

PROTEIN TRANSLOCATION INTO THE ENDOPLASMIC RETICULUM OF

TRYPANOSOMA BRUCEI

by

JOSHUA GRIFFITH DUFFY

(Under the Direction of Kojo Mensa-Wilmot)

ABSTRACT

Protein entry into the secretory pathway is initiated by translocation into the endoplasmic reticulum. ER signal peptides direct protein import into the organelle. Signal peptide (h-region) variants of a variant surface glycoprotein, VSG-117, and small molecule inhibitors were used to determine the properties of trypanosome signal peptides and factors affecting protein translocation into the ER of *T. brucei*.

h-Regions of signal peptides are required for signal peptide activity. h-Region peak hydrophobicity is thought to be crucial to signal peptide activity. We find that peak hydrophobicity alone does not confer signal peptide activity, and that *T. brucei* h-regions possess tri-component peptide motifs (e.g. L-L-x-[AILV], L-x(1,2)-L-[AILV], and L-x(2,3)-L-[AILPV]). The h-motifs have flexible amino acid requirements, and function with either hydrophobic or serine residues. The order of amino acids in h-motifs is essential to signal peptide activity.

Import of VSG into the ER enables trafficking of the protein to the plasma membrane, and is important for *T. brucei* viability. In hopes of finding new lead candidates for new anti-trypanosome drug discovery we sought to identify compounds that blocked VSG import into *T. brucei* ER microsomes. Sodium azide, but not valinomycin, inhibited VSG-117 translocation

into TbRM, suggesting that an azide-sensitive factor is important for protein translocation into TbRM. We identified pyrimidinone-peptoids and cyclopeptolides as two classes of compounds that block VSG-117 translocation into TbRM, and were found to be trypanocidal. Specifically, both MAL3-101 and NFI028 blocked ER protein import and killed *T. brucei* (IG₅₀ of 1.5 μM for each). We propose pyrimidinone-peptoid and cyclopeptolide derivatives as lead compounds for discovery of new anti-trypanosome drugs.

INDEX WORDS: VSG-117, Signal peptides, h-Region, Endoplasmic reticulum, TbRM, Post-translational translocation, Pyrimidinone-peptoid, cyclopeptolide, MAL3-101, MAL3-51, NFI028, CAM741, Sodium azide, *Trypanosoma brucei*

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JOSHUA GRIFFITH DUFFY
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JOSHUA GRIFFITH DUFFY

Major Professor: Kojo Mensa-Wilmot

Committee: Michael McEachern
Silvia Moreno

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

This thesis is dedicated to my mother and my sister, whose unwavering support and unconditional love have made me the person I am today.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1. *Trypanosoma brucei*

1.1 Overview

Trypanosoma brucei is a vector-borne protozoan parasite that is responsible for the debilitating disease human African trypanosomiasis (HAT), which affects 300,000 people annually. *T. brucei* infects a mammalian host by the bite of the tsetse fly (*Glossina spp.*). Two sub-species infect humans; *Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense*. *T. brucei rhodesiense* is primarily located in eastern and southern Africa and is responsible for the acute form of HAT, in which symptoms can emerge within weeks after infection. *T. brucei gambiense* is found predominantly in the areas of western and central Africa, and is responsible for the chronic form of trypanosomiasis, in which an infected person could go months or years without developing symptoms. Regardless of the type of infection, acute or chronic, if left untreated the parasites can cross the blood-brain barrier, invading the central nervous system, which can lead to neurological/psychiatric disorders, and ultimately death (reviewed in (Gull 2002; Fairlamb 2003; Barrett, Boykin et al. 2007)).

1.2 Life Cycle

T. brucei has a unique life cycle where-in the parasite spends stages of its life in the mid-gut and salivary glands of a tsetse fly and the bloodstream of its mammalian host. During the uptake of a blood meal, an infective, non-dividing metacyclic form of *T. brucei* is transferred from the salivary glands of the tsetse fly to the bloodstream of its mammalian host. In the

bloodstream of the mammalian host, metacyclic trypanosomes can transiently express more than one type of variant surface glycoprotein (VSG), the proteins that comprise its dense protective surface coat (reviewed in (Pays 2006)). In the bloodstream, metacyclics differentiate into a long slender form, allowing the parasite population to proliferate and establish parasitemia. When parasite numbers reach a threshold, monitored by quorum sensing, the parasites secrete a “parasite-derived soluble factor”, which stimulates some of the parasites to arrest at the G1/G0 phase of the cell cycle (Vassella, Reuner et al. 1997). This division arrest is accompanied by activation of the mitochondrion, and changes in cell shape from a long slender to a short stumpy morphology, for which this stage of differentiation is named (Reuner, Vassella et al. 1997; Vassella, Reuner et al. 1997) (reviewed in (Fenn and Matthews 2007)). A subsequent bite by a tsetse fly of a *T. brucei*-infected mammal transfers the short stumpy form to the mid gut of the insect where it sheds its VSG coat, silences its VSG gene expression, and activates expression of procyclin genes. This leads to the production of procyclins, the insect-stage specific surface coat proteins. Here the parasites are said to be in the procyclic form (replicating insect-stage trypomastigotes), and along with the changes in gene expression the parasites are morphologically different and now use cellular respiration through their mitochondrion to obtain ATP instead of solely depending on substrate-level phosphorylation from glycolysis as they did in the bloodstream form. The final life cycle stage occurs when the parasites migrate to the salivary glands of the tsetse fly. Here the cells again change morphologically, but still express their procyclin surface coat and are classified as epimastigotes. From epimastigotes the parasites ready themselves for life in the mammalian bloodstream as metacyclics, by inactivating procyclin gene expression and activating the genes necessary for VSG surface coat expression (Rudenko, Blundell et al. 1994) (reviewed in (Matthews 1999; Fenn and Matthews 2007)).

