediated lysis. It was proposed that lysis occurs due solely to the insertion

of apoL-I into the lysosomal membrane. This leads to an influx of anions from the cytoplasm causing extensive lysosomal swelling with the plasma membrane intact until lysis takes place when the trypanosome finally ruptures [6]. Ultimately, trypanosome lysis being due to intracellular pressure from osmotic swelling of the lysosome. A second lysis mechanism has been proposed to be due to the action of both lytic apolipoproteins of TLF-1—Hpr and apoL-I [8]. Both Hpr and apoL-I are proposed to contribute to cell lysis, based on the individual properties of the proteins. Apolipoprotein L-I causes lysis via its pore-forming activity as previously described. Haptoglobin related protein, with Hb as a co-factor, delivers Fe^{2+} ions to the lysosome where the acidic environment facilitates initiation of Fenton chemistry, resulting in lipid peroxidation and subsequent lysosomal membrane disruption [10, 18].

Given the differences in proposed mechanisms and morphologies of lysis, we re-examined the changes in *T. b. brucei* appearance during TLF-1-mediated lysis in order to resolve the differences reported, as well as to gain more insight into the mechanism of lysis. Here, we describe two distinct cell death morphologies. We observed the rounded, “kite-shaped” cell body associated with the more rapid, high efficiency TLF-1 killing activity, as well as the swollen lysosome morphology associated with a slower, low specific activity of TLF-mediated lysis. Further, serum levels of haptoglobin (Hp) appear to play a role in modulating the specific activity of human serum killing of *T. b. brucei*. Haptoglobin is an acute phase protein that functions as a scavenger of hemoglobin, which is released during hemolysis, as a result of injury or infection [19]. Haptoglobin levels fluctuate in human serum, and have been shown to do so in response to trypanosome infections [20-22]. The

levels of Hp in human serum can also differ based on genomic variation as the prevalence of the protein differs across genetic backgrounds [23, 24].

RESULTS

Variation in Trypanolytic Activity of Normal Human Serum.

The variation of trypanolytic activity of sera from different individuals has been well-documented and consistently reported [25, 26]. In order to characterize the serum-induced changes in cellular structure associated with different donors, the trypanolytic activities of NHS from different healthy, fasted donors were analyzed by determining the percent lysis of bloodstream form *T. b. brucei* 427-221 in both short term and extended lysis assays. Trypanosomes were incubated with increasing concentrations of normal human serum (NHS) for 2 and 14 hours, respectively. In the short term (2hr) lysis assays, 10% NHS from one donor resulted in ~50% lysis and was designated as “high activity serum”, while 10% NHS from a second donor resulted in only <10% lysis during the same time period and was designated as “low activity serum” (Figure 2.1A). In an extended (14 hr) survival assay, 5% of the “high activity serum” resulted in 100% lysis, while 5% of the “low activity serum” resulted in ~50% trypanosome lysis (Figure 2.1B). In order to analyze the morphology associated with cell death, live cell imaging was carried out with untreated cells, as well as cells treated with “high activity” and “low activity” sera. Untreated cells appeared slender and elongated. Cells treated with “high activity” serum appeared swollen after 2hrs and exhibited a “kite-shaped” morphology, while cells treated

with “low activity” serum acquired a large swollen vacuole forming after 4 hrs and continuing to expand during 14 hrs of incubation (Figure 2.1C).

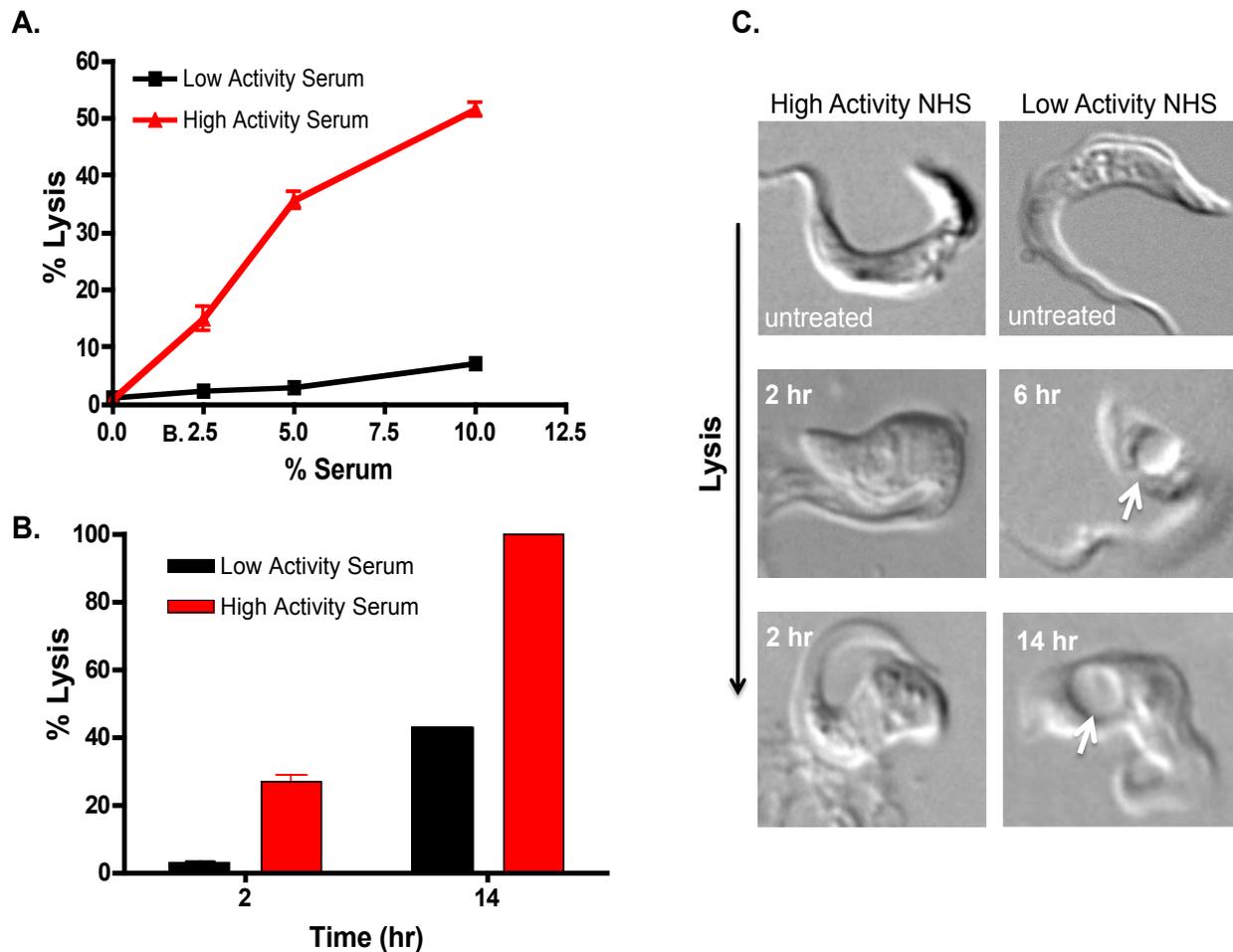


Figure 2.1. Variation in NHS trypanolytic activity leads to differences in lysis morphologies. (A) Comparison of lytic activities of NHS from different donors. *T. b. brucei* cells were incubated with increasing concentrations of NHS at 37°C. Percent lysis was determined after 2 hrs. (B) Trypanolytic activity of NHS was assayed in a short-term (2 hr)

lysis assay and an extended (14 hr) survival assay. (C) DIC images from live cell microscopy videos taken of untreated *T. b. brucei* cells and cells treated with NHS for 2 hrs (20% NHS) and 14 hrs (5% NHS). The white arrow denotes a swollen cytosolic vacuole.

Low activity serum is associated with the formation of the swollen vacuole.

In order to determine whether decreased lytic activity of human serum leads to the formation of swollen vacuoles, we carried out serum lysis assays under conditions previously shown to inhibit serum killing. Treatment of human serum or TLF-1 at 62°C for 30 minutes results in a significant decrease in lytic activity [15]. Following serum treatment at 62°C, less than 5% cell lysis occurred within 2 hrs, while $53 \pm 2.4\%$ cell lysis occurred with untreated serum at the same concentration (Figure 2.2A). Cells incubated with both untreated serum and heat-treated serum were observed at 1 hour intervals for a period of 4 hours to monitor the appearance of swollen vacuoles. The percentage of cells with vacuoles was determined at each time-point. After 1 hour, $4.0 \pm 0.3\%$ of cells incubated with heat-treated serum had swollen vacuoles that localized to the posterior end of trypanosomes (Figure 2.2B). A steady increase in the percentage of vacuoles was determined at each time-point with $25 \pm 2.0\%$ of cells observed with large swollen vacuoles after 4 hours incubated with heat-treated serum (Figure 2.2B). Only rounded, 'kite-shaped' cells without vacuoles were observed in with untreated serum (Figure 2.2C).

In addition to heat treatment, storage of NHS at 4°C also leads to a decrease in lytic activity [15]. We tested the lytic activity of NHS that was stored at 4°C during the course of a week and monitored the change in activity. The relative specific activity was determined for different time points and found to decrease with time stored at 4°C (Figure 2.2D). Live

cell imaging of cells treated with freshly collected NHS that was stored at -80°C showed the rounded, 'kite-shaped' morphology, while cells treated with NHS stored at 4°C , had developed the swollen cytosolic vacuole (Figure 2.2E).

Haptoglobin has also been found to inhibit serum lytic activity and NHS samples with naturally occurring high concentrations of Hp had less trypanocidal activity [5, 26]. Furthermore, despite differences in the lytic activity of sera from different donors, the variation in trypanolytic activity of purified TLF-1 from these same donors was minimal [26]. In order to determine whether the appearance of swollen vacuoles was due to lysis inhibition due to elevated Hp levels, we examined the morphologies of cells treated with normal human serum. Anti-Hp dot blots were used to quantify the haptoglobin levels in our serum samples (Donors A - D).

Consistent with previous findings, Hp levels from different donors were found to differ significantly and the lytic activities of the serum samples decreased with increasing Hp levels (Figure 2.2F, G). Cells treated with serum samples with the highest Hp levels and lowest lytic activities were also found to develop swollen lysosomes that first appeared after 4 hours. There was no cell lysis detected at this timepoint. Cells treated with serum that had low Hp levels consistently assumed a 'kite-shaped' morphology prior to lysis (Figure 2.2H).

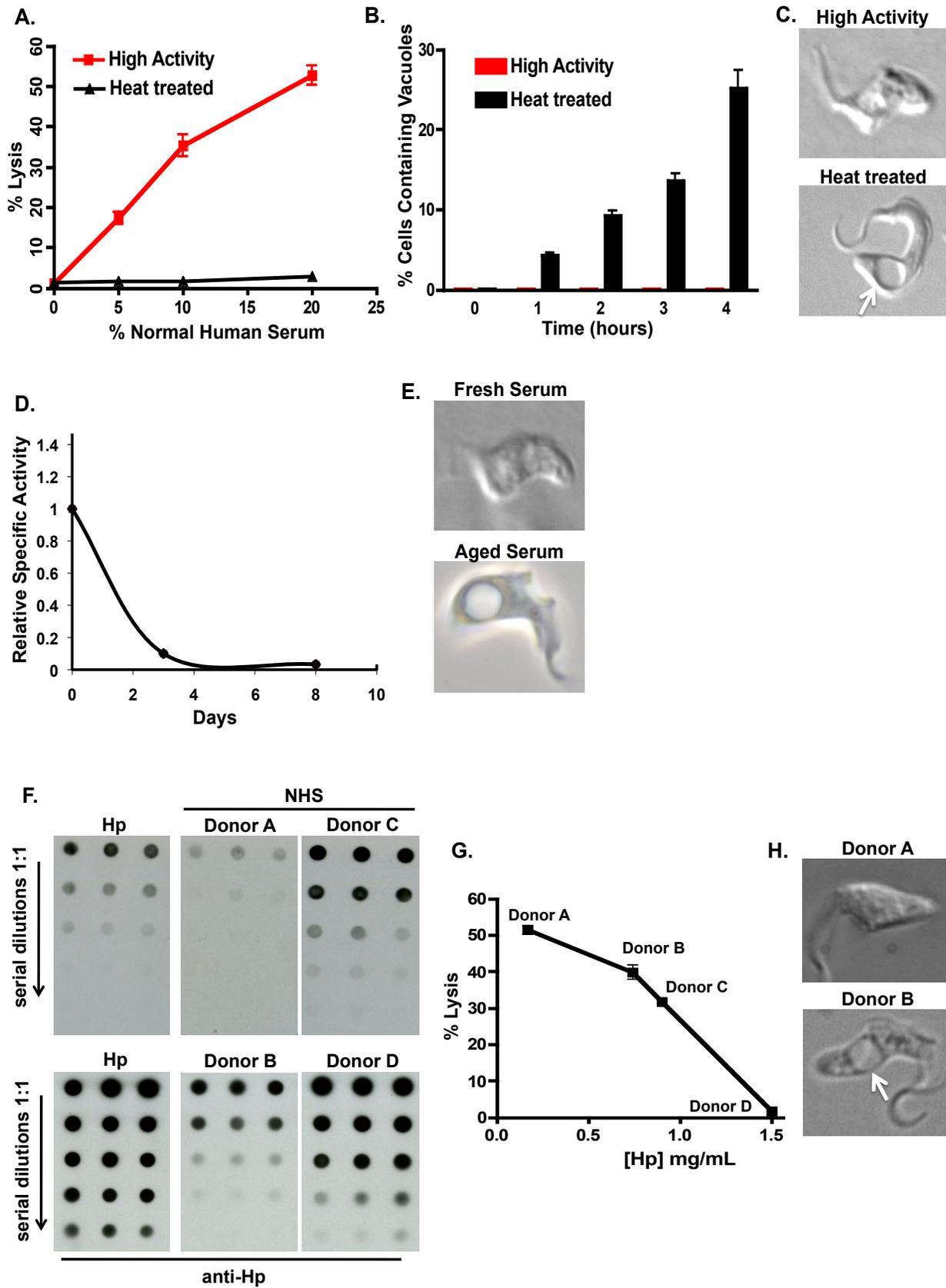


Figure 2.2. Changes in lytic activity of NHS. (A) Heat treatment of NHS (62°C for 30 min) results in a significant decrease in lytic activity of NHS. *T. b. brucei* cells were incubated with increasing concentrations of high activity NHS and heat treated serum at 37°C. Percent lysis was determined after 2 hrs. (B) Decreased lytic activity of NHS due to heat treatment leads to an increase in the appearance of swollen vacuoles over time. Cells were incubated with 20% NHS at 37°C. The percentage of swollen vacuoles was determined at 1 hr time points for a total of 4 hrs. (C) DIC image from live cell microscopy video taken of *T. b. brucei* incubated with high activity or heat treated NHS. The white arrow shows the position of the swollen vacuole. (D) *T. b. brucei* cells were incubated with NHS stored 4°C and changes in relative lytic activity were determined over the course of a week. (E) DIC images from live cell microscopy taken of cells treated with fresh, frozen NHS or NHS stored at 4°C. (F) Dot blot analysis of Hp levels in NHS samples from two different donors. Two-fold serial dilutions of NHS were spotted onto a nitrocellulose membrane and probed with a polyclonal anti-Hp antibody. Serial dilutions of Hp at known concentrations were also probed as a standard for determination of Hp concentration in NHS. (G) Lytic activity of NHS decreases with increasing concentration of Hp. The concentration of Hp in NHS was determined by comparison with Hp standards and plotted against the percent lysis of 10% NHS from different donors in a 2 hr lysis assay. (H) Images of cells incubated with serum samples from different donors.

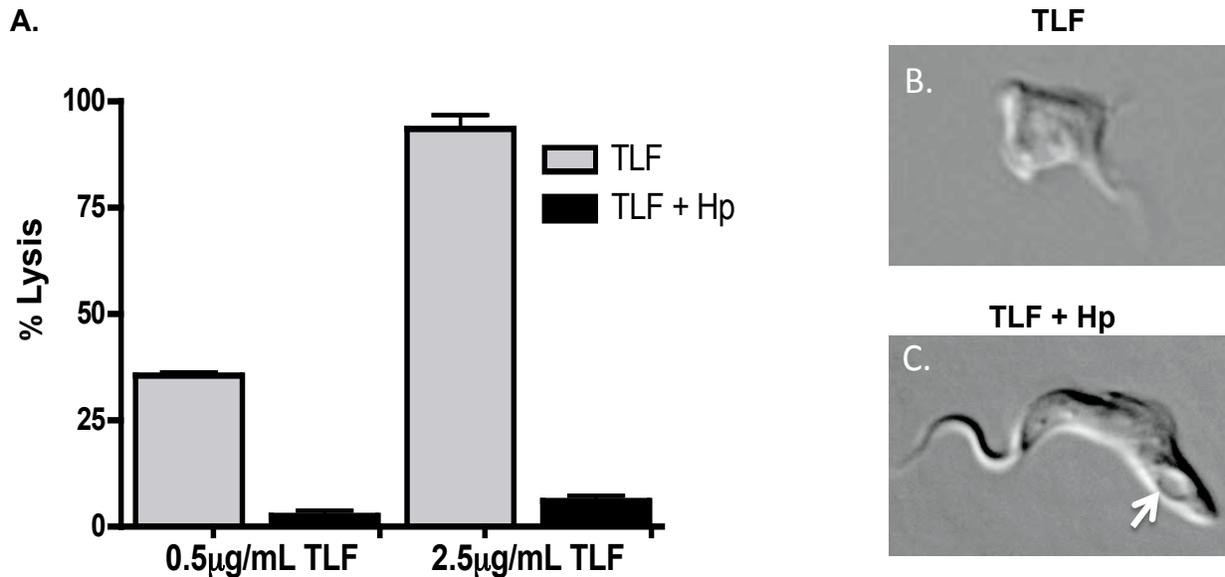


Figure 2.3. Haptoglobin addition to TLF-1 causes swollen vacuole formation. (A) *T. b. brucei* cells were incubated with TLF-1 (0.5 µg/mL; 2.5 µg/mL) in the presence or absence of 0.5 mg/mL Hp. Percent lysis was determined after 2 hrs. (B) DIC image of cell treated with 0.5 µg/mL TLF-1. (C) DIC image of cell treated with 0.5 µg/mL TLF-1 + 0.5 mg/mL Hp. The white arrow denotes the swollen vacuole.

In order to determine whether addition of Hp to TLF-1 would result in the formation of swollen vacuoles, lysis assays and live cell imaging were carried out in the presence of increased concentrations of Hp. At the lower TLF-1 concentration (0.5 µg/mL), $35 \pm 0.6\%$ cell lysis was observed in the absence of Hp. Cell lysis decreased to $2.0 \pm 1.0\%$ with the addition of Hp (0.5 mg/mL) (Figure 2.3A). Similar results were observed when

assays were carried out at a higher concentration of TLF-1 (2.5 $\mu\text{g}/\text{mL}$) with $93 \pm 3.0\%$ lysis without haptoglobin, and $6.0 \pm 0.8\%$ with haptoglobin present (Figure 2.3A). Live cell imaging of cells treated with TLF-1 only revealed rounded, 'kite-shaped' cells with no swollen vacuoles (Figure 2.3B). Large swollen vacuoles similar to those observed when trypanosomes were treated with "low activity" serum were observed when cells were treated with TLF-1 in the presence of Hp (Figure 2.3C). Since Hp is a competitive inhibitor of TLF-1 that binds to the *Tb*HpHbR receptor, we carried out lysis assays at low TLF-1 concentrations and observed the progression of cell death over time. After a 2-hour incubation with TLF-1 at 50 ng/mL, less than 10% cell lysis was observed and the majority of cells had the same appearance of untreated cells. After 3 hours, $13.0 \pm 1.9\%$ lysis was determined and some cells had taken on a rounded and 'kite-shaped' morphology without swollen lysosomes. At the 4-hour timepoint, $29.9 \pm 1.5\%$ lysis was determined and a mixed population of cells was observed with $14.6 \pm 2.9\%$ being 'kite-shaped' and $72.0 \pm 3.6\%$ with swollen lysosomes.

Perez-Morga *et. al.* previously reported that the swollen vacuole formed during lysis localized to the lysosomal compartment [6]. In order to determine whether the swollen vacuoles observed under our conditions were also lysosomal, we performed immunofluorescence localization studies on fixed cells treated with TLF-1 (250 ng/mL) for 8 hours, apoL-I (15 $\mu\text{g}/\text{mL}$) for 4 hours, or Hpr (15 $\mu\text{g}/\text{mL}$) for 1 hour. Cells treated with TLF-1 were fixed, permeabilized and immunostained with anti-p67, a marker for the lysosome. Immunostaining of the lysosome localized the swollen vacuole to this compartment, as anti-p67 staining of the lysosomal membrane revealed a ring-like structure that colocalized with the periphery of the swollen vacuole (Figure 2.4). Cells

treated with apoL-I and Hpr were also fixed, permeabilized and stained for the lysosomal compartment. For cells treated with apoL-I, lysosomal staining showed colocalization with a large swollen vacuole, while Hpr-treated cells did not develop swollen vacuoles and a discrete single lysosome was detected (Figure 2.4).