1.2 Antigenic Variation

Fatal consequences of untreated HAT occur because although *T. brucei* are recognized and destroyed by the host's immune system the parasites are never fully eradicated from the host. Populations of *T. brucei* persist within their host as a result of "antigenic variation". Antigenic variation occurs when subpopulations of *T. brucei*, apart from the majority population in the blood, express a different variant surface glycoprotein that is not recognized by the host-mediated antibody response. Therefore, while most parasites are killed this small set of parasites escapes eradication in the host. This new subpopulation will survive until it increases to numbers that trigger an immune response, upon which it will be destroyed and yet another subpopulation will survive as a consequence of antigenic variation. This rise and fall in parasite population due to proliferation and eradication causes waves of parasitemia in the host (reviewed in (Turner 1999; Pays 2006)).

Antigenic variation is accomplished by changing the type of VSG expressed in the cells. In the bloodstream form (BSF), *T. brucei* have a repertoire of some 1700 VSG genes (Berriman, Ghedin et al. 2005), but only express one VSG at a time, a process called "mono-allelic" expression (Navarro and Gull 2001) (reviewed in (Pays 2006)).

VSG genes are located mainly on minichromosomes or in the telomeric regions of larger chromosomes. When it is time for a trypanosome to switch its VSG surface coat from one VSG type to another it can accomplish this by two distinct mechanisms. Gene conversion and telomeric exchange are VSG switching mechanisms accomplished by DNA recombination where the site of VSG expression remains the same but the VSG gene being transcribed is changed to a different VSG variant or exchanged for another VSG variant, respectively. Active VSG expression sites are susceptible to DNA recombination events because they are unpackaged and

exposed on the chromatin. Moreover, VSG genes contain homologous flanking regions upstream (70 base-pair repeats) and downstream (telomeric repeats) of the VSG gene, which may allow for high rates of homologous and reciprocal recombination to occur (reviewed in (Pays 2006; Taylor and Rudenko 2006)).

The second mechanism by which antigenic variation occurs is *in situ* activation. In this mechanism, changes in VSG expression occur by silencing one expression site while concurrently activating another expression site (reviewed in (Pays 2006)).

1.3 Variant Surface Glycoproteins

Variant surface glycoproteins (VSG) are glycosylated proteins that adopt a coiled-coil conformation in their N-terminus and are bound to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor in its C-terminus (reviewed in (Taylor and Rudenko 2006)). Approximately 10^7 molecules of VSG cover the African trypanosome in a homogenous layer (Jackson, Owen et al. 1985). Neighboring VSGs closely interact with one another, forming a dense protein coat that protects the parasite's plasma membrane from lytic factors (*e.g.* complement) found in the host's bloodstream (reviewed in (Turner 1999; Pays 2006)). VSG synthesis is essential for *T. brucei* viability *in vivo* and growth *in vitro*, and accounts for approximately 10% of the total protein made by the cell (Cross 1975; Bohme and Cross 2002; Sheader, Vaughan et al. 2005) (reviewed in (Taylor and Rudenko 2006)).

2. Human African Trypanosomiasis

2.1 Disease Effects and Symptoms

HAT has two distinct stages demarcated by parasite infection before and after invasion of the host's central nervous system (CNS). Early stage HAT, before the involvement of the host's CNS, includes symptoms generally observed with many infections, but with some exceptions.

Infected patients exhibit fever, headache, malaise, itching of the skin, edema, amenorrhea in women, and impotence in men. In late stage HAT, after the parasites invade the CNS, symptoms become severe and more psychological and neurological in nature. The most noticeable symptom of late stage HAT, and from where the disease derives its common name of “sleeping sickness”, is the disruption of patients’ circadian rhythms. On average, the total amount of time spent asleep is similar in patients with and without HAT. Patients with HAT sleep at different times of the day, sleeping more during the day while exhibiting nighttime insomnia. Other late stage symptoms include extremely heightened sensitivity to pain, memory loss, dementia, depression, agitation, mania, irritability, hallucinations, and death if there is no chemotherapy (Buguet, Bert et al. 1994; Buguet, Bourdon et al. 2001) (reviewed in (Mhlanga, Bentivoglio et al. 1997; Enanga, Burchmore et al. 2002)).

2.2 Current Treatments

For those that it affects, human African trypanosomiasis is not a death sentence because it is an untreatable disease; it is a death sentence because the efficacy of available treatments is very low, and the ability to administer these treatments is not efficient or effective. Currently, chemotherapy regimens for early and late stage HAT use drugs that are highly toxic and require daily to weekly intramuscular injections or intravenous transfusions (reviewed in (Fairlamb 2003)).

Early stage HAT treatment options are limited to only two drugs that have been in use for over 70 years (reviewed in (Fairlamb 2003)). Suramin is used to treat early stage *T. brucei rhodesiense* infections, but it cannot cure *T. brucei gambiense* infections. It is administered to patients by five intravenous injections every 3-7 days for four weeks (reviewed in (Barrett, Boykin et al. 2007)). Side effects from Suramin can be life threatening and include nausea,

vomiting, shock, erythroderma, hemolytic anemia, jaundice, diarrhea, and agranulocytosis (reviewed in (Fairlamb 2003)). Treatment of early stage *T. brucei gambiense* infections has been confined to pentamidine although it is unreliable against *T. brucei rhodesiense* infections (reviewed in (Fairlamb 2003)). In *T. brucei gambiense* HAT, pentamidine is administered in 4mg kg^{-1} intramuscular injections daily (or on alternate days) for 7-10 days. Like suramin, pentamidine is quite toxic and can cause serious side effects that can drastically lower white blood cell counts, damage the liver, cause nephrotoxicity, and cause damage to the pancreas (Sands, Kron et al. 1985) (reviewed in (Barrett, Boykin et al. 2007)).