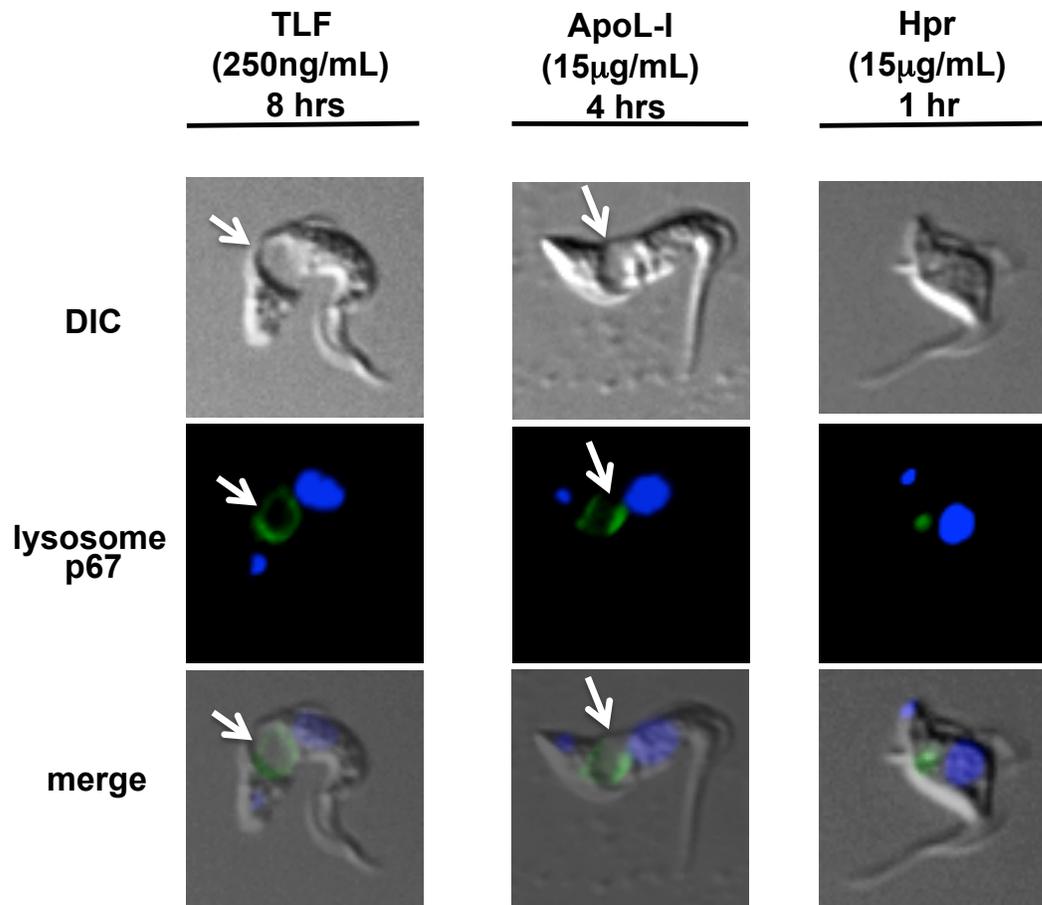


Figure 2.4. Low activity TLF-1 and apoL-I induces lysosomal swelling. *T. b. brucei* cells were treated with TLF-1 (250 ng/mL; 8 hrs), apoL-I (15 μ g/mL; 4 hrs) or Hpr (15 μ g/mL; 1hr). Cells were fixed (1% paraformaldehyde), permeabilized (95% methanol) and immunostained for the lysosomal marker (green, mouse anti-p67). The nucleus and kinetoplast are stained with DAPI. The white arrow denotes the swollen vacuole.

DISCUSSION

Studies on the characterization of the susceptibility of *T. b. brucei* to the lytic properties of NHS have been ongoing since Laveran's initial discovery of this innate immune activity in 1902. The variation of trypanocidal activity and species selectivity of NHS has also been consistently described by several research groups [3, 25-28]. The earliest reports on the morphology associated with trypanosome lysis are consistent with observations previously reported by the Hajduk lab [13, 14] and describe the series of changes that is characteristic of serum-mediated cell death. These changes include an initial 30-minute lag phase prior to swelling and rounding of the trypanosome cell body, followed by the breakdown of the plasma membrane [13, 14]. Hawking and co-workers (1973) reported similar findings from studies with *Trypanosoma equiperdum* and described a half hour-long latent period during which trypanosomes appeared normal. Subsequently, the cells became "distorted" and took on a "globular" appearance. In addition, transmission electron micrographs revealed a breakdown of cytoplasmic structures [29]. Consistent with Hawking's findings, Rifkin's morphological studies of *T. b. brucei* lysis due to NHS also noted a lag phase preceding lysis, followed by the "rounding" of the cell body [12]. Transmission electron microscopy of *T. b. brucei* incubated with NHS showed rounded cells with less dense looking cytoplasm, which is consistent with the disintegrated cytoplasm observed by Hawking [29]. Furthermore, Rifkin also describes a "fraying" of the trypanosome surface coat that was attributed to "perturbation at the plasma membrane level" [12]. Thus, the defining features of the morphology associated with NHS-mediated cell lysis have been consistently reported by independent research groups for several decades.

Contrary to these reports, one group of researchers has reported an alternate set of changes to cellular appearance induced by NHS lysis. This group describes the appearance of a single, cytosolic vacuole that progressively increases in size until the entire cell ruptures. No cellular swelling preceded vacuole formation [6]. The vacuole, identified as the lysosome, typically swells until the trypanosome appears as a large empty vacuole with a partially visible, actively beating flagellum [6, 16]. Another key difference in this lysis morphology is the lack of detection of plasma membrane disruption. This group suggests that the previous reports on plasma membrane breakdown were due to fixation artifacts [17]. It should be mentioned that prior to this, no other research group has reported observations of this swollen lysosome cell death morphology. In order to clarify these discrepancies, we revisited trypanosome lysis and analyzed the cell death-associated structural changes using live cell imaging and immunofluorescence localization studies. We have now characterized the two distinct sets of changes in cellular appearance that were previously documented and have identified the conditions under which these morphologies are observed. These studies resolve some of the long-standing discrepancies reported for *T. b. brucei* killing by NHS.

In order to determine whether the different cell lysis morphologies could be due to the variation in trypanolytic activity of NHS, we assayed the lytic activities of sera from several different donors and carried out imaging studies to investigate morphological changes that accompanied lysis. Since the swollen lysosome was observed in extended survival assays [16], rather than in short-term lysis assays [4], we tested serum activity in both assays. We noted significant variation in lytic activity between donors in both short-term and extended survival assays and identified sera that were designated as having “high

activity” or “low activity”. From live cell imaging of trypanosomes treated with “high activity” NHS in both assays, we observed the general swelling of the cell and the rounded, kite-shaped morphology previously described [12-14, 29]. The vacuole formation was observed only when cells were treated with “low activity” serum in the extended survival assays (Figure 2.1). These findings suggest that the formation of the swollen lysosome is associated with NHS that kills slower, and has lower lytic activity. Because of the longer incubation time required to see lysosomal swelling, it is likely that the morphology would not be seen in the short-term assays [13].

Since the lysosome swelling was observed only when low activity NHS was used, we carried out serum lysis assays under conditions previously shown to decrease serum lytic activity [15, 26]. Heat treatment at 62°C for 30 minutes, storage of serum at 4°C, and elevated Hp levels in NHS have been found to cause a significant decrease in trypanolytic activity [15, 26]. When trypanosomes were incubated with heated NHS, lytic activity decreased 10-fold relative to untreated NHS. These cells exhibited the extensive lysosomal swelling previously observed in trypanosomes treated with “low activity” NHS. The vacuoles increased in size and frequency over time with the largest vacuoles occupying the entire cell body and typically appearing after 4 hours. Storage of NHS at 4°C for 1 week also caused a decrease in lytic activity and resulted in the formation of swollen vacuoles. Consistent with a previous report, analysis of Hp levels of different NHS samples revealed that “low activity” sera correlated with elevated Hp levels when compared to “high activity” sera [26]. Cells incubated with NHS with higher Hp levels also developed swollen lysosomes (Figure 2.2). Thus, this cell death morphology was observed when lytic activity of NHS was reduced, regardless of the method of inhibition used.

Previously, it was demonstrated that in the purification of TLF-1 from human serum, the removal of Hp eliminates significant variations in NHS toxicity to *T. b. brucei*, while the addition of Hp to TLF-1 reduces trypanosome lysis [26]. Haptoglobin-Hb inhibits TLF-1 by competing with the toxin for the *TbHpHbR* [5, 10, 11]. Thus, Hp-Hb is a competitive inhibitor of TLF-1 that decreases the efficiency of TLF-1 endocytosis, and subsequently lysis. Here, we treated cells with TLF-1 in the presence of Hp to determine whether the resulting decrease in TLF-1 lytic activity would lead to the generation of the swollen lysosome. In the absence of Hp, TLF-1 treated cells took on the rounded, kite-shaped appearance, while Hp addition led to the formation of enlarged lysosomes (Figure 2.3). These findings suggest that the swollen lysosome may be formed due to decreased uptake of TLF-1. To determine whether decreased availability of TLF-1, in the absence of an inhibitor would result in lysosomal swelling, we carried out lysis assays at significantly reduced levels of TLF-1. As we observed with Hp inhibition, reducing the concentration of TLF-1 resulted in lysosomal swelling.

The observation of two cell lysis morphologies, with one morphology associated with high specific activity killing and the other with low specific activity killing, indicates that two different lytic mechanisms are involved in TLF-1 lysis. Since there are two lytic proteins in TLF-1, we therefore treated cells with either purified Hpr or apoL-I, as well as with low concentrations of Hb-bound TLF-1. At low concentrations, TLF-1 mediated-lysis was significantly reduced and slowed, with no detection of appreciable lysis occurring until 6 – 8 hours after incubation of cells with TLF-1. The majority of cells observed had large swollen lysosomes but the ‘kite-shaped’ morphology was not observed. Cells treated with apoL-I also exhibited the swollen cytosolic vacuole morphology. As previous studies had

shown [6], the vacuole colocalized with the lysosomal marker, p67. The formation of vacuoles by treatment with apoL-I, independently of Hpr, indicates that this morphology is likely due to apoL-I killing, which involves pore-formation due to apoL-I insertion into the lysosomal membrane, resulting in Cl⁻ ion influx and extensive osmotic swelling of the lysosome [6]. Since vacuole formation was also observed when trypanosomes were incubated with low concentrations of TLF-1, it is possible that the Hpr-Hb lytic mechanism may require higher concentrations of the toxin, so that only apoL-I mediated killing activity is observed under these conditions. Trypanosomes treated with purified Hpr developed the 'kite-shaped' morphology, with significant lysis occurring within one hour of Hpr addition. Haptoglobin related protein-Hb is proposed to mediate lysis through the delivery of Fe²⁺ ions to the acidic, peroxide-rich lysosomal environment, where Fenton chemistry and lipid peroxidation occur, resulting in lysosomal membrane disruption [9]. At high concentrations of Hb-bound TLF-1 and Hpr-Hb, the level of lipid peroxidation and oxidative damage may be too extensive to be ameliorated by the antioxidant defense mechanisms of *T. brucei* resulting in the rapid, high specific activity cell lysis and the 'kite-shaped' morphology. These antioxidant defense mechanisms include the reduction of oxidation products by trypanothione, which is a glutathione derivative that is unique to kinetoplastid organisms [30]. At lower concentrations of TLF-1, the degree of oxidative damage due to Hpr-Hb lysis may be repairable by trypanothione, resulting in the neutralization of the Hpr-Hb lytic mechanism, so that the lysis morphology associated with the more rapid lipid peroxidation reaction is not observed. Instead, the slower apoL-I pore-forming activity and lysosomal swelling becomes the dominant lysis mechanism.

The studies reported here show that there are two distinct lysis morphologies associated with human serum-mediated lysis of *T. brucei* and these changes in cellular appearance are likely to be associated with different mechanisms of lysis. We propose that human serum-mediated lysis of *T. brucei* occurs by both mechanisms. At high concentrations of TLF-1, both trypanolytic apolipoproteins are likely to function efficiently through their individual lytic mechanisms. However, the apoL-I mediated lysosomal swelling may be masked by the more rapid kinetics of the previously proposed synergistic activities of both Hb-bound Hpr and apoL-I [8]. This rapid and highly efficient lysis is associated with the development of rounded, 'kite-shaped' cells. At lower concentrations of TLF-1, the contribution of apoL-I mediated killing is most likely the predominant lytic mechanism. This may be due to trypanosome antioxidant defense against low-level Hpr-Hb peroxidation activity, thus only apoL-I killing and the swollen lysosome morphology, which develops more slowly is observed.

MATERIALS AND METHODS

Cell culture.

Bloodstream form *T. b. brucei* cultures were grown in HMI-9 medium (FBS, Gemini Bio-products, West Sacramento, CA); Serum Plus (SAFC Biosciences, Lenexa, KS). All studies were carried out using *T. b. brucei* 221-427 cells.

Live cell microscopy and immunofluorescence assays.

Live cell microscopy. Cells were resuspended in PBS containing 1% glucose (PBS-G) then spotted onto a glass slide. An equal volume of warmed agarose (2% agarose in PBS) was added to the drop of cells in order to slow cell motility and facilitate imaging. Videos were taken through oil immersion at 63x magnification, using 50ms acquisition times. Individual trypanosomes were centered in each video using digital tracking software (Final Cut, Apple Inc.).

Immunofluorescence. All cells were fixed in 1% paraformaldehyde (PFA) on ice for 15 minutes followed by a brief methanol treatment at -20°C. Fixed cells were washed once in PBS, then resuspended in PBS, 10% FBS for 1 hr. Primary antibody staining was carried out for 45 minutes using mouse anti-p67 (1:1000) as the lysosomal marker or mouse anti-Ty (1:1000) as the late endosomal marker. For secondary antibody staining, slides were incubated with Alexa Fluor 488 mouse immunoglobulin G (Invitrogen, Eugene, OR) for 30 minutes. 4', 6'-Diamidino-2-phenylindole (DAPI) was added after final washes. Serial image z-stacks were acquired through oil immersion optics at 63x magnification, with exposure times kept constant for each experiment. Imaging was carried out using a Zeiss Axio Observer inverted microscope equipped with an AxioCam HSm camera and analyzed with the AxioVision v4.6 software (<http://www.zeiss.com>). A single stack is shown for each experiment with individual channels contrasted to the same extent for each image set and merged using Adobe Photoshop CS2 v9.0.

Normal human serum, TLF-1 and apoL-I purification and lysis assays.

Serum and plasma purification. Human blood was obtained from healthy, fasted donors and allowed to clot at 4°C, before serum was collected. Plasma was separated from whole blood by low-speed centrifugation (3500 rpm, 10 min), and subsequent high-speed centrifugation (10,000 rpm, 5 min) of the supernatant to pellet residual red blood cells. Serum and plasma were stored in single used aliquots at -80°C. Lytic activity of serum and plasma was determined using 2-hr lysis assays as previously described

TLF-1 and apoL-I Purification. HDLs were purified as previously described [4, 8]. Briefly, after collection of the HDL fraction of plasma (1.26 g/ml), TLF-1 was isolated from this fraction by affinity purification using monoclonal antibodies against Hpr. For Hpr and apoL-I purification, HDLs were first solubilized with mild, anionic detergent treatment before affinity purification with a monoclonal antibody against either Hpr or apoL-I [8]. Antibodies were coupled to Pierce Affigel resin based on the manufacturer's recommendations (Thermo Fisher Scientific, Rockford, IL). Eluates were dialyzed against phosphate-buffered saline, 75 µM EDTA (PBSE) at 4°C and stored in single-use aliquots at -80°C. Susceptibility to TLF-1 was determined using 2-hr lysis assays as previously described [4].

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CHAPTER 3

ENDOSOMAL LOCALIZATION OF THE SERUM RESISTANCE ASSOCIATED PROTEIN IN
AFRICAN TRYPANOSOMES CONFERS HUMAN INFECTIVITY¹

¹ **Stephens, N.A.**, and Hajduk, S.L. 2011. *Eukaryotic Cell*. 10(8): 1023-33.

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ABSTRACT

Trypanosoma brucei rhodesiense is the causative agent of human African sleeping sickness. While the closely related subspecies, *T. brucei brucei* is highly susceptible to lysis by a subclass of human high density lipoproteins (HDL) called trypanosome lytic factor (TLF), *T. b. rhodesiense* is resistant and therefore able to establish acute and fatal infections in humans. This resistance is due to expression of the serum resistance associated (SRA) gene, a member of the variant surface glycoprotein (VSG) gene family. Although much has been done to establish the role of SRA in human serum resistance, the specific molecular mechanism of SRA-mediated resistance remains a mystery. Thus, we report the trafficking and steady-state localization of SRA in order to provide more insight into the mechanism of SRA-mediated resistance. We show that SRA traffics to the flagellar pocket of bloodstream form *T. brucei*, where it localizes transiently before being endocytosed to its steady-state localization in endosomes and demonstrate that the critical point of colocalization between SRA and TLF occurs intracellularly.

INTRODUCTION

T. b. brucei causes the veterinary disease, Nagana, but is unable to establish infections in humans. Human resistance to *T. b. brucei* infection is due to the presence of a trypanolytic component of human serum, which provides innate immunity against infection. This component is a minor subfraction of high-density lipoproteins (HDL), called trypanosome lytic factor-1 (TLF-1) [1, 2]. Like all HDLs, TLF-1 contains apolipoprotein A-I (apoA-I), as well as two unique, primate-specific proteins, apolipoprotein L-I (apoL-I)[3] and haptoglobin-related protein (Hpr) [4] that confer lytic activity to the particle. This toxic class of HDLs is internalized in *T. b. brucei* via receptor-mediated endocytosis and is ultimately targeted to the lysosome, where it initiates low pH-dependent killing [3, 5-9].

While TLF-1 is toxic to *T. b. brucei*, *T. b. rhodesiense* is resistant to TLF-mediated killing and causes the acute form of Human African Trypanosomiasis (HAT). The mechanism of resistance to TLF-1 remains to be fully elucidated; however, it is well established that the resistance phenotype of *T. b. rhodesiense* is due to the expression of the serum resistance associated (SRA) protein. Most human isolates of *T. b. rhodesiense* have been found to express SRA [10], and loss of SRA expression leads to susceptibility to TLF-1 toxicity [11]. Furthermore, transfection of the *SRA* gene into susceptible *T. b. brucei* cell lines confers resistance to TLF-1 killing [12, 13].

SRA is a member of the VSG gene family and is predicted to share similar structures and post-translational modifications with VSGs and the trypanosome transferrin receptor (TfR), another VSG family member [11, 14]. Trypanosome VSGs and TfR are glycosylated, cell surface proteins that are anchored to the plasma membrane via the GPI lipid anchor

[15, 16]. Both VSGs and TfR are continually trafficked to and from the cell surface via the flagellar pocket by robust secretory and recycling pathways [17-19]. The GPI anchor attachment is typically associated with cell surface proteins and has been shown to be involved in the trafficking of these proteins [20, 21]. Previous findings have reported that SRA is intracellularly localized, despite being a VSG family protein with a predicted GPI-anchor attachment site [3, 13]. SRA has also been found to bind TLF-1 via direct interaction with apoL-I and to colocalize intracellularly [3, 13].

In this study, we show for the first time that SRA traffics to the flagellar pocket before rapid uptake into cytoplasmic vesicles, which we now identify as early endosomes. We also find that lysosomal localization of SRA is fleeting and is detectable only when protein degradation is inhibited. Deletion of the GPI anchor addition site disrupts flagellar pocket localization of the protein but is not required for trafficking to the endosomes or colocalization with TLF-1. Furthermore, loss of SRA trafficking to the flagellar pocket does not result in increased susceptibility to TLF-1, suggesting that the critical point of interaction of toxin and inhibitor is not at the cell surface. Finally, we show that a trypanosome cysteine protease is involved in rapid TLF-1 turnover in SRA-expressing *T. b. brucei* transfectants, indicating that the mechanism of SRA-mediated resistance to TLF-1 killing may involve accelerated degradation and destabilization of the TLF-1 particle.

RESULTS

SRA traffics to the flagellar pocket. Since VSG family proteins (VSG and TfR) localize to the trypanosome cell surface membrane, this led us to re-examine the cellular localization

of SRA. Previous localization studies showed that SRA does not localize to the trypanosome cell surface coat like VSG, but was distributed within intracellular vesicles localized between the kinetoplast and nucleus [3, 13]. We used the previously characterized cell line in which Ty epitope-tagged SRA was expressed (*T. b. brucei* SRA-Ty) [13] (Figure 3.1A). Northern blot analysis of SRA-Ty mRNA transcript levels in *T. b. brucei* SRA-Ty transfectants were found to be comparable to SRA transcript levels in *T. b. rhodesiense* (Supplemental Figure S3.1.A). Intracellular localization may be the result of direct sorting to a stable vesicular pool or secondary sorting following transient localization at the cell surface. We therefore examined the flagellar pocket, a specialized region of the cell surface membrane to which the trypanosome transferrin receptor localizes. In two-dimensional images, the flagellar pocket can best be delineated as the region between the kinetoplast DNA and the posterior end of the paraflagellar rod (PFR). The basal bodies are directly adjacent to the kinetoplast and are also useful as organellar markers for the flagellar pocket (Figure 1B). In order to examine this specific cell surface region, we carried out live cell binding studies at 3°C using Alexa 594-conjugated transferrin and Alexa 594-conjugated anti-Ty (SRA). At this temperature, we find that endocytosis is strongly inhibited and receptor-bound transferrin was detected in the flagellar pocket (Figure 1B). Under 3°C binding conditions, $93.8 \pm 5.3\%$ of transferrin-labeled cells showed distinct flagellar pocket localization of transferrin (Table 3.1). To determine whether SRA localized to the flagellar pocket, we incubated live *T. b. brucei* SRA-Ty cells at 3°C with Alexa 594-conjugated anti-Ty followed by fixation with paraformaldehyde and visualized the localization by fluorescence microscopy. As with transferrin, these live cell binding studies also showed flagellar pocket

localization of bound Alexa 594 anti-Ty indicating that SRA traffics to the cell surface of trypanosomes but its distribution is limited to the flagellar pocket (Figure 3.1C).

In addition to live cell binding studies, we examined cells that were fixed under non-permeabilizing conditions for flagellar pocket-localized SRA. Immunostaining of fixed, non-permeabilized cells with anti-Ty revealed SRA localization to be restricted to the flagellar pocket (Figure 3.1D). Cells were also immunostained with the anti-*Tb*Cathepsin L (*TbCatL*) antibody, an intracellular marker for the trypanosome lysosomal protease *Tb*Cathepsin L (trypanopain) [22, 23], which colocalizes with the known lysosomal marker, p67 (Supplemental Figure S3.1.B). Lysosomal staining was not detectable, thereby confirming that the fixed cells were impermeable to antibodies under those conditions and the observed anti-Ty staining was therefore extracellular and flagellar pocket-localized (Figure 3.1D). Upon methanol permeabilization and anti-Ty immunostaining of fixed *T. b. brucei* SRA-Ty cells, intracellular SRA-containing vesicles were observed between the kinetoplast and nucleus (Figure 3.1E). Although lysosomal staining was also detectable under permeabilizing conditions, SRA did not colocalize with this marker (Figure 3.1E). Quantitation of SRA distribution showed flagellar pocket localization in only 37.9 ± 9.6 % of cells, while all anti-Ty labeled cells showed intracellularly localized SRA (Table 3.2). These results confirm that SRA traffics to the flagellar pocket but that the steady state distribution is mainly within non-lysosomal intracellular vesicles.

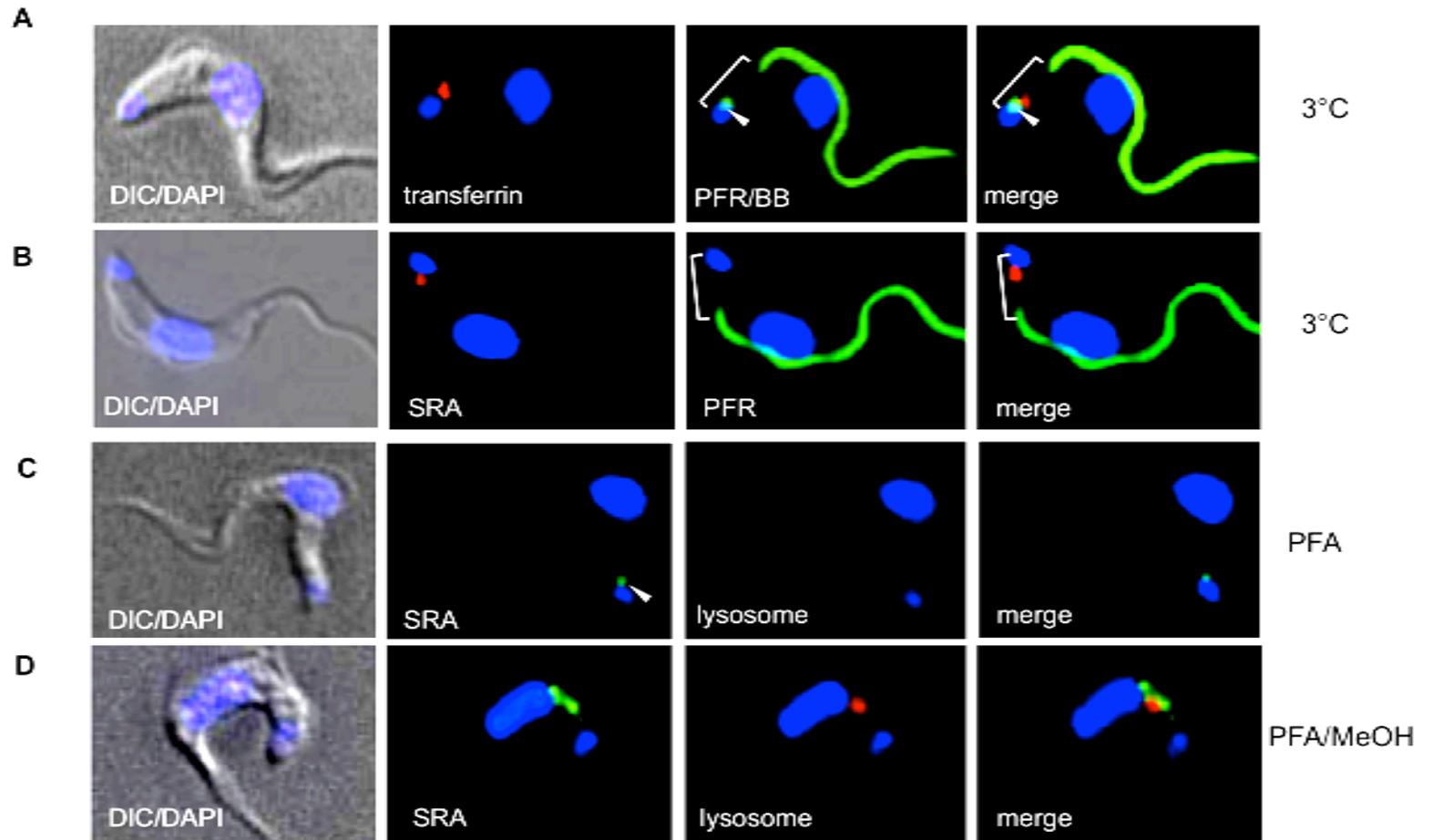


Figure 3.1. SRA traffics to the flagellar pocket. (A) Live cell binding studies at 3°C with Alexa 594-transferrin (red) or (B) Alexa 594 anti-Ty (SRA) (red). Post-binding and fixation, (A) basal bodies were stained with anti-YL1/2 (green) and (A, B) the paraflagellar rod was stained with anti-PFR (green). The flagellar pocket is shown by the white bracket and the arrowheads denote the position of the basal bodies. Cells were fixed under (C) non-permeabilizing and (D) permeabilizing conditions and stained for SRA with anti-Ty (green). (C, D) Cells were also stained for the lysosomal compartment with anti-*TbbCatL* (red). (C, D) Image acquisition was carried out at the same exposure and images were contrasted to the same extent.

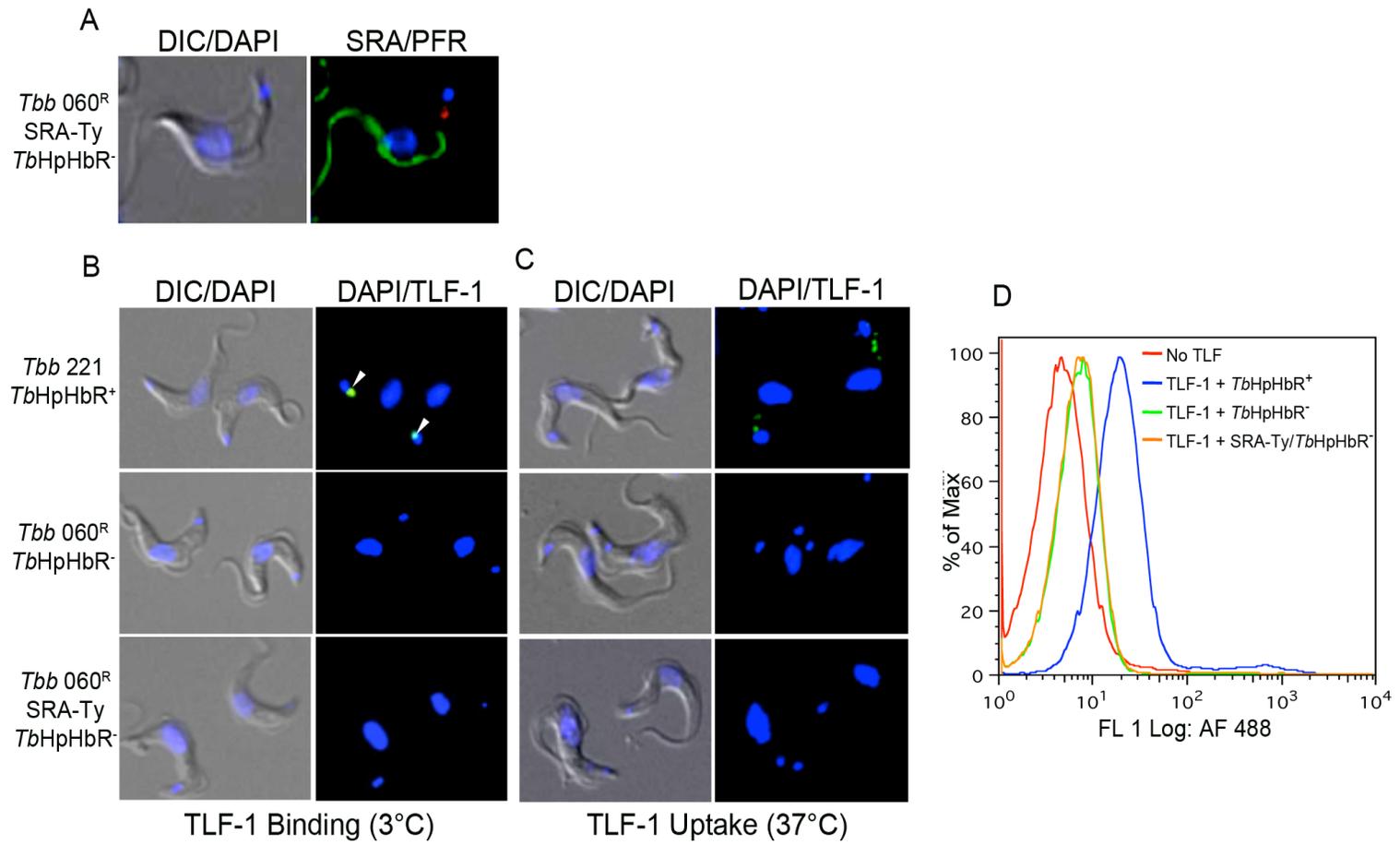


Figure 3.2. The initial interaction between SRA and TLF does not take place at the flagellar pocket. (A) Binding and (B) uptake of Alexa 488-TLF (green) in *T. b. brucei* 221 (*TbHpHbR*⁽⁺⁾), *T. b. brucei* 060^R (*TbHpHbR*⁽⁻⁾) and *T. b. brucei* 060^R SRA-Ty (*TbHpHbR*⁽⁻⁾) cells. White arrowheads show binding in flagellar pocket. (C) Flow cytometry analysis of Alexa 488-TLF uptake; red – No TLF, blue – *T. b. brucei* 221, green – *T. b. brucei* 060^R, orange – *T. b. brucei* 060^R SRA-Ty. (D) Binding of Alexa 594 anti-Ty (SRA) (red) in the flagellar pocket of *T. b. brucei* 060^R SRA-Ty cells. Anti-PFR staining is shown in green.

The initial interaction between TLF-1 and SRA does not occur within the flagellar pocket. Previous studies have demonstrated that SRA binds TLF-1 via apoL-1 [3] and that these proteins colocalize in intracellular vesicles [13]. Since SRA traffics to the flagellar pocket, which is also the site of receptor-mediated uptake of TLF-1 by the haptoglobin/hemoglobin receptor (*TbHpHbR*) [24], we investigated whether the initial colocalization between SRA and TLF-1 could occur within the flagellar pocket. To examine this possibility, we expressed Ty-tagged SRA in a cell line that does not express *TbHpHbR* (Figure 3.2A, Supplemental Figures S3.1.C, S3.1.D) [25]. In these cells, SRA-Ty localized to the flagellar pocket based on localization studies at 3°C with anti-Ty (Figure 2A). The SRA-Ty expressing transfectants, *T. b. brucei* 427 060^R-SRA-Ty, were also assayed for binding and uptake of Alexa 488-conjugated TLF-1 by fluorescence microscopy. *T. b. brucei* 427-221 cells expressing the *TbHpHbR* exhibited TLF-1 binding and uptake (Figures 3.2B-C, Supplemental Figures S3.1.D and S3.2) while *T. b. brucei* 427-060^R cells that did not express the receptor, based on the lack of detectable mRNA by RT-PCR (Supplemental Figure S3.1.D) did not bind or endocytose TLF-1 (Figure 3.2B-C). In addition to fluorescence microscopy, 427-221 (*TbHpHbR*-positive) and 427-060^R (*TbHpHbR*-negative) cell lines were also assayed for Alexa 488 TLF uptake by flow cytometry. Consistent with the localization studies, *T. b. brucei* 427-221 cells bound Alexa 488 TLF at 37°C. *T. b. brucei* 427-060^R and 427-060^R-SRA-Ty cells had low levels of cell-associated TLF-1, which is likely to be due to non-specific cell surface labeling. Despite the localization of TLF-1 and SRA individually at the flagellar pocket, we were unable to detect the colocalization of these two infectivity factors at the flagellar pocket in the *TbHpHbR*-negative cells. These findings indicate that expression of SRA in the absence of the *TbHpHbR* is not sufficient to allow for

TLF-1 binding at the surface of the trypanosome suggesting that the proteins independently enter the endocytic pathway.

SRA is resident within an early endosomal compartment. Extensive characterization of trafficking vesicles in bloodstream form *T. brucei* has defined several classes of endosomes by their associated Rab GTPases, as well as cargo such as transferrin, for which the endocytic trafficking pathway is well-documented [18, 26]. The Rab5a GTPase has been identified as a marker for early endosomes and has been shown to colocalize with transferrin within minutes of receptor-mediated uptake [26, 27]. In order to localize the early endosomal compartment, we transfected *T. b. brucei* 427-221 and *T. b. brucei* 427-221 SRA-Ty cells with haemagglutinin (HA) epitope tagged Rab5a. Anti-HA western blot showed a 33 kDa band in transfectant cell lines that was absent in untransfected cells (Figure 3.3A). Using anti-HA immunofluorescence microscopy, we localized HA-tagged Rab5a endosomes distributed between the kinetoplast and nucleus in both cell lines (Figure 3.3B-C). This discrete non-lysosomal distribution of HA-tagged Rab5a endosomes is consistent with previous reports on Rab5a localization in *T. brucei* [27].

To validate HA-tagged Rab5a as an early endosomal marker, we carried out live cell binding and uptake studies with Alexa 594-transferrin, a known cargo of early endosomes. Both *T. b. brucei* 427-221 Rab5a-HA and *T. b. brucei* 427-221 SRA-Ty transfectant cell lines were allowed to bind Alexa 594-transferrin at 3°C, then shifted to 37°C to permit resumption of vesicle trafficking. Vesicle trafficking was halted by fixation after 1 minute at 37°C, followed by permeabilization and either immunostaining with anti-Ty (SRA-Ty transfectants) or anti-HA (Rab5a-HA transfectants). In *T. b. brucei* 427-221 Rab5a-HA

transfectants, transferrin bound discretely to the flagellar pocket at 3°C (Figure 3.3D). After 1 minute post-uptake, Alexa 594-transferrin colocalized intracellularly with Rab5a-HA (Figure 3.3E). The same pattern of Alexa 594-transferrin localization was observed in *T. b. brucei* 427-221 SRA-Ty transfectants, with flagellar pocket colocalization of transferrin and SRA at 3°C (Figure 3.3F) and colocalization with SRA-containing intracellular vesicles after 1 minute at 37°C (Figure 3.3G). In addition to endosomally-localized Alexa 594-transferrin, a fraction of the labeled cargo is also visible within the flagellar pocket (Figure 3.3G). Post fixation and permeabilization, the steady-state localization of SRA by anti-Ty staining revealed the distribution of SRA from the flagellar pocket throughout the early endosomal compartment (Figure 3.3F). Finally, anti-Ty and anti-HA staining of *T. b. brucei* 427-221 SRA-Ty; Rab5a-HA transfectants showed colocalization of SRA-Ty with Rab5a endosomes, thereby identifying the intracellular SRA-Ty containing vesicles as early endosomes (Figure 3.3H).

Previous studies have presented conflicting observations on the intracellular localization of SRA and TLF-1 [3, 13]. Vanhamme *et al.*, (2003) found that SRA and TLF-1 colocalized in the lysosome, while Oli *et al.*, (2006) reported a non-lysosomal localization. To address this discrepancy, we carried out live cell binding and uptake studies with Alexa 488 conjugated-TLF-1 in *T. b. brucei* SRA-Ty; Rab5a-HA transfectants. Cells were incubated with Alexa 488-TLF at 3°C for binding studies then shifted to 37°C for 1 minute to permit endocytosis and vesicle trafficking. After 1 minute at 37°C, Alexa 488-TLF colocalized with both anti-HA staining (Figure 3.3I) and anti-Ty staining (Figure 3.5A), indicating that both TLF-1 and SRA traffic through the early endosomes—the earliest site of observed colocalization of these virulence factors.

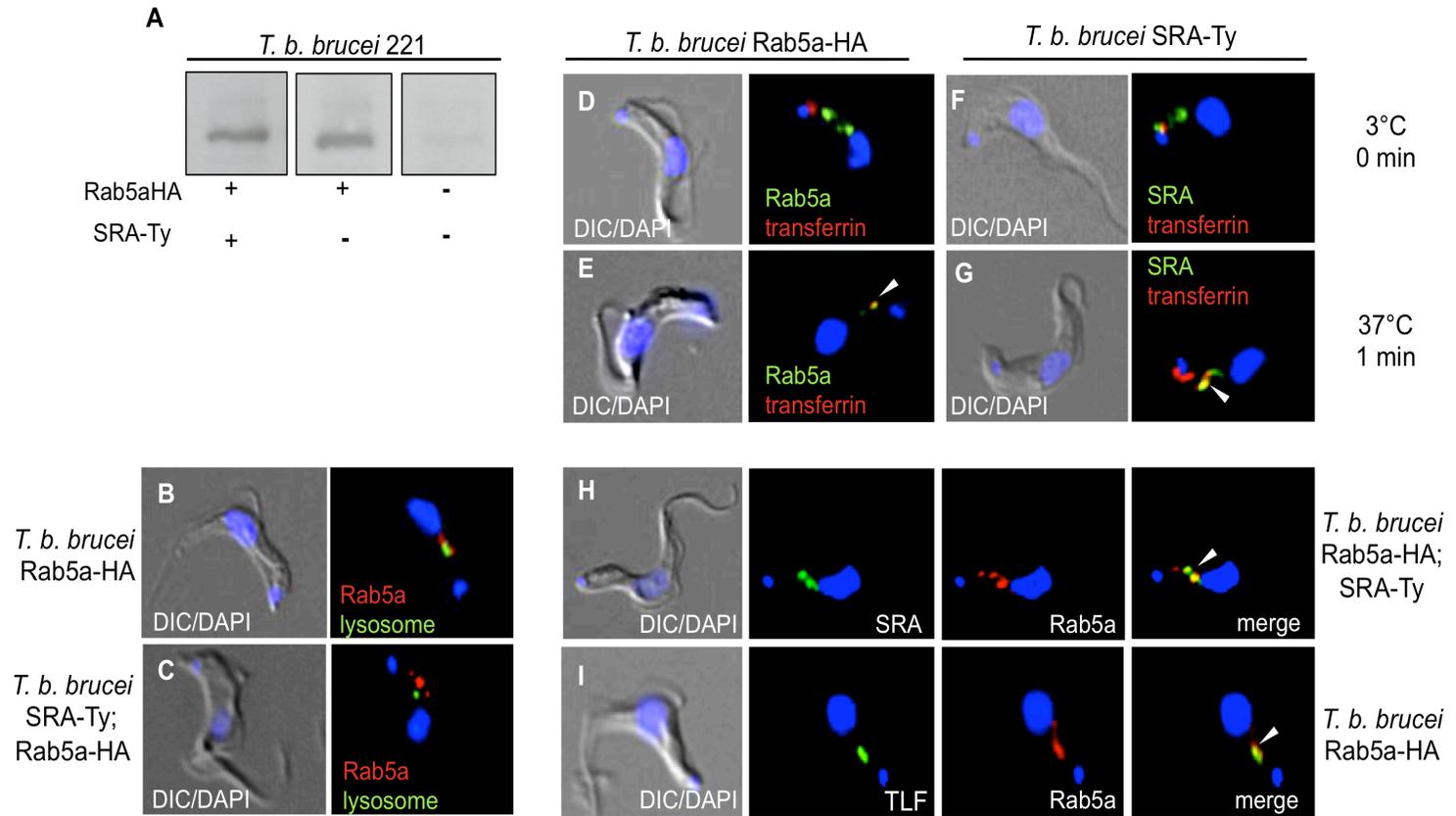


Figure 3.3. Endosomal localization of SRA. (A) Western blot and (B, C) immunofluorescence staining with anti-HA (Rab5a) (red) of *T. b. brucei* 221 *TbRab5aHA* transfectants. (B, C) Lysosomal (anti-*TbbCatL*) staining is shown in green. Binding and uptake studies using (D, E) *T. b. brucei* 221 *TbRab5aHA* and (F, G) *T. b. brucei* 221 SRA-Ty transfectants. (D, F) Live cell binding of Alexa 594-transferrin (red) at 3°C, 0 minutes, followed by (E, G) temperature shift to 37°C for 1 minute, then fixation with 1 % paraformaldehyde to halt vesicle trafficking. Post fixation, (D, E) *TbRab5aHA* was stained with anti-HA (green), and (F, G) SRA was stained with anti-Ty (green). (H) Steady-state localization of SRA-Ty and *TbRab5aHA*. Fixed, permeabilized *T. b. brucei* 221 *TbRab5aHA*; SRA-Ty transfectants were stained with anti-Ty (SRA) (green) and anti-HA (Rab5a) (red). (I) Uptake of Alexa 488-TLF (green) in *T. b. brucei* 221 *TbRab5aHA* transfectants. Rab5a endosomes are shown by anti-HA (red) staining. The nucleus and kinetoplast are localized by DAPI staining. White arrowheads indicate colocalization.

The GPI anchor of SRA is necessary for flagellar pocket, but not endosomal localization.