Because late stage HAT involves parasitic invasion of the host's CNS, drugs used to treat HAT patients at this stage must cross into the cerebrospinal fluid (CSF) and accumulate in amounts that are trypanocidal. Currently, two such clinically approved drugs exist. Melarsoprol is an arsenical compound used to treat both *T. brucei rhodesiense* and *T. brucei gambiense* late stage HAT. Melarsoprol is completely immiscible in water and must be dissolved in propylene glycol for intravenous delivery to patients. These intravenous injections are administered daily for 10 days, and must be delivered in glass syringes because the melarsoprol solution can melt plastic (Burri, Nkunku et al. 2000; Pepin and Mpia 2006) (reviewed in (Barrett, Boykin et al. 2007)). Patients scream and writhe in pain because of the extreme burning sensation felt when melarsoprol is injected into the blood. Because the structure is highly non-polar it is able to passively diffuse across cell membranes and can be readily absorbed and accumulate to trypanocidal concentrations in the CSF. Although valuable for its capacity as a trypanocidal agent of last resort, melarsoprol contains arsenic and is a highly toxic and can cause reactive encephalopathy in 5-10% of patients (reviewed in (Pepin and Milord 1994; Fairlamb 2003)). Aside from the increased chance of death, other side effects include vomiting, severe abdominal

colic, headache, fever, inflammation of the veins, thrombocytopenia, and increased risk of cardiac failure (reviewed in (Pepin and Milord 1994; Fairlamb 2003; Barrett, Boykin et al. 2007)).

Eflornithine is used to treat late stage *T. brucei gambiense* HAT, but is ineffective against late stage *T. brucei rhodesiense* HAT (Matovu, Iten et al. 1997). Eflornithine is administered intravenously, at $400 \text{ mg kg}^{-1} \text{ day}^{-1}$ in 100 mg kg^{-1} doses every 6 hours for a 14-day period (Haeghele, Alken et al. 1981; Griffin, Slavik et al. 1987). Eflornithine activity against *T. brucei gambiense* in humans is predominantly cytostatic and thus requires a properly functioning immune system to rid the parasites from the body. Side effects of eflornithine include fever, headache, hypertension, rash, neuropathy, gastrointestinal pain, and diarrhea (Chappuis, Udayraj et al. 2005).

2.3 The future of HAT treatment and Drug Development

By examining the benefit to detriment ratio of the currently available drugs for the treatment of early and late stage HAT it is evident that there is a strong need for better chemotherapeutic options for HAT (reviewed in (Fairlamb 2003; Barrett, Boykin et al. 2007)). Preferably, these new drugs will be administered orally and have decreased severity of adverse side effects. Innovation in drug delivery is very important because the majority of the HAT treatment facilities in rural Africa are not clinics; they are ill equipped to provide the sterile multiple intravenous and intramuscular injections necessary for proper administration of the current drugs.

Advances in HAT drug discovery research are being stimulated largely due to initiatives like the Bill and Melinda Gates Foundation, Drugs for Neglected Disease initiative, and the

Wellcome Trust (reviewed in (Barrett, Boykin et al. 2007)). While these initiatives have revived interest in HAT drug development no compound is close to approval for use in humans.

The future of drug development for HAT will depend on research focusing on discovering better drugs. Tailoring chemotherapies to the unique biochemical and physiological processes of *T. brucei* will lead to more efficient destruction of the parasite while leaving the human host largely unharmed.

2.4 VSG Biogenesis as a Possible Drug Target

One possible area of interest for future HAT drug discovery is variant surface glycoproteins (VSGs), which mediate antigenic variation. VSG synthesis accounts for approximately 10% of the total protein in the cell (Cross 1975; Bohme and Cross 2002; Sheader, Vaughan et al. 2005). Discovery of drugs that target key steps in VSG protein synthesis or its targeting to the plasma membrane could lead to breakthroughs in the discovery of new anti-trypanosome drugs.

3. Protein Translocation into the Endoplasmic Reticulum

3.1 Overview

Proteins arrive at the cell surface via the secretory pathway. In eukaryotes, the secretory pathway transports proteins to a variety of destinations within the cell via multiple routes. Proteins can be processed through the endoplasmic reticulum, Golgi apparatus, endosomes, and secretory vesicles before they are delivered to the plasma membrane or secreted into the extracellular space. Regardless of the route a protein takes to its final destination, the first step for entry into the secretory pathway for many proteins starts with translocation into the endoplasmic reticulum (ER).

Protein translocation into the ER can occur concurrently with the elongation of the polypeptide, termed co-translational translocation, or it can take place after a protein has dissociated from the ribosome, termed post-translational translocation (reviewed in (Corsi and Schekman 1996; Rapoport 2007)). Each translocation pathway achieves protein import using different mechanisms and machinery, but the general steps of each mechanism are very similar. For accurate translocation into the ER both pathways require recognition of an ER signal peptide at the N-terminus of the preprotein and correct interaction of this signal peptide with the translocation machinery.