The GPI-anchor modification has previously been shown to play a role in the trafficking and localization of glycoproteins in *T. b. brucei* [21, 28]. To investigate the role of the GPI anchor in SRA trafficking, we engineered a Ty-tagged deletion mutant of SRA to be translated without the C-terminal hydrophobic peptide and the GPI anchor addition site, thus preventing post-translational addition of the GPI anchor (Figure 3.4A). Western blot analysis of the mutant *T. b. brucei* 427-221 SRA Δ GPI and *T. b. brucei* 427-221 SRA-Ty cell lysates revealed comparable levels of SRA protein expression; however, SRA Δ GPI migrated faster than soluble, GPI-PLC cleaved wild type SRA-Ty. (Figure 3.4B). Under the solubilization conditions (1% NP-40) used here we expect wild type SRA-Ty to be released by GPI-phospholipase C activity. Therefore, neither the mutant SRA Δ GPI nor soluble wild type SRA-Ty is membrane-bound [29]. The small apparent size of the SRA Δ GPI mutant is due to the complete lack of the amino acid anchor addition site, as well as the phosphoethanolamine, glycan, inositol and diacylglycerol moieties that comprise the GPI anchor. Wild type SRA-Ty retains all these components except diacylglycerol following GPI-PLC cleavage. SRA Δ GPI therefore lacks the cleaved GPI-anchor fragment present on soluble wild type SRA-Ty, which is likely to account for the 5kDa difference in migration of the proteins. Deglycosylation of SRA Δ GPI by PNGase F treatment led to a shift in migration indicating that the GPI-anchorless protein traffics through the ER where N-linked glycans are added (Supplemental Figure S3.1.E).

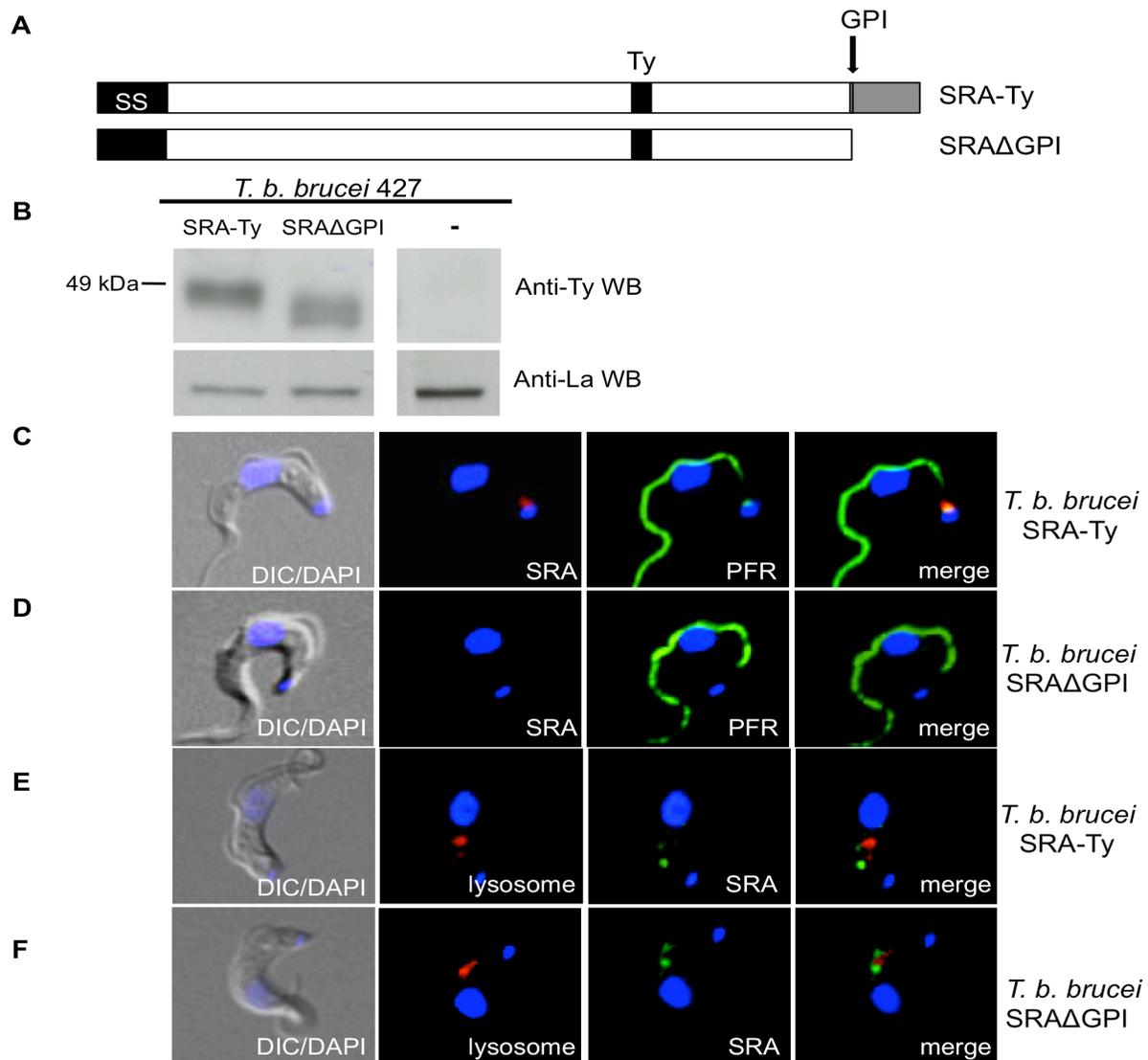


Figure 3.4. Loss of the GPI anchor disrupts flagellar pocket localization. (A) Schematic diagram of SRA-Ty and SRAΔGPI. (B) Western blot of SRA-expressing *T. b. brucei* transfectants probed with anti-Ty (SRA) and anti-La (La protein loading control). (C, D) Binding of Alexa 594 anti-Ty (SRA) antibody at 3°C in (C) SRA-Ty cells and (D) SRAΔGPI cells is shown in red. PFR staining is shown in green. (E) SRA-Ty and (F) SRAΔGPI cells were stained for SRA (green) and lysosomal localization (red).

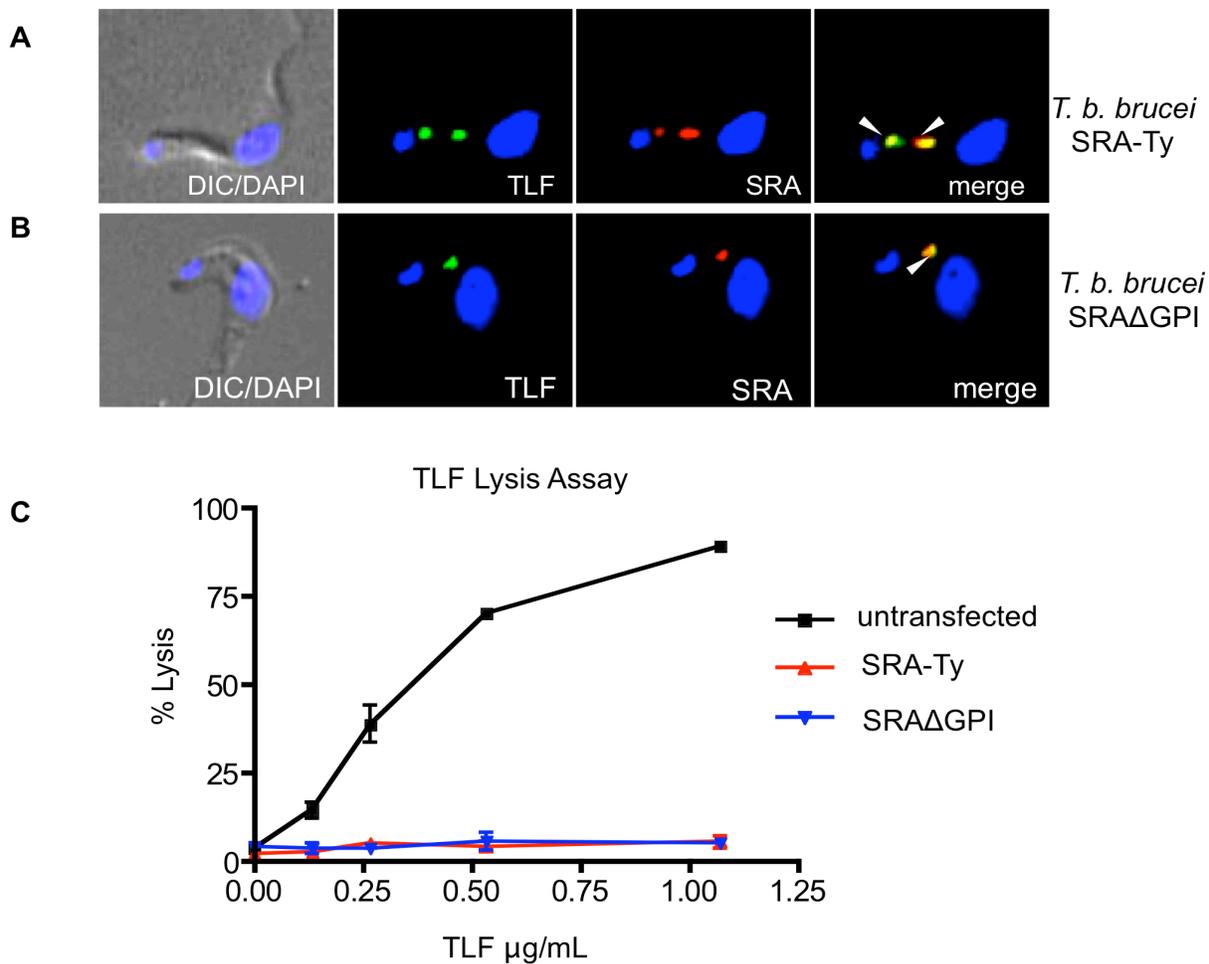


Figure 3.5. The critical interaction between TLF and SRA occurs endosomally. Uptake of Alexa 488-TLF (green) at 37°C for 1 minute post-binding in (A) SRA-Ty and (B) SRA Δ GPI cells. SRA is visualized by anti-Ty staining (red). White arrowheads indicate colocalization. (C) Untransfected *T. b. brucei* 221 (black), *T. b. brucei* 221 SRA-Ty (red) and *T. b. brucei* 221 SRA Δ GPI cells (blue) were incubated with increasing concentrations of purified TLF. Percent lysis was determined after 2 hrs at 37°C.

To examine the cellular localization of SRA Δ GPI, we performed 3°C binding studies with Alexa 594 anti-Ty and found that 100% of SRA Δ GPI cells observed had a marked absence of fluorescence in the flagellar pocket region (Figure 3.4D) in contrast to cells expressing wild type SRA-Ty (Figure 3.4C). To determine whether SRA Δ GPI was being secreted into the culture media, we conducted anti-Ty immunoprecipitation on media but were unable to detect SRA Δ GPI despite being able to immunoprecipitate SRA Δ GPI protein from cell lysates (Data not shown). Despite the loss of cell surface localization, intracellular localization of SRA Δ GPI showed a similar distribution of SRA-containing vesicles as was observed with wild type SRA-Ty (Figures 3.4E, 3.4F). Consistent with this finding, TLF-1 was found to colocalize with SRA within endosomes in both wild type SRA (Figure 3.5A) and in deletion mutant cells (Figure 3.5B), respectively. Furthermore, upon *in vitro* incubation with TLF-1, untransfected cells were highly sensitive to TLF-1-killing, while transfectants expressing wild type SRA and SRA Δ GPI were equally resistant to TLF-1 toxicity (Figure 3.5C).

TLF does not accumulate within the lysosome of SRA-expressing cells in the absence of protease inhibition.

The endosomal localization of SRA that we observe differs from reports by others that SRA is a lysosomal resident protein [3] (Figure 3.6A). To address the discrepancy in lysosomal localization of SRA, we treated *T. b. brucei* 427-221 SRA-Ty cells with the lysosomal protease inhibitor, FMK024, in order to inhibit protein turnover [22]. Under these conditions, we were able to observe an accumulation of SRA in the lysosome that was specifically dependent on inhibition of protein degradation by this thiol protease inhibitor

(Figures 3.6B, 3.6C). This lysosomal accumulation was more complete in some cases, with only lysosomal SRA detected in $35.8 \pm 16.4\%$ of cells (Figure 3.6C, Table 3.2), while in others, both endosomal and lysosomal SRA was detected by anti-Ty staining (Figure 3.6B, Table 3.2) in $44.6 \pm 8.7\%$ of cells. This lysosomal accumulation was also observed by anti-Ty western blot of whole cell SRA-Ty lysates, with an increase (1.4-fold) in SRA in FMK-treated cells compared to untreated cells (Figure 3.6D). Thus, while SRA traffics to the trypanosome lysosome, abundance is limited due to rapid degradation, suggesting that the major intracellular pool of SRA is endosomal.

In a previous study, we reported that TLF-1 was not observed in the lysosome of SRA-expressing cells, thus SRA was thought to alter the trafficking pathway of TLF-1 [13]. Here we have found that while TLF-1 accumulates in the lysosome of untreated susceptible *T. b. brucei* 427-221 cells (Figure 3.6E), a similar accumulation is not observed in SRA-expressing transfectants (Figure 3.6F) unless protein turnover is inhibited with FMK024 treatment (Figure 3.6G). In *T. b. brucei* 427-221 cells, $81.5 \pm 3.7\%$ of cells showed TLF-1 localized only to the lysosome, while $74.2 \pm 2.5\%$ of SRA-expressing cells showed a similar distribution of TLF-1 upon treatment with FMK024 (Table 3.3). The protease inhibitor-dependent accumulation of TLF-1 in the lysosome of SRA expressing cells indicates that TLF-1 may be more rapidly turned over in the presence of SRA, or that the rate of trafficking from endosome to lysosome may be slowed in the presence of SRA. These results suggest that the mechanism of SRA-mediated resistance to TLF-1 may therefore be due to accelerated lysosomal proteolysis of the TLF-1/SRA complex or slowed trafficking between endocytic compartments.

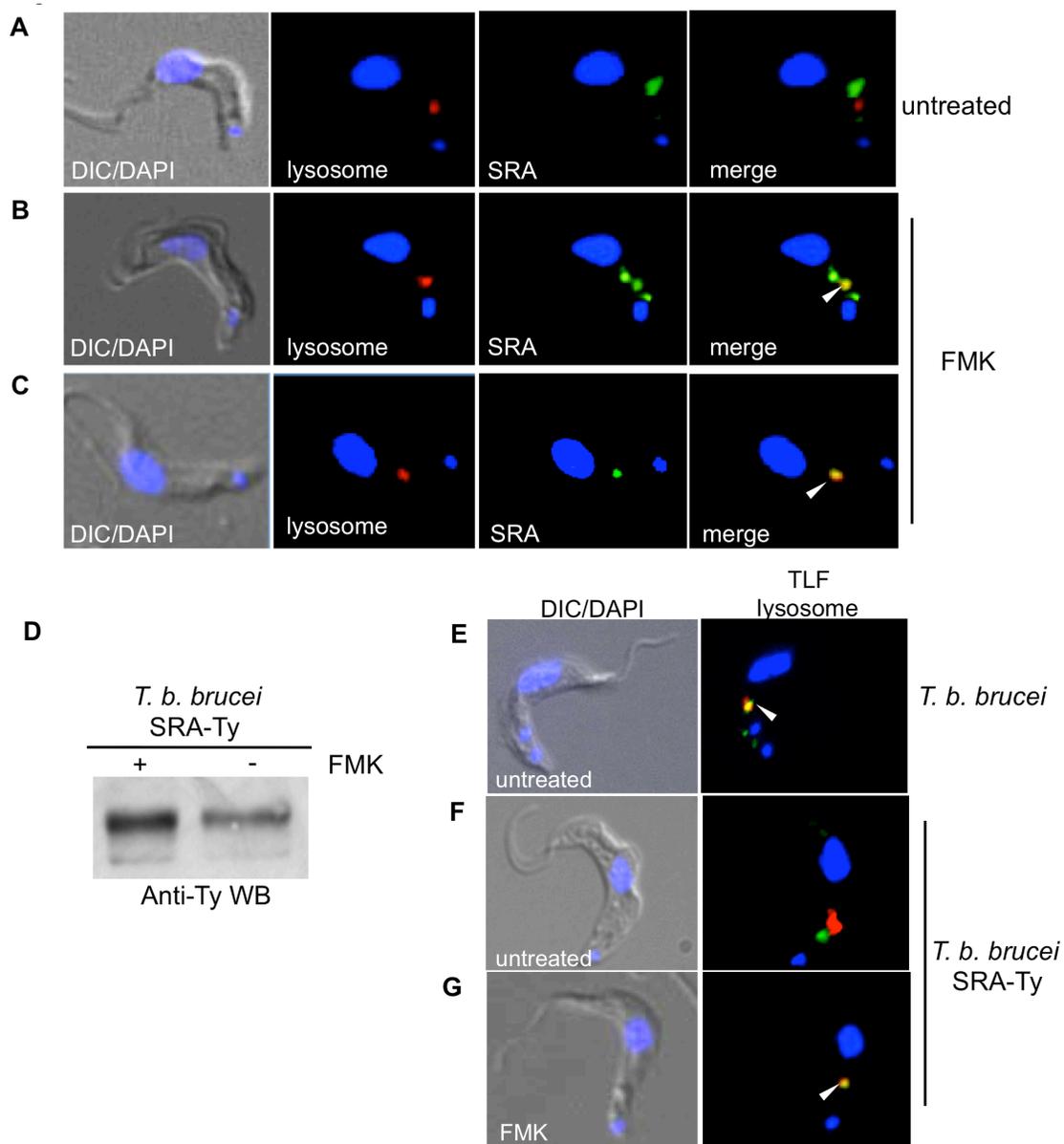


Figure 3.6. SRA is rapidly turned over by lysosomal proteolysis. Immunofluorescence staining of fixed, permeabilized *T. b. brucei* 221 SRA-Ty transfectants. Cells were (A) untreated or (B, C) FMK024-treated prior to fixation ($20\mu\text{M}$, 37°C , 1hr). Anti-Ty (SRA) (green) and anti-*TbCatL* (red) staining show SRA and lysosomal localization, respectively.

(A-C) Image acquisition was carried out at the same exposure and images were contrasted to the same extent. (D) Anti-Ty (SRA) western blot of untreated and FMK024-treated *T. b. brucei* 221 SRA-Ty transfectants showing accumulation of SRA due to protease inhibition. (E-G) Uptake of Alexa 488-TLF (green) in (E) *T. b. brucei* 221 and (F, G) *T. b. brucei* 221 SRA-Ty cells. Cells were either (E, F) untreated or (G) treated with FMK024 (20 μ M, 37°C, 1hr) during TLF-1 endocytosis. Anti-*TbCatL* (lysosomal) staining is shown in red. The nucleus and kinetoplast are localized by DAPI staining. White arrowheads indicate colocalization.

DISCUSSION

Human infectious *T. b. rhodesiense* are able to resist TLF-1 killing through the expression of the VSG-like protein, SRA. The molecular basis of resistance remains unclear. However, it is evident that the intracellular colocalization of SRA and TLF-1 is required for neutralization of the trypanolytic activity [3]. In an effort to gain insight into the mechanism of human serum resistance, we carried out a thorough characterization of SRA by examination of its cellular trafficking and steady-state localization. In this study, we have for the first time defined the steady state localization of SRA and its point of initial colocalization with TLF-1. Furthermore, we report that in the absence of protease inhibition, TLF-1 accumulates in the lysosomes of *T. b. brucei* cells, but not in SRA-expressing transfectants. This finding suggests a difference in the stability of TLF-1 or a difference in the rate of trafficking of TLF-1 from endosomes to lysosome, in resistant SRA-expressing cells compared to sensitive *T. b. brucei* cells. Thus we propose that accelerated

turnover of TLF-1 or altered kinetics of trafficking is involved in the mechanism of SRA-mediated resistance.

Bioinformatic analysis of the amino acid sequence of SRA has reported the presence of post-translational features such as an N-terminal signal sequence, N-linked glycosylation sites and a GPI anchor attachment site [14] that are characteristic of cell surface-localized VSG family proteins, such as VSG and the trypanosome transferrin receptor [16, 30, 31]. VSGs exist as densely-packed homodimers that coat the entire plasma membrane, while transferrin receptor is more discretely localized to the flagellar pocket [32]. Given the shared features of the VSG family proteins, it was therefore surprising that SRA exhibited a primarily intracellular localization.

Binding studies with live cells at 3°C and immunofluorescence microscopy of fixed, non-permeabilized cells, showed that a portion of steady-state SRA localized to the cell surface (Figure 1). The cell surface distribution of SRA was observed solely in the flagellar pocket region of the cell, delineated by several previously characterized markers. Both the kinetoplast and basal bodies are directly associated with the pocket, while the paraflagellar rod extends along the flagellum of the trypanosome to the point of entry of the flagellum into the pocket [33, 34]. The region between the kinetoplast and the posterior end of the paraflagellar rod therefore includes the flagellar pocket. In addition to these cellular markers, we also utilized binding of fluorescently labeled transferrin as a flagellar pocket marker. Immunostaining of SRA in fixed, non-permeabilized cells revealed that SRA colocalized with receptor-bound transferrin within the flagellar pocket region, thus we show conclusively that SRA traffics to the flagellar pocket. Prior to these studies, the

apparent lack of SRA in the flagellar pocket is most likely due to the difficulty of visualizing SRA as a consequence of the remarkably high rate of endocytosis that is a hallmark of bloodstream form *T. brucei* cell biology [17, 35]. Flagellar pocket localization of SRA is therefore transient as the protein is likely to be rapidly endocytosed almost immediately upon transport to the cell surface.

Since SRA was found to traffic to the flagellar pocket, which is also the site of receptor-mediated endocytosis of TLF-1, we examined the possibility that the initial point of colocalization of SRA and TLF-1 would take place in this specialized region of the plasma membrane. We expressed SRA in a cell line that does not express *TbHpHbR* in order to determine whether SRA colocalizes with TLF-1 at the cell surface. Although SRA localized to the flagellar pocket, in the absence of the TLF-1 receptor we did not detect binding or uptake of Alexa488 TLF, indicating that SRA is not sufficient for binding or uptake of TLF-1. Based on proposed models of VSG and SRA protein structure[14], SRA appears to be a shorter molecule and may therefore be inaccessible to TLF-1 on the trypanosome cell surface. This unavailability of cell surface SRA may be due to steric hindrance by the larger, more abundant VSGs that are continually endocytosed via the flagellar pocket.

In a previous study, we reported the steady-state distribution of SRA within cytoplasmic vesicles localized between the kinetoplast and nucleus [13]. Characterization of trafficking vesicles in bloodstream form *T. brucei* has defined several classes of endosomes by their associated Rab GTPases and cargo, including the early endosomal Rab5a GTPase [18, 26, 27, 36-39]. Taking advantage of these classifications, we generated a cell line that expressed an epitope-tagged Rab5a as an early endosomal marker.

Immunostaining and fluorescence microscopy revealed that SRA colocalized with the early endosomal marker (Figure 3.3). Given that the other known VSG family proteins, VSG and TfR, are resident cell surface proteins, SRA therefore has an atypical localization and is the first example of a resident early endosomal VSG family protein in *T. brucei*. This localization raises important questions about trafficking and retention of GPI-anchored proteins. While both VSG and TfR traffic as homodimers and heterodimers respectively, no binding partner has been identified for SRA to date. Campillo and Carrington (2003) have proposed models that indicated SRA is likely to exist as a dimer. SRA may therefore be retained in the endosomal compartment through protein-protein interactions within these trafficking vesicles. In addition, studies by Wang and Cross (2003) have shown that unlike VSG and TfR, SRA is not released but remains cell-associated upon GPI-PLC activation, which suggests that the GPI-anchor of SRA may not be susceptible to GPI-PLC activity and may differ from that of VSG and TfR in a way that leads to the retention of the protein in the endosomes.

When Alexa 488-TLF was incubated with live cells, it localized to SRA-containing Rab5a endosomes. Given that a previous report showed that SRA binds to ApoL-I [3], it is likely that SRA interacts with TLF-1 via ApoL-I binding within this compartment. Thus, the initial point of interaction between TLF-1 and SRA may occur early on in the trafficking pathway, rather than later in the terminal lysosomal compartment. The shared trafficking pathway via the early endosomes was also observed for Alexa 594-transferrin, an independent cargo, unrelated to TLF-1 susceptibility. This overlap of independent cargo is not surprising as both transferrin and TLF-1 are endocytosed by GPI-anchored receptors that localize to the flagellar pocket. The transient flagellar pocket localization and the

steady state, intracellular accumulation of SRA within endosomes may be accounted for by the high rates of endocytosis of proteins associated with the flagellar pocket [17].

In order to determine whether altering of SRA trafficking would have an effect on trypanosome susceptibility to lysis, we generated a deletion mutant of SRA that lacks the GPI anchor modification (Figure 4). The loss of the GPI anchor resulted in the disruption of the flagellar pocket localization of SRA and the protein was no longer detectable in the flagellar pocket. Despite the loss of cell surface localization, the endosomal localization of SRA Δ GPI was unaltered. We were also unable to detect SRA Δ GPI in cell growth media suggesting that the GPI deletion mutant is not secreted. These findings are consistent with studies by Triggs and Bangs (2003), as well as by Bohme and Cross (2002). Neither set of investigators detected secretion of GPI-minus VSG, but instead found delayed ER exit to be a consequence of GPI anchor deletion. Some fraction of SRA Δ GPI may therefore be ER localized, however SRA Δ GPI also colocalized with endocytosed TLF-1 indicating that at least some SRA Δ GPI localizes to the endosomal compartment. This endosomal localization may therefore be the result of direct targeting by an alternative sorting pathway to the endosomes. Cell surface localization of SRA did not appear to be critical to TLF-1 resistance as SRA Δ GPI mutants were resistant to TLF-1-mediated lysis. These data were consistent with the finding that SRA does not facilitate binding or uptake of TLF-1, but mediates human serum resistance via an interaction, which begins with endosomal colocalization.

Contrary to previous reports [3], we were not able to localize SRA to the lysosome in the absence of protease inhibitors. However, in cells treated with the thiol protease inhibitor, FMK024, we were able to detect an increase in SRA by western blot, as well as

observe SRA staining that colocalized with the lysosomal marker. We therefore attribute this localization to accumulation of the protein due to inhibition of the major trypanosome lysosomal protease, *T. b. brucei* Cathepsin L [23]. Since we could only observe SRA in the lysosomal compartment when protein turnover was disrupted, SRA appears to be highly unstable in the lysosomal environment and is therefore likely to be rapidly degraded once it reaches the protease-rich organelle. This susceptibility to rapid lysosomal degradation is consistent with findings on other VSG family proteins—TfR and VSGs [19, 21, 28, 40].

In a previous study, we reported that TLF-1 was not observed in the lysosomal compartment of SRA-expressing cells and SRA was thought to alter the trafficking pathway of TLF-1 [13]. The localization of SRA and TLF-1 to the lysosome in FMK024-treated cells clearly shows that altered trafficking to the lysosome in SRA expressing cells is not the mechanism of resistance. Rather, the apparent loss of lysosomal accumulation of TLF-1 in SRA-expressing cells indicates that the mechanism of resistance may instead be due to accelerated turnover of TLF-1 in the presence of SRA. Alternatively, the rate of TLF-1 trafficking from the endosomes to the lysosome may be slower in the presence of SRA, therefore TLF-1 accumulation within the lysosome is not observed in the absence of protease inhibition. In the absence of SRA however, rapid trafficking of TLF-1 into the lysosomal compartment allows for accumulation and subsequent detection. Thus, SRA-mediated resistance may also involve differential rates of trafficking between endosomes and the terminal lysosomal compartment.

The studies reported here allow definitive description of the complete trafficking pathway of SRA from synthesis to degradation. Upon translation of the N-terminal signal

sequence, SRA is targeted to the ER where it is post-translationally modified [3, 14]. The mature protein then moves through the Golgi for additional processing of N-linked glycans [3] and is then targeted to the flagellar pocket. SRA is only transiently localized at the cell surface before it is rapidly endocytosed into early endosomes, where it initially encounters TLF-1. The interaction between TLF-1 and SRA may occur within the endosomal compartment, or TLF-1 and SRA may traffic independently to the lysosome before binding and subsequently degradation takes place. Neutralization of the trypanosome toxin may be a consequence of accelerated degradation or slowed trafficking from endosomes to lysosomes. Furthermore, the identification of the lysosomal cysteine protease, *Tb*Cathepsin L, as at least one class of proteases involved in turnover, implicates the lysosome as the final site of detoxification of TLF-1. While the molecular changes that lead to SRA-mediated inhibition of TLF-1 remain to be elucidated, the findings presented here have brought us closer to resolving the mechanism of inhibition of TLF-1 and human infectivity by *T. b. rhodesiense*.

MATERIALS AND METHODS

Cell culture

Bloodstream form *T. b. brucei* cultures were grown in HMI-9 medium (FBS, Gemini Bio-products, West Sacramento, CA); Serum Plus (SAFC Biosciences, Lenexa, KS).

Construction of reporter genes

All SRA gene constructs were cloned into the pURAN trypanosome expression vector as previously described [13]. The SRA-Ty construct was transfected into both *T. b. brucei* 427-221 cells and *T. b. brucei* 060^R cells, to generate *T. b. brucei* 427-221 SRA-Ty transfectants and *T. b. brucei* 060^R SRA-Ty transfectants, respectively. The *T. b. brucei* 427-221 SRA-Ty cell line was previously generated and characterized [13]. *T. b. brucei* 060^R cells were derived from a TLF-1-resistant cell line, lacking the *T. brucei* haptoglobin/hemoglobin receptor (*TbHpHbR*). *T. b. brucei* 221 SRAΔGPI cells were generated by transfection into the *T. b. brucei* 221 cells. The construct is shown schematically in Figure 4A. The sequence was generated by PCR amplification of the full length SRA-Ty coding sequence using the following primers—(5' *SRAΔGPI* CCTGCAGG **ATG** CCC CGA AAT TCG GGC CGG; 3' *SRAΔGPI* GGCGCGCC **TTA** TTT GGA TTC TTT TCC TTC CC). PCR amplified products were ligated into the TA cloning vector (Invitrogen, Eugene, OR). The SRAΔGPI insert was digested from the cloning vector, using the EcoRI restriction endonuclease. The gel-purified insert was then ligated into the pURAN expression vector, which was linearized by BsiWI restriction digest prior to transfection. To generate an epitope-tagged *T. brucei* Rab5a construct, trypanosome genomic DNA was amplified using the following primers—(5' *Rab5aHA* CCTGCAGG **ATG** *TAC CCA TAC GAC GTC CCA GAC TAC GTC* CCA GAC TAC GCT ATG TCG GTG TCA GCG ACA CCA; 3' *Rab5aHA* GGCGCGCC **TTA** TCA GCA GGC ACA CCC GCC TTC). The full length coding sequence of *TbRab5a* (accession no. U24678) was engineered with the HA epitope (Y P Y D V P D Y A) at the N-terminus. Primers were synthesized with 5' SbfI and 3' AscI overhangs for directional cloning into the pTub-phleo expression vector [41]. Restriction sites are shown underlined, with start and termination codons shown in boldface, and the epitope tag sequence italicized.

Transfections. Transfections were performed using the Lonza nucleofection system according to the manufacturer's instructions. (<http://www.lonzabio.com/>). 1×10^7 cells were resuspended in 100 μ L of the Lonza nucleofection solution containing 10 μ g of linearized plasmid DNA. The cell suspension was subjected to nucleofection using the pre-programmed setting X-001. Transfected cells were then placed in HMI-9 media for 24 hours before selection of clonal cultures (2.5 μ g/mL—G418, SRA constructs; 3 μ g/mL—phleomycin, Rab5a construct). The solutions provided with the Lonza nucleofection kit are of unknown composition.

Immunoblotting

Western blotting. Total cell lysates for western blotting were obtained and analyzed as previously described [13]. The anti-Ty antibody was directly conjugated to horseradish peroxidase (HRP) using Pierce EZ-link activated peroxidase (Thermo Fisher Scientific, Rockford, IL) and used at a dilution of 1:5000. Rat monoclonal Anti-HA-biotin (Roche Diagnostics, Indianapolis, IN) was used at a dilution of 1:1000, with Streptavidin HRP conjugate (Invitrogen, Camarillo, CA) used for secondary detection at 1:5000.

Immunofluorescence assays

All cells were fixed in 1% paraformaldehyde (PFA) on ice for 15 minutes followed by a brief methanol treatment at -20°C , unless otherwise stated. For non-permeabilizing conditions, cells were fixed in 1% PFA in HMI-9 medium, pH 7.5 for 15 minutes on ice. Fixed cells were washed once in PBS, then resuspended in PBS, 10% FBS for 1 hr. The following antibody dilutions were used—mouse anti-Ty 1:1000; rabbit anti-*TbCatL* 1: 4000; mouse anti-parafagellar rod (PFR) 1:1000, rat anti-HA-biotin 1:100. Primary antibody staining was

carried out for 45 minutes. For secondary antibody staining, slides were incubated with the appropriate Alexa Fluor 488- or Alexa Fluor 594 mouse or rabbit immunoglobulin G (Invitrogen, Eugene, OR) for 30 minutes, for all primary antibodies except, anti-HA-biotin. Secondary detection for anti-HA-biotin was carried out using streptavidin, Alexa Fluor 594 conjugate (Invitrogen, Eugene, OR). 4', 6'-Diamidino-2-phenylindole (DAPI) was added after final washes. Serial image z-stacks were acquired through oil immersion optics at 63x magnification, with exposure times kept constant for each experiment. Imaging was carried out using a Zeiss Axio Observer inverted microscope equipped with an AxioCam HSm camera and analyzed with the AxioVision v4.6 software (<http://www.zeiss.com>) A single stack is shown for each experiment with individual channels contrasted to the same extent for each image set and merged using Adobe Photoshop CS2 v9.0. Polyclonal anti-*TbCatL* was kindly provided by Dr. Jay Bangs, University of Wisconsin, Madison. The anti-paraflagellar rod antibody was generously provided by Dr. Diane McMahon-Pratt, New Haven.

Binding and uptake assays

Binding assays. Cells were collected and resuspended in chilled HMI-9 medium, 10µg/ml hemoglobin at 3×10^7 cells/ml and allowed to equilibrate to a 3°C waterbath for binding assays. Alexa fluor-conjugated ligands were added to cell suspensions and incubated for 15 minutes at 3°C then washed 3 times in ice-cold PBS-G. Cells were fixed by resuspension in 1% PFA for 15 minutes on ice. Cells were then processed for immunofluorescence as previously described. Alexa fluor-conjugated ligands were labeled according to the manufacturer's directions (Invitrogen, Eugene, OR) and used at the following

concentrations—Alexa 594 anti-Ty - 0.2 $\mu\text{g}/\text{mL}$; Alexa 594 transferrin - 50 $\mu\text{g}/\text{ml}$; Alexa 488 TLF - 5 $\mu\text{g}/\text{ml}$.

Uptake assays. Cells were collected and resuspended in HMI-9 media, 10 $\mu\text{g}/\text{ml}$ hemoglobin at 3×10^7 cells/ml. Cells were incubated with Alexa 488 TLF at 37°C for 30 minutes then washed 3 times in ice cold PBS-G, fixed and analyzed by microscopy as previously described. Cells were also analyzed by flow cytometry using a Beckman Coulter CyAn ADP flow cytometer (<http://www.coulterflow.com>) and FlowJo v7.5 software (<http://www.flowjo.com>).

Binding-chase assays. Binding was carried out as previously described. After washing, cells were resuspended in warm HMI-9 media then placed in a 37°C water-bath for 1 minute. 2% PFA was added to cell suspensions for a final concentration of 1% PFA, in order to fix cells and halt uptake at 1 minute. Cells were processed for immunostaining as previously described.

Protease Inhibition Assays

Cells were treated with 20 μM FMK024 or left untreated for 1 hour at 37°C. For immunofluorescence assays, cells were then fixed with 1% PFA and prepared for immunostaining as previously described. For western blotting, protease inhibition was halted by cell lysis through addition of an equal volume of 10% trichloroacetic acid (TCA) for TCA precipitation. Lysates were incubated overnight at 4°C, precipitated by addition of isopropanol, then centrifugation at 13000 rpm for 15 minutes. Pellets were washed 3 times with cold (-20°C) acetone. Protein pellets were then dissolved in PBS and protein

concentrations determined by Bradford assay. Samples were prepared for western blotting as previously described. 15 μ g of protein was analyzed for each condition.

TLF-1 purification and lysis assays

Human blood was obtained from healthy, fasted donors and plasma was separated by low-speed centrifugation (3500 rpm, 10 min), and subsequent high-speed centrifugation (10,000 rpm, 5min) of the supernatant to pellet residual red blood cells. HDLs were purified as previously described [2]. After collection of the HDL fraction of plasma (1.26 g/ml), trypanolytic HDLs were isolated from this fraction by affinity purification using monoclonal antibodies against Hpr. Antibodies were coupled to Pierce Affigel resin based on the manufacturer's recommendations (Thermo Fisher Scientific, Rockford, IL). Eluates were dialyzed against phosphate-buffered saline, 75 μ M EDTA (PBSE) at 4°C and stored in single-use aliquots at -80°C. Susceptibility to TLF-1 was determined using 2-hr lysis assays as previously described [2].

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ABBREVIATIONS

GPI, glycosyl phosphatidylinositol; TLF-1, trypanosome lytic factor; HDL, high-density lipoprotein; apo, apolipoprotein; Hb, hemoglobin; Hp, haptoglobin; Hpr, haptoglobin related protein; SRA, serum resistance associated gene; ApoL-1, Apolipoprotein L-1; ApoA-1, Apolipoprotein A-1; VSG, variant surface glycoprotein

SUPPLEMENTAL FIGURES

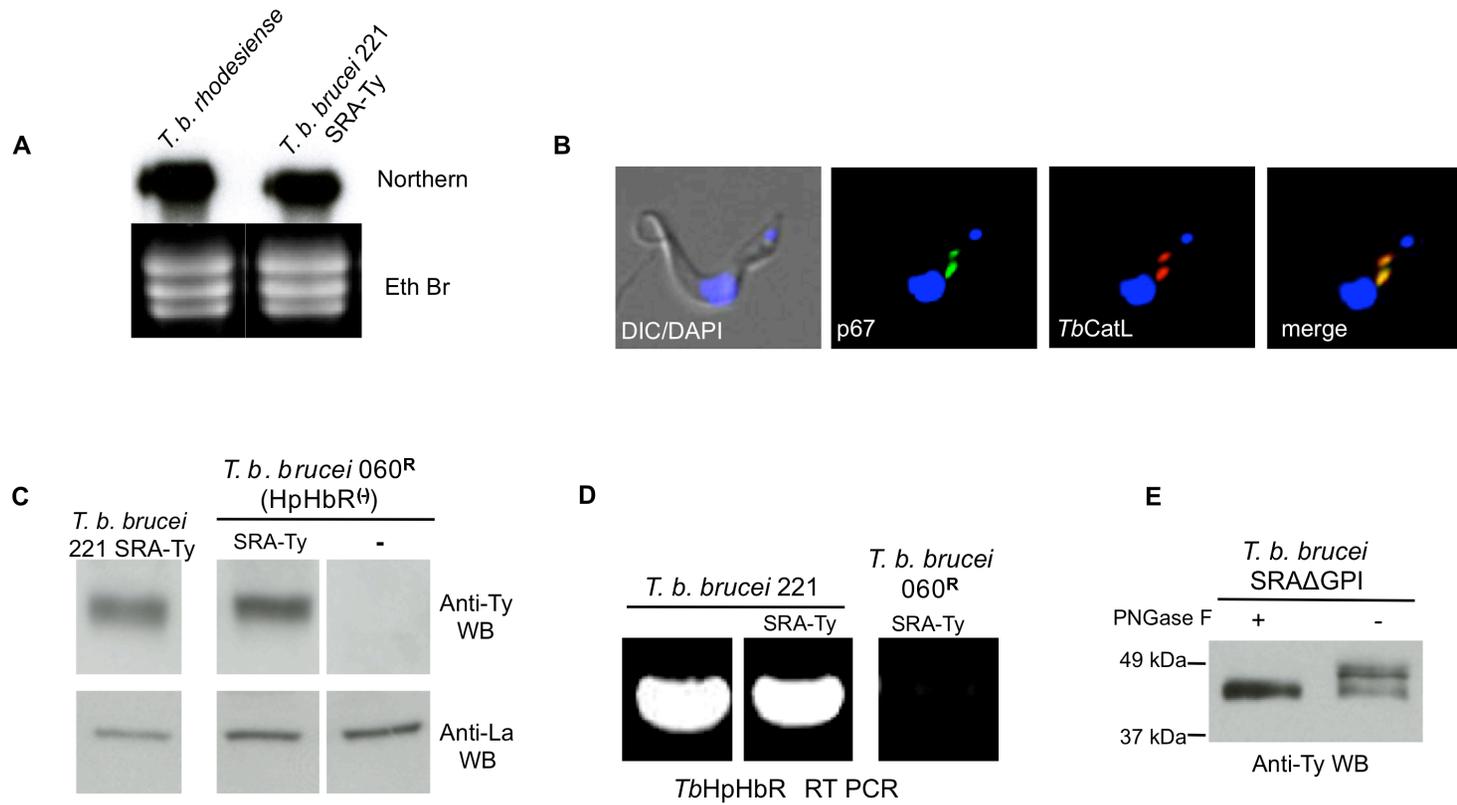


Figure S3.1. mRNA transcript levels of SRA in *T. b. brucei* SRA-Ty cell lines are comparable to transcript levels in *T. b. rhodesiense*. (A) Northern blot and ethidium bromide staining showing SRA mRNA transcript levels in *T. b. rhodesiense* and *T. b. brucei* SRA-Ty transfectants. (B) Anti-p67 and anti-*TbCatL* are both markers for the trypanosome lysosome. Anti-p67 (green) and anti-*TbCatL* (red) staining colocalized in fixed, permeabilized *T. b. brucei* 221 cells. The nucleus and kinetoplast are stained with DAPI (blue). (C) Anti-Ty and anti-La western blots (WB) of total cell lysates from untransfected and SRA-Ty-transfected cell lines. (D) RT PCR analysis showing *TbHpHbR* mRNA transcript levels in untransfected *T. b. brucei* 221 and *T. b. brucei* 221 SRA-Ty transfectants. *TbHpHbR* transcript levels in TLF-resistant *T. b. brucei* 060^R and *T. b. brucei* 060^R SRA-Ty cell lines were undetected. (E) Anti-Ty western blot (WB) of untreated and PNGase F-treated SRAΔGPI cell lysates.