3.2 Co-Translational Import

Co-translational import is a pathway by which secretory precursor proteins are translocated across the ER membrane at the same time as protein synthesis is taking place. It is mediated by the signal recognition particle (SRP).

Eukaryotic SRP is a multimeric protein-RNA complex that, in mammals, consists of six protein subunits (SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72) and a 300 nucleotide 7SL RNA scaffold (Walter and Blobel 1981) (reviewed in (Wild, Weichenrieder et al. 2002; Egea, Stroud et al. 2005)).

As a signal peptide of the nascent chain emerges from the ribosome it is bound by the SRP54 subunit of SRP, and upon binding of the signal peptide by SRP the Alu domain (SRP9/14 and 5' end of the RNA) of SRP causes a “translational pause” of the ribosome, delaying elongation of the nascent chain (Siegel and Walter 1986; Siegel and Walter 1988; Bernstein, Poritz et al. 1989). Next, the ribosome stimulates an exchange of GDP for GTP on SRP54, causing SRP to bind the SRP-receptor (GTP-bound SR α subunit) on the ER membrane (Rapiejko and Gilmore 1992; Bacher, Lutcke et al. 1996). Next, the ribosome-nascent chain

complex is loaded onto the Sec61 translocon. Subsequently, SRP dissociates from SR after GTP hydrolysis by both SRP54 and SR α , allowing SRP to recycle into the cytoplasm (Miller, Wilhelm et al. 1993). Once the ribosome is docked on the Sec61 translocon and if the signal sequence is recognized by the translocon, translation resumes, and the protein is concurrently pushed by translation of the protein on the translation complex and “pulled” into the ER lumen by the ER-luminal Sec63p/Kar2p (BiP) complex (Matlack, Misselwitz et al. 1999). Upon termination of translation the ribosome dissociates from the translocon, recycling back into the cytoplasm (reviewed in (Corsi and Schekman 1996; Rapoport 2007)).

3.3 Post-Translational Import

Post-translational import pathway is an alternative pathway in which secretory proteins enter the ER after they have been released from the ribosome. In yeast, post-translational and co-translational import pathways use similar translocation machinery at the ER membrane and ER lumen (*e.g.* Sec61 translocon, Sec63 complex, and BiP). Post-translational import does not depend on SRP and SR. However, SRP may act as a chaperone in post-translation delivery of tail-anchored membrane proteins to the ER membrane (Abell, Pool et al. 2004) (reviewed in (High and Abell 2004)).

Post-translational import requires the interaction of the nascent protein chain with cytosolic chaperones from the Hsp family of proteins (McClellan, Endres et al. 1998; Ngosuwan, Wang et al. 2003). Hsp70s bind secretory precursor proteins tightly upon hydrolysis of ATP, keeping cargo in an “import-competent” conformation (Caplan, Cyr et al. 1992; McClellan, Endres et al. 1998; McClellan and Brodsky 2000). This partially unfolded conformation is necessary because a fully folded protein would preclude it from passing through the small translocation pore at the ER membrane. Also, cytoplasmic Hsp70s interact with Hsp40 co-

chaperones, which can stimulate the intrinsic ATPase activity of Hsp70 and deliver proteins to the peptide-binding domain of Hsp70 (reviewed in (Laufen, Mayer et al. 1999; Han and Christen 2003)).

Upon recruitment to the ER membrane this “import-competent” precursor is recognized and translocated through the Sec61 translocation pore, mediated by Sec63p. In *S. cerevisiae*, the Sec62/63p complex is comprised of the integral membrane proteins Sec62p, Sec63p, and Sec71p and the peripheral associated protein Sec72p. It is a major complex adjacent to the Sec61p complex that is necessary for co- and post-translational import into the ER in *S. cerevisiae* (Brodsky, Goeckeler et al. 1995; Panzner, Dreier et al. 1995; Young, Craven et al. 2001) (reviewed in (Rapoport 2007)). Mammals only possess Sec62 and Sec63, but the extent to which they utilize this complex may differ from yeast since mammals use co-translational import almost exclusively (Meyer, Grau et al. 2000). BiP, a luminal hsp70 acts as an ATP-dependent ratchet to facilitate translocation, “pulling” or “trapping” the precursor into the ER lumen (Brodsky and Schekman 1993; Brodsky, Goeckeler et al. 1995; Matlack, Misselwitz et al. 1999) (reviewed in (Schatz and Dobberstein 1996)).

3.4 Sec61 Translocon

Regardless of import pathway, all secretory precursors enter the ER through a Sec61 translocon. The Sec61 translocon is a heterotrimeric complex composed of Sec61 α , Sec61 β , and Sec61 γ in mammals (Sec61p, Sbh1p, and Sss1p, respectively in *S. cerevisiae*) (reviewed in (Rapoport, Jungnickel et al. 1996; Rapoport 2007)).