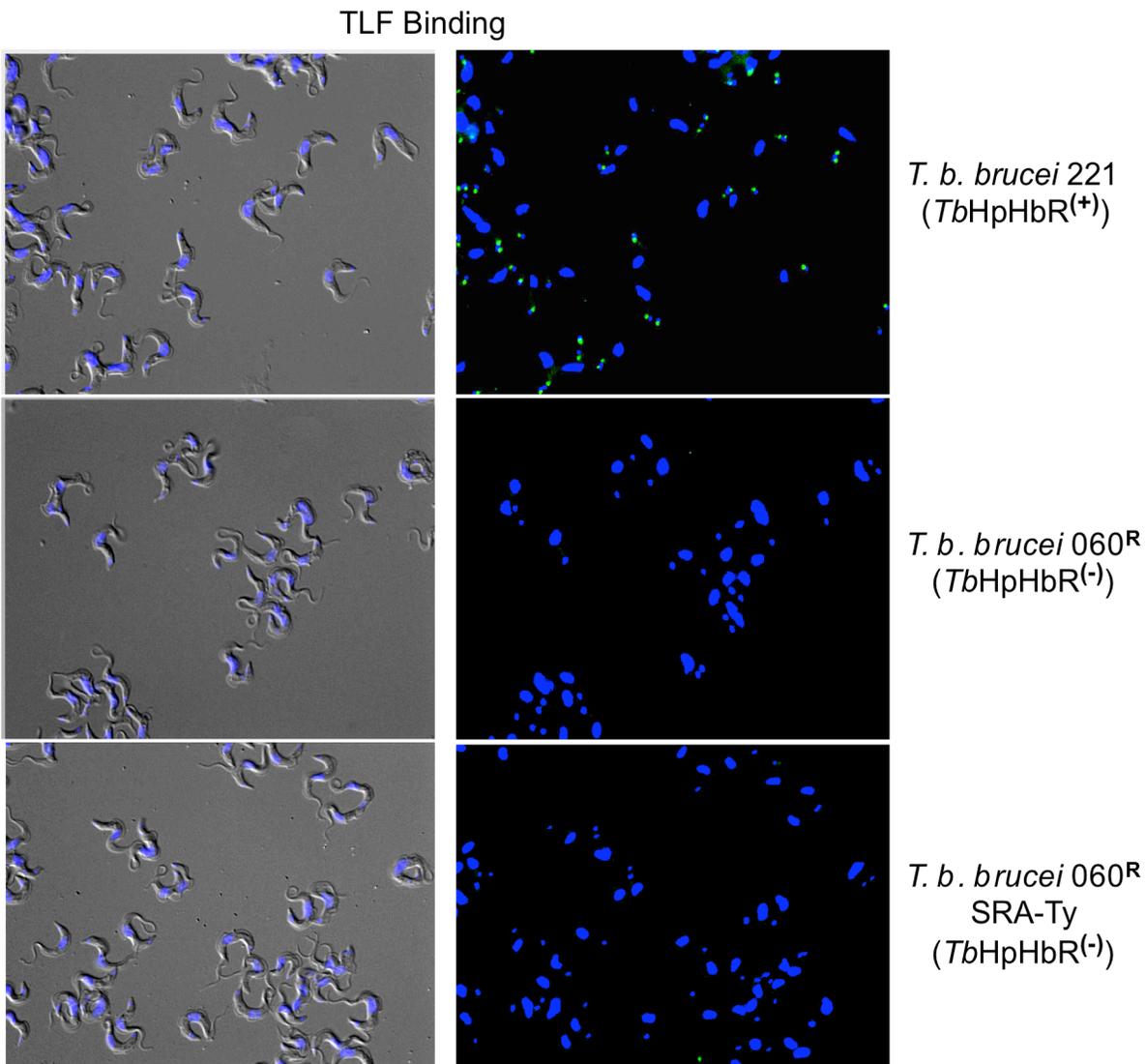


Figure S3.2. Field view of *T. brucei* cells with bound Alexa 488-TLF within the flagellar pocket. TLF-1 binding is detected in *T. b. brucei* 221 cells, but not in *T. b. brucei* 060^R or *T. b. brucei* 060^R SRA-Ty cells.

SUPPLEMENTAL TABLES

Table 3.1. Alexa 594-Transferrin Labeling of Trypanosomes at 3°C

Distribution of Labeled Cells	Result (%)
Labeled*	91.1 ± 4.7
Unlabeled*	8.9 ± 4.6
Flagellar Pocket [§]	93.8 ± 5.3
Internal [§]	6.2 ± 5.3

Quantitation of Alexa 594-transferrin labeling of cells at 3°C. Results are presented as the mean of 3 binding assays. Mean ± SEM. (n=3). Total number of cells = 100. *Percentage of total cells. [§]Percentage of transferrin-labeled cells.

Table 3.2. Distribution of SRA cellular localization

Untreated	Percentage	FMK-Treated	Percentage
FP/Endosomal	37.9 ± 9.6	Endosomal only	19.6 ± 8.0
Endosomal	100	Lysosomal only	35.8 ± 16.4
Lysosomal	-	Endosomal/Lysosomal	44.6 ± 8.7

Quantitation of cellular distribution of untreated and FMK-treated SRA-expressing cells. Percentages for untreated cells are presented as Mean ± SEM (n=4). Flagellar Pocket (FP). Percentages of FMK-treated cells are presented as Mean ± SEM (n=3). Total number of untreated cells = 102; Total number of FMK-treated cells = 115.

Table 3.3. Distribution of TLF-1 cellular localization in FMK-treated cells

<i>T.b.brucei</i> (untransfected)	Percentage	<i>T.b.brucei</i> SRA-Ty	Percentage
Endosomal	4.1 \pm 0.5	Endosomal	3.2 \pm 0.8
Lysosomal	81.5 \pm 3.7	Lysosomal	74.2 \pm 2.5
Endosomal/Lysosomal	14.4 \pm 5.4	Endosomal/Lysosomal	22.6 \pm 3.1

Quantitation of cellular distribution of TLF-1 in FMK-treated untransfected *T. b. brucei* cells and *T. b. brucei* SRA-Ty cells. Percentages are presented as Mean \pm SEM (n=3). Total number of *T. b. brucei* cells = 303. Total number of *T. b. brucei* SRA-Ty cells = 108.

CHAPTER 4

THE POST-TRANSLATIONAL MODIFICATIONS OF THE SERUM RESISTANCE
ASSOCIATED PROTEIN¹

¹ **Stephens, N.A.**, Lim, J. M., Wells, L., Hajduk, S.L. 2011.

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ABSTRACT

The Serum Resistance Associated protein (SRA) is a member of the variant surface glycoprotein (VSG) gene family of *Trypanosoma brucei*. This VSG-like protein confers resistance to an innate immune trypanocidal component of human serum and facilitates the infectivity of *T. b. rhodesiense*, a human infectious subspecies of *T. b. brucei*. SRA has been recently shown to be a resident endosomal protein, while all other VSG family proteins have been shown to localize to the trypanosome plasma membrane. Despite the difference in localization, SRA is predicted to be post-translationally modified in the same manner as other VSG family members, by the addition of N-linked glycans and a glycosyl phosphatidylinositol (GPI) anchor attachment. Although analysis of the amino acid sequence of SRA indicates that the protein has post-translational modifications that are associated with cell surface localization, the presence of the GPI anchor modification was previously unconfirmed. Here, we show that the resident endosomal protein, SRA, is indeed a glycosylated, GPI-anchored protein.

INTRODUCTION

The trafficking and sorting of glycosyl phosphatidylinositol (GPI)-anchored proteins to the cell surface is a unifying characteristic of these lipid-modified proteins across all eukaryotes ranging from the unicellular protozoan *Trypanosoma brucei brucei* to higher eukaryotes [1, 2]. While these proteins traffic transiently through intracellular compartments, it is with rare exception, that a GPI-anchored protein does not localize as a steady-state cell surface protein. To our knowledge, the only known exception is the Golgi-resident protein, GREG, which is involved in maintenance of Golgi structure [3]. *T. brucei* has been used as a model organism in the study of GPI-anchor structure and biosynthesis, largely due to the abundance of the variant surface glycoprotein (VSG). VSGs represent ~10% of the total cellular protein in bloodstream form African trypanosomes, thus, allowing for extensive characterization of the modification, trafficking and localization of this GPI-anchored protein family [2].

Structural analysis of VSG, GPI anchors led to the resolution of the first complete GPI anchor structure [4-6]. Since then, it has been shown that the core structure of GPI anchors is highly conserved across a wide range of eukaryotic species [7, 8]. This core structure consists of a phosphoethanolamine moiety linked on one end to the C-terminus of a glycoprotein and on the other end to a branched glycan attached to an inositol phospholipid [4-6]. The heterogeneity in GPI anchor structures stem mainly from the variation of the glycan groups and the acyl chains linked to the phosphoinositol. The amino acid sequences of proteins that are designated for this mode of lipid modification contain an N-terminal signal sequence, a C-terminal hydrophobic peptide and an anchor attachment residue—three key features that target the protein for the GPI lipid

modification. The N-terminal signal sequence targets the protein to the endoplasmic reticulum (ER), where it is translated and translocated into the ER lumen [9]. The C-terminal hydrophobic peptide is cleaved off during translocation and the GPI anchor is transferred *en bloc* in a transamidation reaction [10, 11] to the amino acid attachment site called the ω residue [12].

The amino acid sequence of SRA was found to have an N-terminal signal sequence (residues 1- 30), a C-terminal hydrophobic peptide (residues 389 - 410) and a GPI anchor attachment site (D388)—all the requisite features of a GPI-anchored protein [13]. Despite the identification of sequence elements that are consistent with GPI-anchoring and cell surface localization, we showed that SRA is a resident early endosomal protein, that traffics only transiently to the cell surface [14]. Given the unusual intracellular localization of SRA, we sought to determine whether this VSG-like protein was in fact modified with the predicted GPI anchor. In this study, we confirm that SRA is a glycosylated, GPI-anchored protein. This is the first report of a GPI-anchored protein that has been shown to be primarily resident within a defined compartment in the endocytic pathway and thus raises important questions concerning mechanisms of GPI-anchor recognition and retention.

RESULTS

Analysis of the amino acid sequence of SRA predicts that the protein is translated with an N-terminal signal sequence (residues 1-30), three N-linked glycans (N86, N321 and N320) and a single GPI-anchor addition site (D388) (Figure 4.1A) [13]. To confirm the presence of the predicted glycans, we used the previously characterized cell line in which

Ty epitope-tagged SRA was expressed (*T. b. brucei* SRA-Ty) (Figure 4.1A) [15]. Total cell lysates from SRA-expressing cells were treated with PNGase F, a glycosidase that catalyzes the cleavage of N-linked glycans. Western blot analysis revealed an approximate 5 kDa shift in migration of deglycosylated SRA, consistent with three predicted glycosylation sites, as a 2 kDa shift is typically associated with the loss of each glycan [16] (Figure 4.1B).

Since SRA is a VSG-like protein, the presence of the GPI-anchor has long been speculated upon, though not confirmed. Differential detergent lysis of cells results in activation or inhibition of an endogenous glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) that catalyzes the cleavage of GPI-anchors from glycopeptides [17]. Cell lysates were prepared by nonionic detergent lysis (1% NP-40) to activate GPI-PLC, as well as by anionic detergent lysis (2% SDS) to inhibit GPI-PLC. Differential detergent extraction allowed us to identify a potentially GPI-anchored membrane form of SRA, as well as a slower-migrating, GPI-minus soluble form of SRA (Figure 4.1C). The shift in migration due to cleavage of the GPI anchor under nonionic detergent lysis conditions was consistent with reports on migration of soluble VSG (GPI minus) [17] and suggested that SRA is GPI-anchored. We observed a doublet with a prominent upper band that exhibited the characteristic change in migration when compared to membrane form SRA (GPI-containing) extracted with SDS. The less intense, lower band of the doublet is likely to be a minor fraction of unconverted membrane form SRA, as it co-migrated with the band detected in SDS cell lysates (Figure 4.1C).

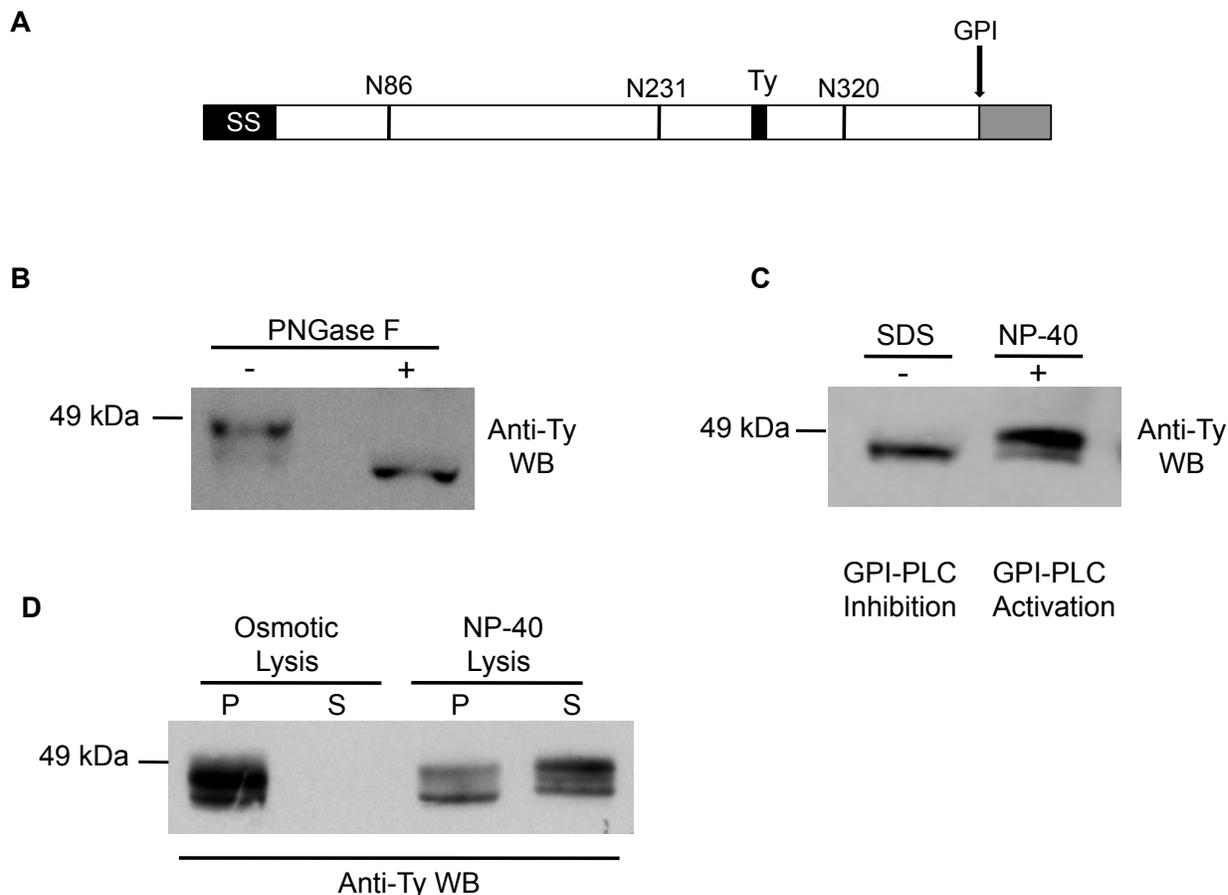


Figure 4.1. SRA is modified by N-linked glycans. (A) Diagram of SRA indicating the predicted positions of the N terminal signal peptide (SS), N-glycosylation sites (N86, N231, N320), GPI-anchor addition site (D388), C terminal peptide (grey) and Ty epitope tag. (B) Cell lysates were left untreated (-) or treated (+) with the glycopeptidase, PNGase F for 1 hr at 25°C. (C) Cells were lysed for 1 hr at 25°C in 1% NP-40 (GPI-PLC activation) or 2% SDS (GPI-PLC inhibition). Western blots (WB) were probed with a monoclonal antibody against the Ty epitope. (D) *T. b. brucei* SRA-Ty cells were lysed osmotically (H₂O), then either solubilized by nonionic detergent treatment (1% NP-40) or left unsolubilized. Cell lysates

were fractionated into pellets (P) and supernatants (S), then analyzed by SDS-PAGE and anti-Ty western blot.

The endogenous GPI-PLC is also activated by osmotic cell lysis as evidenced by the release of GPI-minus VSG [17, 18]. However, a previous study reports detection of a membrane-associated form of SRA in the pellet of osmotically lysed cells [18]. Consistent with these findings, we were also unable to detect the soluble, GPI-minus form of SRA when cells were lysed osmotically (Figure 4.1D). It has been reported that GPI-PLC localizes to the flagellar membrane [19, 20], while SRA, which predominantly localizes to intracellular vesicles may not be accessible to GPI-PLC using this lysis method. In order to release GPI-PLC from the flagellar membrane, we solubilized hypotonically lysed SRA-expressing cells by addition of nonionic detergent (1% NP-40), shown to be permissible for GPI-PLC activity (Figure 4.1C). Upon solubilization of cell lysates, we were able to detect both the membrane-bound and the soluble forms of SRA in the pellet and supernatant fractions respectively (Figure 4.1D). Consistent with the change in migration of SRA due to differential detergent treatment of cell lysates (Figure 4.1C), the soluble form of SRA observed in the supernatant fraction migrated slower than the membrane form of SRA detected in the pellet fraction (Figure 4.1D).

Anti-Ty immunoprecipitation from the supernatant fraction of *T. b. brucei* SRA-Ty cell lysates was performed and the immunoprecipitated material was resolved under non-reducing conditions on a 12% SDS-PAGE Coomassie blue stained gel. To determine whether SRA was present in the anti-Ty precipitated material, Coomassie stained bands were excised and processed for liquid chromatography-mass spectrometric (LC/MS/MS)

analysis (Figure 4.2A). Two unique peptides were identified that correspond to the amino acid sequence of SRA, thereby confirming the identity of the immunoprecipitated material to be SRA (Figure 4.2A, Supporting Table 4.1). Under non-reducing conditions, SRA migrates at ~42 kDa (Figure 4.2A). VSG 221 was also identified and found to correspond to a 47 kDa band (Figure 4.2A, Supporting Table 4.1).

Cleavage of the GPI-anchor by GPI-PLC results in the formation of a distinct moiety called the cross-reacting determinant (CRD), which has been used to verify the GPI-anchor modification [16, 21-24]. Using this known immunoreactivity against the CRD epitope, we confirmed the presence of the GPI anchor of SRA. Anti-Ty immunoprecipitated SRA was also analyzed by western blot and probed with HRP-conjugated anti-Ty or with anti-CRD. Both HRP-conjugated anti-Ty and anti-CRD reacted with a 42 kDa protein in only the SRA-Ty transfected cells, confirming the GPI-anchor modification of SRA (Figure 4.2B). No cross-reactivity was detected with untransfected *T. b. brucei* 221 cell lysates that were also subjected to anti-Ty immunoprecipitation and probed by anti-Ty western blot (Figure 4.2B). Collectively, these findings indicate that SRA is synthesized at the endoplasmic reticulum where it is N-glycosylated, and GPI-anchor modified. These post-translational modifications are consistent with those of VSG family proteins and with forward trafficking to the cell surface.

DISCUSSION

In an effort to gain insight into the unusual endosomal localization of a VSG family protein, we carried out an examination of post-translational modifications of SRA. In this

study, we have for the first time confirmed the predicted modifications of SRA proposed to be involved in trafficking and sorting of the mature protein.

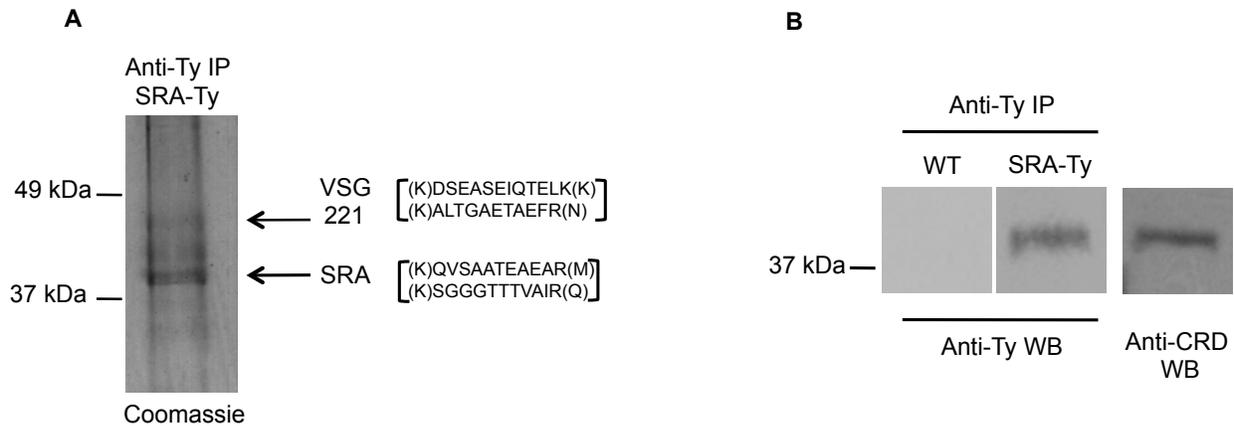


Figure 4.2. SRA is a GPI-anchored protein. Cells were lysed by nonionic detergent treatment (1% NP-40), followed by fractionation into pellet and supernatant. Anti-Ty immunoprecipitation was carried out from the supernatants. (A) Anti-Ty immunoprecipitated (IP) material was run out under non-reducing conditions on a 12% SDS-PAGE gel stained with Coomassie Brilliant blue R-250. LC/MS/MS analysis was carried out on the bands indicated by black arrows. Two representative peptides from analysis of each band are shown in brackets []. (B) Anti-Ty immunoprecipitated material from *T. b. brucei* cells and *T. b. brucei* cells SRA-Ty cells was also analyzed by western blot (WB) and probed with HRP-conjugated anti-Ty and anti-CRD.