Sec61 α is the major constituent of the translocation channel. It has ten transmembrane (TM) regions with each predicted to adopt a helical conformation in the ER membrane. Sec61 α can be divided into two halves with TMs 1-5 connected to TMs 6-10 by a loop to form an

hourglass-shaped molecule (Van den Berg, Clemons et al. 2004) (reviewed in (Clemons, Menetret et al. 2004)). This translocation channel can adopt a “closed” or “open” state to regulate entry into the ER lumen. It is suggested that TM2a of Sec61 α acts to “plug” the translocation channel, keeping the channel closed to prevent the loss of small molecules from the ER lumen (Van den Berg, Clemons et al. 2004; Junne, Schwede et al. 2006) (reviewed in (Clemons, Menetret et al. 2004)). To switch from a “closed” to an “open” conformation it is likely that the signal peptide is recognized at the junction of TM2b and TM7. Binding of the signal peptide to these transmembrane regions induces a conformational change within Sec61 α , allowing the TM2a, or the “plug”, to rotate angularly away from the center of the translocation channel. After full import of the secretory precursor and/or release of the signal peptide from TM2b and TM7 the “plug” is proposed to rotate back into the center of the translocation channel. Much debate remains regarding the role that the “plug” plays in sealing/gating the luminal face of the translocation channel because evidence in mammals implicates the luminal chaperone BiP in this role (Haigh and Johnson 2002; Alder, Shen et al. 2005; Junne, Schwede et al. 2006). Furthermore, yeast mutants with partial or full deletions in the “plug” domain exhibited normal growth and viability, but showed defects in signal anchor protein orientation, limited defects in co-translational import, and significantly lowered efficiency in post-translational import (Junne, Schwede et al. 2006).

The structure of Sec61 β contains a small cytosolic loop and one transmembrane region positioned in close proximity to Sec61 γ and TMs 1 and 4 of Sec61 α . The positioning of the β -subunit suggests that it has limited interaction with Sec61 α and therefore is not crucial for proper Sec61 function (Van den Berg, Clemons et al. 2004) (reviewed in (Clemons, Menetret et al. 2004)).

Sec61 γ is responsible for clamping the two halves of the Sec61 α “hourglass” together. Sec61 γ consists of two helical domains: the N-terminal helix is positioned at the cytoplasmic side of the ER membrane and is connected to its other domain, a transmembrane helix that traverses the ER membrane. By making contacts on both sides of Sec61 α , Sec61 γ effectively “clamps” the two halves of Sec61 α together (Van den Berg, Clemons et al. 2004) (reviewed in (Clemons, Menetret et al. 2004)).

3.5 Translocon Complex Associated Machinery

In addition to the Sec61 translocon there are other peripheral and integral membrane proteins at the ER membrane that aid in the translocation of secretory precursor proteins.

In yeast, Sec62p spans the ER membrane two times and has both its N-terminus and C-terminus positioned out into the cytosol. Here, these two extreme ends of the protein serve important functions to properly align secretory precursors for post-translational import. Deletions mutants showed that when amino acids were removed from the N-terminal, C-terminal, or both ends, the Sec-complex (Sec61p complex and Sec62/63p complex) lost its function (Wittke, Dunnwald et al. 2000). From this study it was proposed that the extreme C-terminus of Sec62p, or effector domain, serves to recognize the signal sequence of secretory precursors. Signal sequence recognition at the effector domain prompts the N-terminus of Sec62p to interact with the Sec63p C-terminus, and readies the luminal domain of Sec63p for translocation of the secretory precursor through the translocation pore (Wittke, Dunnwald et al. 2000).

Sec63p, like Sec62p, is an essential integral membrane protein of the ER membrane in *S. cerevisiae*. Sec63p possesses a conserved J-domain on the luminal side of the ER membrane (Sadler, Chiang et al. 1989) (reviewed in (Rapoport, Jungnickel et al. 1996; Rapoport 2007)).

Upon activation from the cytosolic side of Sec63p, the J-domain of Sec63p interacts with an ER luminal Hsp70, BiP (Brodsky and Schekman 1993). BiP (Kar2p in yeast) is recruited to the luminal side of the ER membrane where it binds the protein being translocated, and acts as an ATP-dependent “molecular ratchet”, pulling the secretory precursor through the Sec61 translocation channel into the ER lumen (Matlack, Misselwitz et al. 1999). In yeast, BiP is essential for both co-translational and post-translational import pathways (Brodsky, Goeckeler et al. 1995).

3.6 ER Protein Import and Associated Translocation Machinery in Trypanosomes

In contrast to *S. cerevisiae* and mammals, very little is known about protein import into the ER in *T. brucei*. However, data suggests that both co-translational and post-translational import pathways direct protein import into the ER in *T. brucei* (Liu, Liang et al. 2002; Lustig, Vagima et al. 2007; Goldshmidt, Sheiner et al. 2008).

The trypanosome SRP differs from yeast and mammalian SRP in that it possesses only four of the six proteins found in yeast and mammals (SRP19, SRP68, SRP72, and SRP54). Also, *T. brucei* SRP possesses two RNA components: the 7SL RNA and a special tRNA-like molecule, sRNA-76, whose involvement in SRP is specific only to trypanosomes (Liu, Ben-Shlomo et al. 2003). In *T. brucei*, this tRNA-like molecule has been suggested to be a functional analog of the Alu domain found in yeast and mammals (SRP9/14/7SL RNA complex), a domain to which no homology exists in *T. brucei* (Lustig, Goldshmidt et al. 2005; Lustig, Vagima et al. 2007). Knockdown of SRP expression in *T. brucei* results in cells that mislocalize a number of important membrane proteins, implicating SRP in proper membrane protein biogenesis (Liu, Liang et al. 2002; Lustig, Vagima et al. 2007). Specifically, depletion of SRP68 and SRP72 was lethal to the cells (Lustig, Goldshmidt et al. 2005). Although depletions in

SRP68 and SRP72 were lethal, proteins were able to cross the ER membrane and be glycosylated properly. This suggests the existence of an alternate route, independent of SRP, by which proteins can enter the trypanosome ER (Liu, Liang et al. 2002; Lustig, Vagima et al. 2007).