Analysis of the amino acid sequence of SRA revealed that the protein is translated with an N-terminal signal sequence, as well as a C-terminal hydrophobic peptide. In addition, three N-glycosylation sites and a GPI anchor attachment site were predicted [13,

25]. Our findings confirm the presence of the predicted N-linked glycans, consistent with an earlier report [26]. Differential detergent extraction of total cellular protein allowed us to identify a GPI-anchored membrane form of SRA and a soluble form produced by endogenous GPI-PLC cleavage. The membrane attachment of SRA via a GPI anchor was supported by detection of the CRD epitope, following GPI-PLC activation (Figure 4.2). These protein modifications and presence of an N-terminal signal sequence are characteristic of the family of major cell surface antigens of *T. brucei*—the VSGs and the trypanosome transferrin receptor [6, 27, 28]. VSGs exist as densely-packed homodimers that coat the entire plasma membrane, while transferrin receptor is more discretely localized to the flagellar pocket [29]. Given the shared features of the VSG family proteins, it was therefore surprising that SRA exhibited a primarily intracellular localization. Findings by our group (Figure 4.1D), as well as by Wang and Cross [18], showed that SRA was not susceptible to GPI-PLC activation by osmotic lysis. Soluble SRA was released only after membrane solubilization by nonionic detergent treatment following osmotic lysis. These findings indicate that SRA may remain membrane-associated even after GPI-PLC cleavage. This may be due to protein-protein interactions or sequestering of soluble SRA within the lumen of endosomal vesicles, which would prevent release of the soluble protein. The data may also suggest that the GPI anchor of SRA differs from that of VSGs, and may also account for the differences in localization of the proteins. The retention of SRA in the early endosomal compartment, rather than being recycled to the cell surface may therefore be an indication of unique GPI structure.

Previous studies in mammalian cell lines have explored the effect of GPI structure on the localization and lateral diffusion of GPI-anchored GFP constructs. GPI anchors that

were chemically synthesized to have variant glycan cores resulted in altered protein mobility and speed of lateral diffusion [30]. This effect on the movement of GPI anchored proteins through membranes based on the structure of the GPI anchor indicate that the structure of the GPI anchor may influence the sorting of the protein within membrane microdomains or lipid rafts. This in turn may influence sorting and packaging into vesicles, as well as targeting to cellular compartments. Another factor that may be involved in the localization of GPI anchored proteins is the ω site, to which the GPI anchor is linked. Research on the localization of GPI-anchored cell wall proteins in *Saccharomyces cerevisiae* revealed that the mutation of amino acids upstream of the ω site led to changes in the class of GPI anchor added, and subsequently to the localization of the modified proteins [31, 32]. These findings suggest that amino acid sequence upstream of the GPI anchor attachment site may therefore influence the class of GPI anchor attached to the protein and subsequently determine its localization.

We previously examined the effect of GPI anchor deletion on SRA localization and function and reported that the presence of the GPI anchor is not required for intracellular localization or SRA resistance [14]. This suggests that the GPI anchor of SRA is not involved in retention of the protein within the endosomal compartment. Based on the requirement for detergent solubilization for SRA release following GPI-PLC activation (Figure 4.1D), it is more likely that the endosomal localization of SRA may be due to protein-protein interactions or other membrane interactions other than that of GPI anchoring.

The studies reported here allow definitive confirmation of the attachment of a previously predicted post-translational modification to the SRA protein. This is also the

first report of a resident endosomal GPI-anchored protein in *T. brucei*. Given the well-established role of GPI anchors in protein sorting and targeting, the unusual localization of SRA may be due to a unique feature of the SRA GPI anchor. Biochemical analysis of potential protein-protein interactions, as well as the structure of this membrane anchor and the features that direct lipid anchoring will provide further insight into protein localization and may also lead to the development of novel tools for manipulation of protein targeting.

MATERIALS AND METHODS

Cell culture and cell lines

Bloodstream form *T. b. brucei* cultures were grown in HMI-9 medium supplemented with 10% fetal bovine serum (FBS, Gemini Bio-products, West Sacramento, CA) and 10% Serum Plus (SAFC Biosciences, Lenexa, KS). The *T. b. brucei* 427-221 SRA-Ty cell line was previously generated and characterized. All experiments were carried out using this cell line.

GPI-PLC Activation Assays

Detergent lysis. Cells were collected and resuspended in 10mM sodium phosphate, pH7.5 containing 1% octylphenoxypolyethoxyethanol (NP-40, Accurate, Westbury, NY) (nonionic; GPI-PLC activation) or 2% sodium lauryl sulfate (SDS, Sigma Aldrich, St. Louis, MO) (ionic; GPI-PLC inhibition). For nonionic detergent lysis, cells were incubated at 25°C for 30

minutes. For ionic detergent lysis, cell lysates were placed at 95°C for 5 minutes. Cell lysates were analyzed by anti-Ty western blot analysis.

Hypotonic lysis. Cells were treated as previously described [17]. For solubilization and release of GPI-PLC from the flagellar membrane, 1% NP-40 was added to the supernatant fraction of cell lysates. Pellet and supernatant fractions were analyzed by anti-Ty western blot.

Immunoprecipitation and western blot analyses

Immunoprecipitation. 5×10^8 cells were washed three times in phosphate-buffered saline containing 1% glucose (PBS-G) then lysed in 500 μ l of 50 mM Tris, pH 7.5, 1% NP-40. The lysis buffer contained 20 μ M FMK 024 (morpholinourea-phenylalanine-homophenylalanine-fluoromethylketone; MP Biomedicals, Solon, OH) and 1X Complete Mini; Protease Inhibitor Cocktail, EDTA-free (Roche Biochemicals, Indianapolis, IN). Cell lysates were centrifuged at 13000 rpm for 10 minutes at 4°C. Supernatants were collected and applied to 50 μ l of anti-Ty bound Pierce Protein G beads (Thermo Fisher Scientific, Rockford, IL). Samples were incubated and rotated for 4 hrs at 4°C. Beads were washed 5 times with TEN buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 50 mM NaCl), then boiled in 2% SDS for 5 minutes. Immunoprecipitates were analyzed by western blot.

Western blotting. Total cell lysates for western blotting were obtained and analyzed as previously described. The anti-Ty antibody was directly conjugated to horseradish peroxidase (HRP) using Pierce EZ-link activated peroxidase (Thermo Fisher Scientific, Rockford, IL) and used at a dilution of 1:5000. The polyclonal anti-CRD antibody was

generously provided by Kojo Mensa-Wilmot, University of Georgia, Athens, and was used at 1:4000. Rat monoclonal Anti-HA-biotin (Roche Diagnostics, Indianapolis, IN) was used at a dilution of 1:1000, with Streptavidin HRP conjugate (Invitrogen, Camarillo, CA) used for secondary detection at 1:5000.

Identification of Proteins with Tryptic In-Gel Digestion by Liquid Chromatography-Mass Spectrometry

Protein Assignment by LC/MS/MS. The peptides resulting from the in-gel digest were resuspended with 19.5 μ L of mobile phase A (0.1% formic acid, FA, in water) and 0.5 μ L of mobile phase B (80% acetonitrile, ACN, and 0.1% formic acid in water) and filtered with 0.2 μ m filters (Nanosep, PALL). The sample was loaded off-line onto a nanospray tapered capillary column/emitter (360 \times 75 \times 15 μ m, PicoFrit, New Objective) self-packed with C18 reverse-phase resin (10.5 cm, Waters) in a Nitrogen pressure bomb for 10 min at 1000 psi (~5 μ L load) and then separated via a 160 min linear gradient of increasing mobile phase B at a flow rate of ~200 nL/min directly into the mass spectrometer. LC-MS/MS analysis was performed on a LTQ Orbitrap XL ETD mass spectrometer (ThermoFisher, San Jose, CA) equipped with a nanospray ion source. A full FTMS (Fourier transform mass spectrometry) spectrum at 30,000 resolution was collected at 300 - 2000 m/z followed by 8 data dependent MS/MS spectra in ITMS (Ion trap mass spectrometry) of the most intense ion peaks following CID (36 % normalized collision energy).

Data Analysis. The resulting data was searched against *T. brucei* database including the common contaminant database using the TurboSequest algorithm (BioWorks 3.3.1 SP1, ThermoFisher). DTA files were generated for spectra with a threshold of 15 ions, a TIC of

$1e^3$ and a range of MH^+ 400–6000 m/z . The Sequest parameters were set to allow 20.0 ppm of precursor ion mass tolerance and 0.5 Da of fragment ion tolerance with monoisotopic mass. Tryptic peptides were allowed with up to three missed internal cleavage sites and the differential modifications of 57.0215 Da, 15.9949 Da and 162.0528 Da were allowed for alkylated cysteine and oxidized methionine respectively. Data was filtered to remove all peptide assignments that did not meet the requirement of mass accuracy less than 5 ppm, $Xcorr > 2.0$, and $Sf > 0.8$.

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SUPPLEMENTAL TABLE

Table 4.1. Identification of VSG 221 and SRA-Ty from anti-Ty IP by LC/MS/MS.

No.	Proteins	Z	Mass Accuracy (ppm)	XCorr	Sf	Peptides
1	SRA	2	-2.1	3.19	0.97	(K)QVSAATEAEAR(M)
		2	-2.1	3.50	0.96	(K)SGGGTTTVAIR(Q)
2	VSG 221	2	-3.9	3.46	0.97	(K)DSEASEIQTELK(K)
		2	-3.2	3.43	0.96	(K)ALTGAETAEFR(N)
		2	-3.3	2.98	0.96	(K)AGGTIGGVPC#K(L)
		2	-3.5	2.56	0.96	(K)RPAATYLGK(A)

		2	-3.8	2.37	0.90	(K)LQLSPIQPK(R)
		2	-2.9	2.09	0.85	(K)LVVEALLK(K)

Z, Charge state; Xcorr, Cross correlation; Sf, Final score; #, Alkylated cysteine

A final score of 1.0 indicates absolute certainty of peptide identification.

CHAPTER 5

A PROPOSED MECHANISM OF INHIBITION OF TRYPANOSOME LYTIC FACTOR-1 BY THE
SERUM RESISTANCE ASSOCIATED PROTEIN

INTRODUCTION

Trypanosoma brucei are protozoan parasites that cause widespread disease in sub-Saharan Africa. There are three subspecies of *T. brucei*—*T. b. gambiense* and *T. b. rhodesiense* cause western Human African Trypanosomiasis and eastern Human African Trypanosomiasis (HAT), respectively, while *T. b. brucei* causes the wasting disease, Nagana in cattle. This species selectivity is due to the presence of an innate immune component of human serum that is toxic to *T. b. brucei*, but not to *T. b. gambiense* or *T. b. rhodesiense* [1-3]. This trypanocidal component of serum is a very minor subclass of high-density lipoproteins (HDL) called Trypanosome Lytic Factor-1 (TLF-1) [4]. The cytotoxicity of the TLF-1 particle is due to two primate-specific apolipoproteins associated with TLF-1—apolipoprotein L-I (apoL-I) and haptoglobin related protein (Hpr), which are individually toxic to trypanosomes [5, 6]. In addition to its lytic activity, Hpr binds hemoglobin (Hb) to form the ligand that mediates TLF-1 binding and endocytosis via the high affinity *T. brucei* haptoglobin hemoglobin receptor (*TbHpHbR*) [7-9]. Once endocytosed, TLF-1 traffics to the trypanosome lysosome, where its localization is necessary for initiation of cell lysis [10-13]. Harrington *et. al.* has demonstrated that the proteins of TLF-1 bind negatively-charged liposomes in a pH-dependent manner [14]. The mechanism of TLF-1 lysis is therefore proposed to involve the targeting of TLF-1 and its associated proteins to the anionic

lysosomal membrane, where cell death is mediated through the combined lytic mechanisms of both apoL-I and Hpr [10, 12, 14].

Both *T. b. gambiense* and *T. b. rhodesiense* have evolved mechanisms of resistance that allow them to evade cell lysis by TLF-1 and to establish chronic and acute infections, respectively, in humans. The mechanism of resistance of *T. b. gambiense* was recently found to be due to the modulation of the *T. b. gambiense* HpHbR (*TbgHpHbR*). Through downregulation and mutation of its receptor, *T. b. gambiense* avoids uptake of TLF-1 altogether, and is able to escape lysis by the trypanolytic HDL component of human serum [15].

The ability of *T. b. rhodesiense* to circumvent TLF-1-mediated lysis and survive in the human bloodstream is due to the expression of the Serum Resistance Associated (SRA) protein [16, 17]. Serum resistance is an unstable trait that is directly dependent on SRA expression [16-18]. Computational modeling of the structure of apoL-I led to the identification of an α -helical region at the C-terminus of apoL-I, proposed to be the binding domain for SRA. Analysis of the SRA sequence also led to the identification of an α -helical region at the N-terminus of the protein. SRA and apoL-I are proposed to bind through a coiled-coil protein-protein interaction of the α -helical regions of both proteins [12]. Through this interaction, SRA is thought to mediate resistance to TLF-1 by binding apoL-I and preventing its interaction with the lysosomal membrane [12].

SRA is a member of the variant surface glycoprotein (*VSG*) gene family, and like other *VSG* family proteins, *SRA* is a GPI-anchored protein [19-21]. However, rather than localizing to the trypanosome cell surface, it is a resident early endosomal protein [21].

Unlike *T. b. gambiense*, *T. b. rhodesiense* has a functional *TbHpHbR* and internalizes TLF-1 [15, 21, 22]. Studies on the trafficking and localization of both SRA and TLF-1 revealed the early endosomal compartment to be the initial site of colocalization of these infectivity factors. SRA and TLF-1 were also found to accumulate in the lysosome of resistant SRA-expressing cells when protein degradation was inhibited, while in sensitive *T. b. brucei* cells, this accumulation was observed in the absence of protease inhibition [21]. Consistent with these findings, early studies on TLF-1 turnover in *T. b. brucei* cells showed Hpr to be a stable, degradation-resistant protein in the absence of SRA expression [13].

In this report, we investigate the stability of SRA to determine whether the degradation rates of the protein also reflected rapid turnover. We found that SRA has the shortest half-life recorded to date in comparison to other VSG proteins. These findings are consistent with previous observations that Hpr is stable in sensitive *T. b. brucei* cells, but TLF-1 does not accumulate in resistant SRA-expressing cells. Given the most recent data on the mechanism of TLF-1 lysis, as well as the apparent differential stability of TLF-1 between sensitive and resistant cell lines, we suggest that SRA neutralizes TLF-1 through a two-step mechanism. We therefore propose a model in which SRA-mediated neutralization of TLF-1 occurs through binding of apoL-I and inhibition of TLF-1 interaction with the lysosomal membrane, followed by the accelerated clearance of the toxin through rapid lysosomal degradation.

THE STABILITY OF SRA

We observed in a previous study that fluorescently-labeled Alexa 488 TLF-1 could not be visualized in the lysosomal compartment of SRA-expressing cells unless protein

turnover was inhibited [21]. However, in TLF-1 sensitive cells that did not express SRA, Alexa 488 TLF-1 was found to accumulate in the lysosome. In addition, comparative studies on the turnover of transferrin and TLF-1 in sensitive *T. b. brucei* cells showed that Hpr in particular was more stable than transferrin [13]. Given the apparent difference in TLF-1 accumulation in SRA- vs. non-SRA expressing cells, we examined the stability of SRA, independently of TLF-1, to determine whether the half-life of SRA may provide insight into the role of protein turnover in the resistance mechanism.

In order to examine the turnover rate of SRA, we treated *T. b. brucei* SRA-Ty [21, 22] cells with cycloheximide and quantitated the loss of SRA over time by western blot. Cycloheximide is a bacterial toxin commonly used to inhibit eukaryotic protein translation [23] and therefore facilitates the monitoring of protein turnover. Cells were collected from culture and resuspended in PBS containing 1% glucose and cycloheximide (100 μ M). Cells were collected at 15-minute time-points over the course of 45 minutes. At each time-point, turnover was halted by the addition of a lysis buffer. Samples were prepared for SDS-PAGE and immunoblotted for SRA. Western blot analysis revealed a doublet migrating at 49 kDA that decreased in intensity over time (Figure 5.1A). This band was present in samples from SRA-Ty-transfectants, but not in untransfected *T. b. brucei*. Coomassie staining of protein lysates showed equal sample loading (Figure 5.2B). The intensities of the bands were quantitated and the half-life of SRA was determined to be $t_{1/2} = 26.5$ minutes (Figure 5.2C).

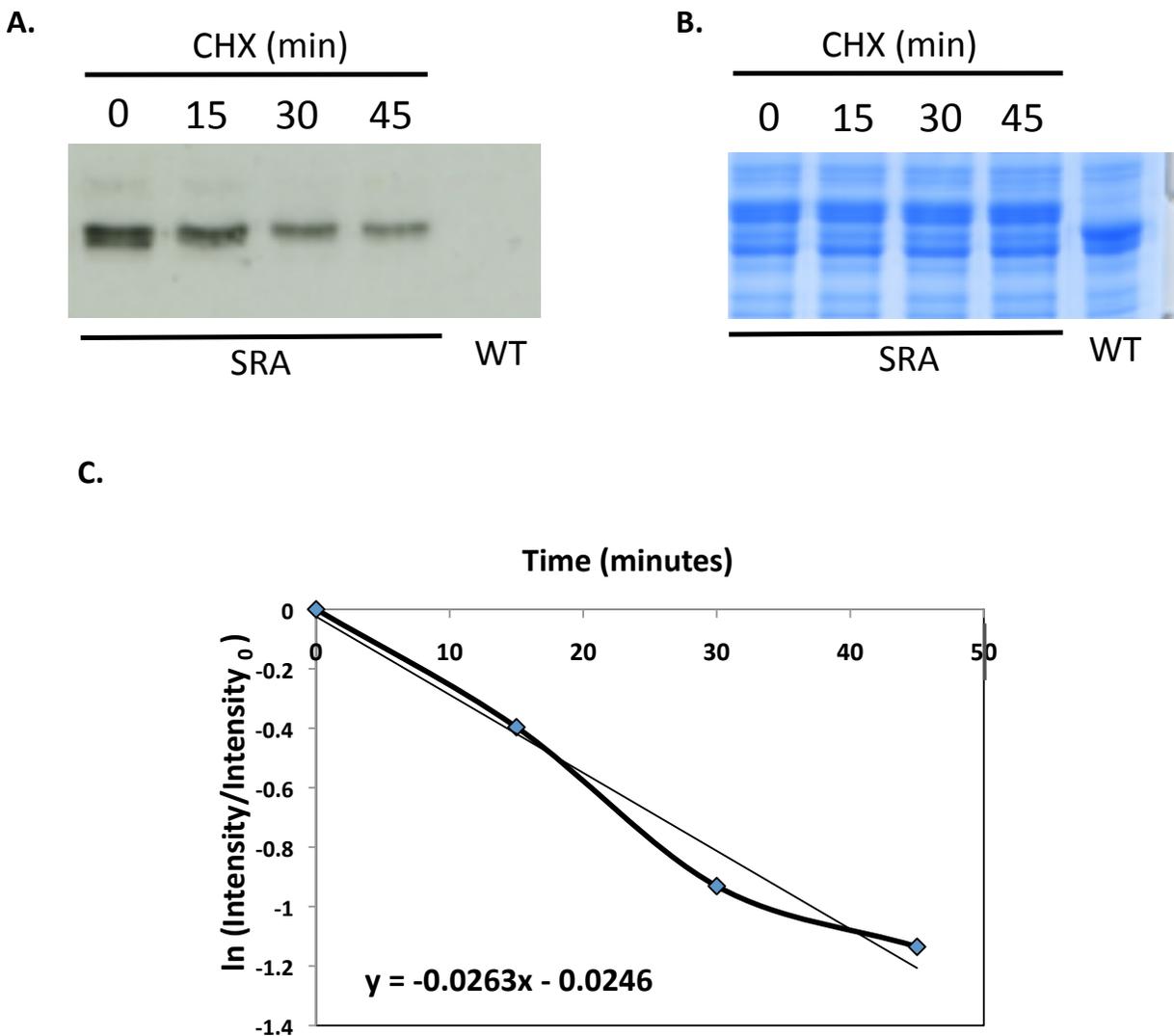


Figure 5.1. SRA is a rapidly degraded protein. (A) Anti-Ty western blot of SRA-expressing transfectants. *T. b. brucei* SRA-Ty cells were treated with CHX (100 μM). Cells were collected at 15-minute intervals for 45 minutes. (B) Coomassie blue-stained gel of cell lysates. 2×10^6 cell equivalents were loaded to each lane. (C) The intensity of each band from the western blot of CHX-treated cells was quantitated and used to determine a rate constant for SRA degradation over time. The half-life of SRA was then calculated.

A MODEL FOR THE INHIBITION OF TLF-1 BY SRA

The resistance of *T. b. rhodesiense* to human serum killing is due to the expression of the endosomal, VSG-like protein, SRA. Since the identification of SRA as a trypanosome resistance factor, much has been done to resolve the mechanism of TLF-1 inhibition. SRA has been shown to interact directly with TLF-1 via binding to apoL-I [6], in order to inhibit the pore-forming activity of the protein. This interaction is likely to occur immediately post-endocytosis in the early endosomal compartment, where colocalization of these infectivity factors was initially detected [21]. Following uptake, SRA and TLF-1 were both found to traffic to the lysosome for degradation. The Hpr protein of TLF-1 was found to be highly stable in turnover assays carried out with *T. b. brucei* cells [13]. However, while TLF-1 accumulated in the lysosomal compartment of sensitive *T. b. brucei* cells, TLF-1 did not accumulate in the lysosomes of SRA-expressing cells, unless protease activity was inhibited [21]. Furthermore, SRA was found to be a rapidly turned over protein with a short half-life. Collectively, these findings have now led us to propose a mechanism of SRA-mediated resistance to TLF-1 trypanosome lysis.