Homologous DNA sequences of many of the Sec61 associated genes present in *S. cerevisiae* and *H. sapiens* are absent in *T. brucei*. Genes encoding Sec61 β , Sec62, Sec63, Sec71, and Sec72 cannot be detected in a search of the *T. brucei* genome (Berriman, Ghedin et al. 2005). Although homology to some of these sequences has not been observed it has been suggested that functional analogues exist in *T. brucei* to account for the non-represented proteins, preserving the biochemical function of these proteins (Patham et al., submitted). This is supported by evidence showing that upon a knockdown of SRP54p, using RNAi, *T. brucei* imports proteins into the ER by an alternate, or SRP-independent pathway. In these studies, proteins were translocated across the ER membrane into the lumen, and in some cases post-translationally modified (Liu, Liang et al. 2002; Lustig, Vagima et al. 2007).

Recently, functional homologues of Sec63 and Sec71 were identified in *T. brucei*, and silenced using RNAi (Goldshmidt, Sheiner et al. 2008). Silencing of Sec63 affected the entry of all secretory precursors tested, and as observed in yeast Sec63 is suggested to be essential to both import pathways in *T. brucei* (Brodsky, Goeckeler et al. 1995; Goldshmidt, Sheiner et al. 2008). Proteins were imported into the ER when Sec71 expression was knocked down, but several proteins (*e.g.* GPI-anchored proteins) exhibited improper localization. Further, Sec71 is essential for *T. brucei* viability (Goldshmidt, Sheiner et al. 2008).

Also, *T. brucei* possesses BiP, which transiently associates with VSG secretory precursors in the ER lumen (Bangs, Uyetake et al. 1993; Bangs, Brouch et al. 1996). Whether or

not *T. brucei* BiP is activated by a Sec63-like DnaJ domain as observed in *S. cerevisiae* remains to be established.

4. ER Signal Peptides

4.1 General Characteristics

Proteins must exist in their designated compartments within a cell to function properly. Because most proteins are made in the cytosol cells possess protein-targeting systems to direct each protein to its correct destination. Essential to many of the complex targeting pathways are different targeting signals present on the precursor proteins (Lingappa and Blobel 1980) (reviewed in (Schatz and Dobberstein 1996)).

Proteins destined to translocate or integrate into the ER membrane have ER-specific signal peptides (Blobel and Dobberstein 1975; Lingappa and Blobel 1980). A signal peptide displays a tri-partite arrangement. It consists of a 20-30 residue segment at the N-terminus of the precursor, containing a positively charged n-region, a hydrophobic h-region, and a polar c-region (von Heijne 1985) (reviewed in (Hegde and Bernstein 2006)).

4.2 n-Region

The n-region is the most N-terminal of the three sub-regions of a signal peptide. As a functional consequence of its positive charge, the n-region could provide electrostatic attraction to the ER phospholipid bilayer (Keller, ten Berge et al. 1996), and facilitate interactions between the signal sequence and the translocation machinery (Puziss, Fikes et al. 1989; Goder and Spiess 2003). An overall positive charge in this region increases import efficiency of some secretory precursors. When an h-region is highly hydrophobic the presence of an n-region and charge intensity of the n-region become dispensable for protein translocation (Izard, Rusch et al. 1996). Also, as a hydrophobic core becomes shorter there is an increasing requirement for a positively

charged n-region (Hikita and Mizushima 1992). Thus, as h-region hydrophobicity/length decreases a positively charged n-region becomes crucial for high import efficiency of proteins (Izard, Rusch et al. 1996).

4.3 h-Region

The h-region is a central hydrophobic core of at least seven non-charged amino acids (von Heijne 1985), and in eukaryotes is leucine-rich (von Heijne 1981; Nielsen, Engelbrecht et al. 1997). This display of hydrophobic residues, uninterrupted by charged amino acids, is highly conserved and considered to be necessary and sufficient for signal peptide function (Kaiser, Preuss et al. 1987) (reviewed in (Hegde and Bernstein 2006)). Since there is no primary sequence homology among signal peptides (reviewed in (Zheng and Gierasch 1996)), the generally accepted paradigm is that the overall hydrophobic nature, or peak hydrophobicity of this region dictates signal peptide function. This is based on experimental data showing some correlation between degree of hydrophobicity and protein import efficiency (Ryan, Duncan et al. 1986; Chou and Kendall 1990; Doud, Chou et al. 1993; Ryan, Robbins et al. 1993; Tomilo, Wilkinson et al. 1994; Ng, Brown et al. 1996). Peak hydrophobicity of an h-region is the area within an h-region that is the most hydrophobic (Kyte and Doolittle 1982). As peak hydrophobicity of an h-region increases the extent of protein import into the ER increases both *in vivo* and *in vitro* (Bird, Gething et al. 1987; Chou and Kendall 1990; Doud, Chou et al. 1993). However, an upper limit of hydrophobicity may exist for eukaryotic signal peptides. Some sequences can become too hydrophobic to function as a signal peptide and thus cause reduced levels of translocation (Tomilo, Wilkinson et al. 1994). Collectively, these data implicitly suggest that h-region peak hydrophobicity must be within a certain range of hydrophobicity for a signal peptide to be functional.

In yeast, h-Region hydrophobicity affects the pathway by which import occurs. Signal peptides with higher hydrophobicities were translocated into the ER co-translationally while signal peptides with relatively lower peak hydrophobicities entered the ER post-translationally (Ng, Brown et al. 1996). It is suggested that highly hydrophobic h-region sequences allow SRP to bind with high affinity to the signal peptide and thus proceed via a co-translational pathway (Ng, Brown et al. 1996).