In order to determine whether the lack of TLF-1 accumulation in SRA-expressing cells would be consistent with SRA turnover independently of the toxin, we treated resistant SRA transfectants with cycloheximide to inhibit protein synthesis, then measured SRA protein turnover over an hour. SRA was found to have a half-life of only 26.5 minutes. The reported half-life of VSG is considerably longer at >30 hours [24, 25], while reports on the half-life of *T. brucei* transferrin receptor range from 0.7 – 7 hours [26-28]. Thus, in comparison to other known VSG family proteins, SRA has the shortest half-life (Figure 5.1).

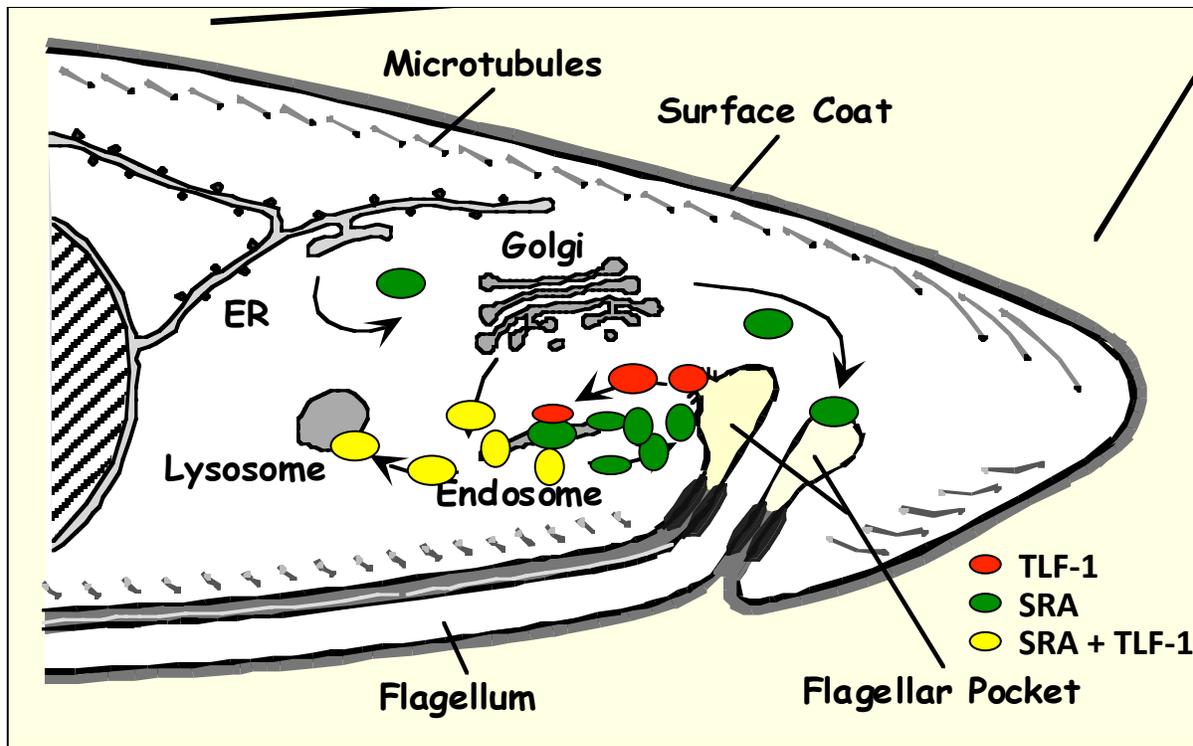


Figure 5.2. The trafficking pathways of TLF-1 and SRA. SRA is translated and modified at the ER, targeted to the Golgi for processing, then trafficked to the cell surface, where it is rapidly endocytosed into early endosomes. TLF-1 binds the *TbHpHbR* in the flagellar pocket and is also internalized into the early endosomes, where it colocalized with SRA. Both SRA and TLF-1 are trafficked to the lysosome for degradation. Endocytic vesicles containing TLF-1 only– red circles; Endocytic vesicles containing SRA only – green circles; Endocytic vesicles containing both SRA and TLF-1 – yellow circles. (Adapted from Bangs, *Curr Opin Microbiol*, 1998).

We previously reported that SRA is a GPI anchored protein that is also a resident endosomal protein. The early endosomes are the initial point of colocalization, and

presumably interaction, of TLF-1 and SRA. The unusual endosomal localization of SRA is therefore likely to be a factor in resistance, as an abundant pool of SRA is conveniently positioned in the compartment of the cell through which TLF-1 must traffic in order to reach its final destination in the lysosome (Figure 5.2). We have also reported that the accumulation of TLF-1 within the trypanosome lysosome differs between sensitive *T. b. brucei* cells and resistant SRA-expressing cells. Uptake assays in both cell lines revealed that Alexa-labeled TLF-1 was detectable in sensitive cells, but not in resistant SRA-expressing cells [21, 22]. Alexa-labeled TLF-1 was only observed in SRA-expressing cells when protease inhibition had been carried out [21]. Furthermore, quantitative studies on TLF-1 turnover in sensitive *T. b. brucei* cells showed Hpr to be a stable protein in comparison to transferrin. No appreciable loss of Hpr was determined to have occurred over the course of an hour [13]. This data is consistent with the accumulation of Alexa-labeled TLF-1 in *T. b. brucei* cells. These findings indicate differential stability of TLF-1 in sensitive cells compared to resistant cells. SRA has been shown to bind TLF-1 via the ApoL-I protein and likely prevents it from being able to insert into the lysosomal membrane and execute its pore-forming activity [6]. This binding is therefore likely to be critical to resistance. The inhibition of the TLF-1 proteins from lysosomal membrane association also means that the toxin is left exposed in the lumen of the lysosome, and likely to be more susceptible to protease activity. The consequence of this may be a more rapid turnover and clearance of TLF-1 proteins in the presence of SRA. Taken together, these findings indicate differential stability of TLF-1 in sensitive *T. b. brucei* cells compared to resistant SRA-expressing cells.

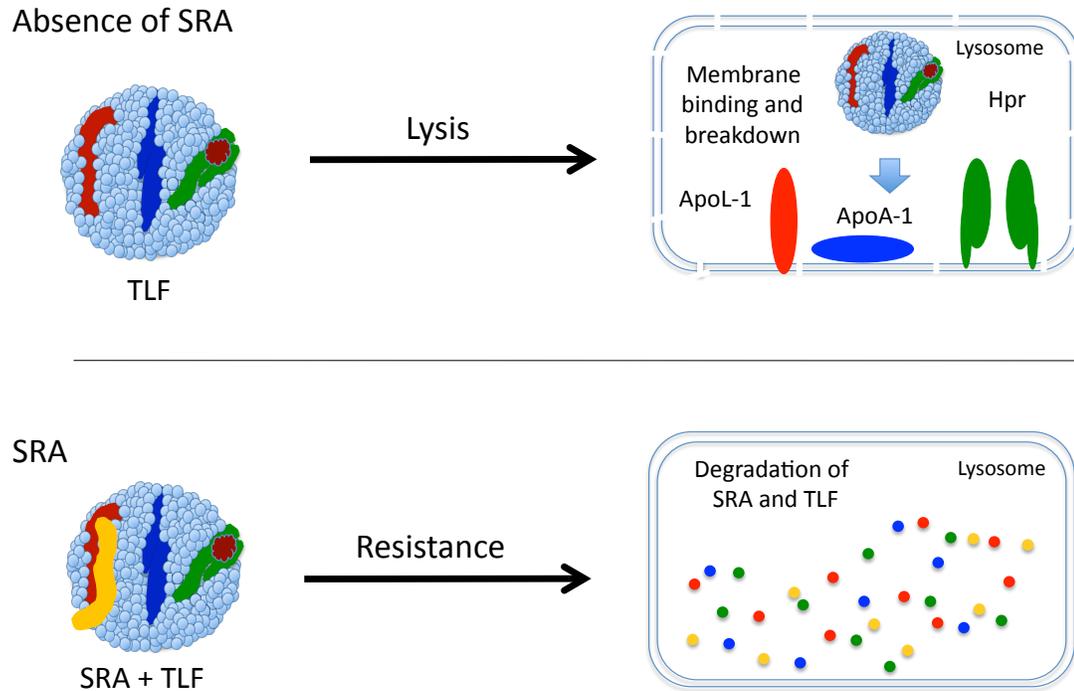


Figure 5.3. Model depicting the mechanism of SRA-mediated inhibition of TLF-1. (A) In the absence of SRA, the proteins of TLF-1 associate with the lysosomal membranes to mediate membrane breakdown via the lytic activities of Hpr and apoL-I. (B) In SRA-expressing trypanosomes, SRA binds apoL-I and prevents membrane interaction of TLF-1 molecules. As a consequence, the proteins of TLF-1 remain in the lysosomal lumen where they are more susceptible to protease activity and accelerated degradation. TLF-1 is therefore rapidly degraded before cell lysis is initiated. (Adapted from Harrington *et. al.*, *J. Biol. Chem.*, 2009).

Collectively, these reports point to an SRA-mediated mechanism of inhibition of TLF-1 that involves three key elements—the binding of TLF-1 via apoL-I and inhibition of membrane association, the endosomal localization of SRA and the increased lysosomal

turnover of the toxin. We therefore propose that SRA arbitrates resistance through the binding and neutralization of TLF-1 immediately post-uptake in the early endosomal compartment, where the unusual intracellular localization of SRA intersects the TLF-1 trafficking pathway (Figure 5.2). Once binding occurs, the SRA/TLF-1 complex traffics to the lysosome for the second phase of the resistance mechanism, which is the inhibition of association of TLF-1 proteins with the lysosomal membranes and clearance of the toxin by accelerated lysosomal degradation (Figure 5.3). The model proposed here has provided an additional element to the previously proposed mechanism of resistance and has brought the field closer to the elucidation of the mechanism of SRA-mediated resistance.

MATERIALS AND METHODS

Cell Culture and Cell lines.

Bloodstream form *T. b. brucei* were maintained in HMI-9 medium (FBS, Gemini Bio-products, West Sacramento, CA); Serum Plus (SAFC Biosciences, Lenexa, KS). *T. b. brucei* 427-221 and *T. b. brucei* 427-221 SRA-Ty cells were used for all experiments. *T. b. brucei* 427-221 SRA-Ty cells were previously characterized [22].

Turnover Over Assay and Immunoblotting

SRA turnover assay. Cells in log phase growth were washed then resuspended in PBS-G (phosphate buffered saline, 1% glucose) containing 100 μ M cycloheximide (Sigma Aldrich, St Louis, MO) at 1×10^7 cells/mL. 1×10^7 cells were collected at 15-minute intervals and immediately added to protein lysis buffer to halt turnover (50mM Tris pH 7.0, 5mM EDTA,

0.5% SDS, 0.1% Triton X-100, 1x cocktail protease inhibitor (Roche Complete mini, Roche Diagnostics, Indianapolis, IN). Cell lysates were then prepared for immunoblotting.

Western blotting. Total cell lysates for western blotting were resolved under non-reducing conditions on a 10% SDS-PAGE gel and transferred to nitrocellulose at 280mA for 1 hour. Anti-Ty staining (1:5000) was used to detect epitope-tagged SRA-Ty. HRP Goat anti-mouse antibody was used for secondary detection (1:5000).

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CHAPTER 6

CONCLUSIONS AND DISCUSSION

Human innate immunity to African trypanosomes is provided by a remarkably efficient subclass of lytic high-density lipoproteins (HDL) that make up less than 0.1% of total HDLs [1, 2]. The mechanism of lysis of the cytotoxic HDL, Trypanosome Lytic Factor-1 (TLF-1), has been the subject of scrutiny for over three decades, with recent developments pointing to a complex set of events that is dependent on the specificity of both the host proteins involved, as well as the parasite membranes. While TLF-1 is highly toxic to *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense* are not susceptible and able to infect humans, causing disease in 36 sub-Saharan African countries. The human infectious subspecies have developed mechanisms of resistance that allow them to successfully persist in the mammalian bloodstream, avoiding eradication by both the innate and adaptive immune systems. While the mechanism of resistance of *T. b. gambiense* was finally resolved recently [3], the means by which *T. b. rhodesiense* neutralizes TLF-1 has not been entirely elucidated although the critical factor in *T. b. rhodesiense* resistance was identified well over a decade ago [4-6].

In an effort to examine the mechanism of TLF-1 mediated lysis from a new perspective, as well as to resolve inconsistencies in the literature, we investigated the morphologies associated with human serum and TLF-1-mediated trypanosome killing. With two distinct mechanisms of lysis and associated cell death morphologies previously

described [7-12], we suggest here that lysis results from a combination of both mechanisms and can lead to both morphologies. The proposed lysis mechanism of haptoglobin related protein (Hpr), one of the protein components of TLF-1, involves Hpr-hemoglobin (Hb) binding for receptor-mediated uptake as well as for delivery of Fe^{2+} ions to the lysosome. The low lysosomal pH facilitates Fenton chemistry and lysosomal membrane disruption by lipid peroxidation [7, 8, 12-15]. This mechanism of killing is thought to lead to a more rapid course of lysis, with the majority of cell lysis occurring within 2 hours, ultimately due to lysosomal membrane breakdown. The second toxic lipoprotein, of TLF-1, apolipoprotein L-I (apoL-I), acts as a pore-forming toxin, which allows the influx of Cl^- ions and H_2O from the cytoplasm, thereby leading to expansive swelling of the lysosome. The extent of lysosomal swelling is such that osmotic pressure ultimately results in rupturing of the cell membrane [9]. These findings, first proposed by the Pays group, were not initially apparent in our earlier observations of lysis, which were typically short 2-hour assays. Since the vacuoles were not visible until around 4 hours, these shorter assays did not allow for observation of this change in morphology.

The lack of detection of the swollen vacuole during the rapid lysis mechanism does not exclude the contribution of apoL-I. At high concentrations of TLF-1, Hpr-Hb killing, which is due to lipid peroxidation is a much more rapid reaction and results in lysosomal membrane perturbation before the swollen lysosome increases to the point where it is detectable. At low concentrations of TLF-1 however, Hpr-Hb peroxidation may be minimal and therefore able to be counteracted by the antioxidant defenses of *T. brucei*. As a result, only apoL-I activity occurs and leads to lysosomal swelling, which then appears as the dominant lysis mechanism under these conditions. We propose that the synergistic

activities of both apoL-I and Hpr bring about the complete and highly efficient trypanolytic activity of TLF-1.

The resistance mechanism of *T. b. rhodesiense* requires the expression of the trypanosome resistance protein, SRA [4-6]. The link between SRA expression and serum resistance is the binding interaction that occurs between apoL-I and SRA [9, 11]. Analysis of the apoL-I amino acid sequence led to the identification of a colicin-like domain at the N-terminus of the protein. As with bacterial colicins, the colicin-like domain of apoL-I was shown to have pore-forming activity. The C-terminus of apoL-I was described as having a membrane addressing domain and an SRA-binding domain that are not required for pore formation. Computational modeling of the C-terminus of the protein revealed an amphipathic α -helical structure that is proposed to interact with the N-terminus of SRA, also proposed to be α -helical. Binding of apoL-I and SRA is therefore thought to lead to the formation of a coiled—coil, protein—protein interaction between the proteins. When this interaction occurs, SRA is proposed to inhibit apoL-I from being able to carry out its pore-forming activity (Figure 5.3) [9, 11]. With SRA bound to the C-terminus of apoL-I, the apolipoprotein may be held away from the membrane at a distance that does not allow for insertion and pore formation. Another possibility is that binding to SRA, may alter protein structure in a way that prevents the extension of the N-terminus of apoL-I into the lysosomal membrane. SRA binding is also likely to inhibit the overall lytic activity of TLF-1 as all apolipoproteins of the particle have been shown to interact with unilamellar liposomes [8]. Furthermore, the membrane interaction of apoL-I is specific to both pH and lipid composition. This specificity suggests that apoL-I targets the TLF-1 particle to the lysosomal membrane [8]. Therefore, both apoL-I pore-formation and Hpr-mediated lipid

peroxidation are likely to be inhibited by SRA-mediated binding of apoL-I and subsequent sequestration of the TLF-1 particle away from the targeted site of action.

While the structure of SRA in its ability to bind apoL-I is critical to the resistance mechanism, the subcellular localization of SRA is also likely to be involved in resistance. The details of the localization of this protein have been controversial, with varying reports of cell surface, lysosomal and non-lysosomal, vesicular distribution [5, 11, 16]. We recently resolved these inconsistencies in a report that detailed the trafficking pathway and steady-state localization of SRA [17]. We have demonstrated that SRA is a GPI-anchored protein with an unusual endosomal localization. This unusual intracellular localization of a GPI-anchored protein is likely to play a role in resistance to lysis. Since TLF-1 traffics via this endosomal compartment in order to reach the lysosome, where lysis initiates, the steady-state localization of SRA to this compartment ensures that TLF-1 encounters the most abundant pool of SRA within the trypanosome immediately post-uptake (Figure 5.2).

The intracellular localization of SRA raises an important question regarding the trafficking of this GPI-anchored resistance protein—How is SRA retained in the endosomal compartment? This endosomal localization may be due to differential protein sorting or to protein-protein interactions within the endosomal compartment. To address the question of differential sorting, an examination of the structure of the SRA GPI anchor and comparative analysis to that of VSG 221 and the *T. brucei* transferrin receptor may provide insight into the observed differences in localization of these VSG family proteins. Since lateral diffusion, and therefore sorting of GPI-anchored proteins into lipid rafts, is influenced by GPI-anchor structure [18], SRA may be sorted to the endosomal

compartment if it has an usual GPI anchor structure. The reported analysis of GPI anchor structure by Mehlert and Ferguson outlines a method for this type of biochemical analysis and provides a guide to carrying out these studies [19]. In addition to GPI anchor structure being a factor in protein sorting, the C-terminal sequence of proteins with this modification also influences the class of GPI anchor to be attached to the glycoprotein [20-22]. Mutational analysis studies may therefore be carried out to determine the role of the class of GPI anchor in protein localization. Finally, to address whether protein-protein interactions are involved in SRA endosomal localization, co-immunoprecipitation studies of SRA can be carried out to search for potential binding partners of SRA.

Studies on apoL-I/SRA interaction reported that this binding occurs at a pH range from pH 5.8 – pH 7.5 [11]. This is consistent with the initial colocalization of SRA and apoL-I being detected in the early endosomal compartment [17] and indicates that binding of SRA to the toxin occurs in the endosomes prior to lysosomal localization. Both SRA and TLF-1 are ultimately trafficked to the lysosome where TLF-1 appears to be more rapidly degraded in SRA-expressing cells [17]. Consistent with this rapid degradation, we have shown in this report that SRA is a rapidly turned-over protein, with the shortest reported half-life of any *T. brucei* protein.

Given these findings, we are now able to propose more complete mechanisms of lysis and resistance, and to establish the connection between them. TLF-1 is able to initiate trypanosome lysis by the action both Hpr and apoL-I. Both toxic components of TLF-1 have dual functions as ligands and lytic agents. Hpr binds Hb to form the first ligand, which binds the *TbHpHb* receptor at the cell surface and enables entry of the toxin into the

endocytic pathway for lysosomal targeting. Apolipoprotein L-I specifically binds negatively charged liposomes at low pH, which model the lipid membrane of the lysosome and indicate that apoL-I acts as the second ligand that targets TLF-1 to the lysosomal membrane. Once TLF-1 has reached this site of action, both Hpr and apoL-I are able to mediate their individual lytic activities, leading to a rapid, synergistic lysis of the cell. In *T. b. rhodesiense*, TLF-1 is intercepted by endosomally-localized SRA, which is able to block the interaction between apoL-I and the lysosomal membrane by binding to the C-terminus of the protein. With the TLF-1 proteins no longer able to associate with the lysosomal membrane, the SRA-bound complex is delivered to the lysosomal lumen where it is more susceptible to proteolysis and is rapidly cleared in the final step of the resistance mechanism (Figure 5.3).

The complexities of lysis and resistance that have evolved due to the interaction of host and parasite have slowly unraveled over time as further research into the mechanisms of lysis and resistance has progressed. In addition, the tools needed to resolve the remaining unanswered questions are currently in place. Harrington *et. al.* has established a well-characterized liposome model that allows a more direct analysis and manipulation of the TLF-1 proteins with model membrane systems [8]. We have now expressed recombinant SRA, which can be used in this liposome model system to elucidate the mechanism of SRA-mediated neutralization of TLF-1.

One of the most important unknowns to be addressed is determination of the biochemical conditions required for the interaction between SRA and apoL-I to occur. What is the optimal pH at which this interaction occurs? What is the stoichiometry of the

binding? Is the interaction salt-sensitive or pH-sensitive? These questions may now be addressed using the *in vitro* liposome system and recombinant SRA. In addition, the affinity and stringency of this binding needs to be determined and compared to the affinity of TLF-1 and the *TbHpHbR* binding. Does SRA have a higher affinity for apoL-I, than the *TbHpHbR* does for Hpr-Hb? Also, since there are two ligands present on the TLF-1 particle, is SRA able to bind TLF-1 while the particle is bound to the receptor?

Another significant aspect of the SRA/TLF-1 interaction that may also be addressed using the liposome model system is the fate of the TLF-1 particle after binding occurs. Does SRA binding disrupt the TLF-1 molecule? More importantly, as our model proposes, does SRA binding to apoL-I actually inhibit the TLF-1 proteins from associating with lipid membranes? Previous studies reported the use of I¹²⁵ protein-labeling in turnover studies of Hpr [10]. This technique can therefore be used to determine the turnover rates of the TLF-1 proteins in the presence and absence of SRA. The data from these comparative turnover studies will address whether the presence of SRA does in fact lead to accelerated degradation of TLF-1. Collectively, the use of the *in vitro* liposome system and protein turnover studies will allow us to investigate the current model of SRA-mediated resistance, and to test these hypotheses in an elegant system.

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