4.4 c-Region

The most C-terminal region of an ER signal peptide is the “cleavage region” or c-region. It is a stretch of 5-8 polar amino acids and contains a cleavage site recognized by signal peptidase I (von Heijne 1985) (reviewed in (Rusch and Kendall 1995)). Although amino acid composition and length of a c-region varies among species, a common characteristic of a c-region is compliance with the (-3,-1) rule at the signal peptide cleavage site. The (-3,-1) rule states that amino acid residues at positions -3 (3 residues N-terminal to the cleavage site) and -1 (1 residue N-terminal to the cleavage site) have small, neutral side chains for proper signal peptidase action (von Heijne 1983; von Heijne 1985).

4.5 Species-Specificity of ER Signal Peptides

Signal peptides from eubacteria and eukaryotes have similar tri-partite organization (reviewed in (Izard and Kendall 1994)), and may be functionally interchanged (Talmadge, Kaufman et al. 1980; Wiedmann, Huth et al. 1984). However, some signal peptides from different species are not exchangeable (Laforet, Kaiser et al. 1989).

Although some translocation machinery can functionally interact with signal sequences from different biological kingdoms, this interchangeability of signal peptide function is not universal (Abrahmsen, Moks et al. 1985; Al-Qahtani, Teilhet et al. 1998). This incompatibility

of signal peptides and translocons could be due to species-specific characteristics of ER signal peptides (Abrahmsen, Moks et al. 1985; Bird, Gething et al. 1987; Laforet, Kaiser et al. 1989; von Heijne and Abrahmsen 1989; Al-Qahtani, Teilhet et al. 1998; Ramirez, Boscardin et al. 1999; Zheng and Nicchitta 1999) (reviewed in (Izard and Kendall 1994)). This idea is supported by several observations: (i) the hydrophobic core of *E. coli* M13 failed to translocate *E. coli* alkaline phosphatase into the *E. coli* periplasm (Laforet, Kaiser et al. 1989); (ii) *E. coli* LamB is not translocated into canine microsomes (Zheng and Nicchitta 1999); (iii) yeast carboxypeptidase Y is not translocated into canine microsomes (Bird, Gething et al. 1987); (iv) a signal sequence from *T. brucei* VSG-117 did not allow translocation into canine ER membranes (Al-Qahtani, Teilhet et al. 1998); (v) *Leishmania chagasi* gp63 is not imported into canine microsomes (Al-Qahtani, Teilhet et al. 1998). These data seemingly upend the dogma that signal peptides are interchangeable across different species. One explanation offered for this apparent species-specificity of signal peptide function is that peak hydrophobicity of these non-functional signal peptides is not within an optimum range to direct import. Another explanation is that n-region charge, h-region hydrophobicity, c-region cleavage site, and the N-terminus of the mature protein must complement one another to direct efficient protein import, suggesting that an overlap in function exists among these regions. While these ideas accurately explain some of the data they do not explain all of the data, and they fail to predict the ability of a signal peptide to be imported. Collectively, this data presents only partial answers to the question: how do ER signal peptides function?

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CHAPTER II

H-MOTIFS: A NEW PERSPECTIVE ON THE IMPORTANCE OF HYDROPHOBIC RESIDUES IN ER SIGNAL PEPTIDES¹

¹ Joshua Duffy and Kojo Mensa-Wilmot. To be submitted to *Proceedings of the National Academies of Sciences*.

Abstract

Protein translocation of secretory proteins into the endoplasmic reticulum (ER) in eukaryotes enables polypeptide entry into the secretory pathway, which is important for the viability of the human protozoan parasite *Trypanosoma brucei*. An N-terminal signal peptide of precursor proteins is essential for entry into the ER. A hydrophobic core, or h-region, of the signal peptide is required for signal peptide activity. h-Regions are thought to be composed of random hydrophobic amino acids, whose peak hydrophobicity governs activity of signal peptides. We have re-evaluated these concepts using a trypanosome microsomal system for import of a variant surface glycoprotein, VSG-117. We find that h-region peak hydrophobicity alone does not dictate signal peptide activity. Moreover, we identified conserved tri-component peptide motifs (e.g. L-L-x-[AILV], L-x(1,2)-L-[AILV], and L-x(2,3)-L-[AILPV]) in h-regions of *T. brucei* signal peptides. Signal peptides frequently contain multiple h-motifs. Functional analysis of h-region motifs (h-motifs) led to the following conclusions: (i) h-motifs have flexible amino acid requirements, and (ii) can operate with either hydrophobic or serine residues; (iii) the order of amino acids in h-motifs governs signal peptide activity. We surmise that h-regions are a repository of h-motifs that are essential for biological activity of signal peptides.

Introduction

Trypanosoma brucei causes human African trypanosomiasis (HAT). *T. brucei* persists in its vertebrate host because of “antigenic variation”, whereby sub-populations of parasites switch expression of variant surface glycoproteins (VSGs) on the cell surface (reviewed in (Pays 2006; Taylor and Rudenko 2006)). VSGs enter the secretory pathway at the ER en route to the plasma membrane (reviewed in (Taylor and Rudenko 2006)). Because VSGs are crucial to antigenic variation understanding their targeting to the cell surface could provide insight into the development of new lead anti-trypanosome drugs.

Protein entry into the ER lumen depends on two components: (i) Sec61 translocon at the ER membrane and (ii) a signal peptide (reviewed in (Rapoport 2007)). Protein translocation into the ER lumen can occur by two mechanisms. In mammals, secretory precursor proteins are brought to and translocated through the Sec61 translocon predominantly by a co-translational mechanism that requires a signal recognition particle (SRP) and its receptor (SR) (Walter and Blobel 1981) (reviewed in (Rapoport 2007)). In a second mechanism, termed post-translational translocation, protein import occurs after translation of the secretory precursor is completed in the cytoplasm. This mechanism involves the Sec71/72 and Sec62/63 complexes as well as the Sec61 translocon (reviewed in (Matlack, Mothes et al. 1998; Rapoport 2007)). Furthermore, cytosolic Hsp70 and Hsp40 chaperones are necessary for post-translational protein import into the ER (Caplan, Cyr et al. 1992; Becker, Walter et al. 1996; McClellan and Brodsky 2000; Ngosuwana, Wang et al. 2003) (reviewed in (Rapoport 2007)). In both mechanisms, post-targeting recognition of a signal peptide at the Sec61 translocon is necessary for protein translocation into the ER (Jungnickel and Rapoport 1995). Recognition of signal peptides at the

ER involves transmembrane segments TM2 and TM7 of Sec61 α (Plath, Mothes et al. 1998; Van den Berg, Clemons et al. 2004) (reviewed in (Clemons, Menetret et al. 2004)).

An ER signal peptide has a charged n-region, an h-region composed of approximately 7-13 non-charged or hydrophobic amino acids, and a polar c-region (von Heijne 1985; Haeuptle, Flint et al. 1989) (reviewed in (Izard and Kendall 1994; Hegde and Bernstein 2006)). The hydrophobic core is necessary for signal peptide function (Kaiser, Preuss et al. 1987), and the peak hydrophobicity of this region is a major factor governing the ability and pathway by which a protein enters the ER (Chou and Kendall 1990; Doud, Chou et al. 1993; Ng, Brown et al. 1996) (reviewed in (Hegde and Bernstein 2006)).

ER signal peptide h-regions lack sequence conservation (reviewed in (Zheng and Gierasch 1996)), and some have been replaced by apparently “random” hydrophobic sequences selected to promote protein import into the ER (Kaiser, Preuss et al. 1987). Nevertheless, several studies suggest that signal peptides of equivalent peak hydrophobicity can be species-specific (Abrahmsen, Moks et al. 1985; Bird, Gething et al. 1987; Al-Qahtani, Teilhet et al. 1998; Zheng and Nicchitta 1999), suggesting that h-region sequences may not be composed of random hydrophobic amino acids. For example, *E. coli* β -lactamase and *T. brucei* VSG-117 h-regions are strikingly similar in composition and peak hydrophobicity, yet *E. coli* β -lactamase is imported into canine microsomes (CfRM) whereas *T. brucei* VSG-117 is not translocated into CfRM (Al-Qahtani, Teilhet et al. 1998). Furthermore, h-region sequences are not truly random on a gross level because all of the possible hydrophobic amino acids are not equally represented (von Heijne 1985; von Heijne and Abrahmsen 1989). Different species have preference for specific amino acids in their h-regions; *Saccharomyces cerevisiae* shows preference for serine while *E. coli* favors alanine (von Heijne 1981; Nielsen, Engelbrecht et al. 1997) (Patham and

Mensa-Wilmot, unpublished). Finally, recent re-evaluation of h-regions from *E. coli*, *S. cerevisiae*, and humans has revealed that each species has a unique set of h-motifs in its signal peptides (Patham and Mensa-Wilmot, unpublished).

While it cannot be denied that high hydrophobicity contributes to translocation of signal peptides into the ER, we find that peak hydrophobicity alone is not sufficient for signal peptide activity. Moreover, the arrangement of key amino acid residues (hydrophobic or polar) into motifs in an h-region (h-motifs) imparts physiological activity to a signal peptide.

Materials and Methods

Materials

Taq DNA polymerase, amino acid mixture minus methionine and cysteine, and nuclease treated rabbit reticulocyte lysate were obtained from Promega (Madison, Wisconsin). dNTPs were obtained from Sigma (St. Louis, Missouri). DEPC-treated water was from USB Corporation (Cleveland, Ohio); QIAquick PCR purification kit was from Qiagen (Valencia, California); Ampliscribe T7 *in vitro* transcription kit was from Epicentre (Madison, Wisconsin); TRAN35S - LABEL™ No-thaw metabolic labeling reagent was from MP Biomedicals (Solon, Ohio); cycloheximide was from Calbiochem (La Jolla, California); proteinase K and leupeptin were from Roche (Indianapolis, Indiana); PMSF was from Boehringer Mannheim (Mannheim, Germany); ethanol and urea were from Fisher (Norcross, Georgia); Acrylagel and Bis-Acrylagel were from National Diagnostics (Atlanta, Georgia); TEMED and ammonium persulfate were from Bio-Rad (Hercules, California); DE52 was from Whatman (Hillsboro, Ohio). All other chemicals and reagents were obtained from Sigma (St. Louis, Missouri).

Plasmids

All VSG-117 mutants were made through PCR mutagenesis (Innis, Myambo et al. 1988) of a plasmid template, pVSG-117 (Bangs, Brouch et al. 1996), provided by Dr. James D. Bangs (University of Wisconsin).

Cell Strain and Isolation

Monomorphic *Trypanosoma brucei* strain 427 were grown in and purified from Sprague Dawley® rats, using chromatography on DE52 (Cross 1975).

Primer Construction For VSG-117 h-Region Mutants

PCR mutagenesis was used to create VSG-117 h-region mutants from the template, pVSG-117, using variations of the forward primer *TAATACGACTCACTATAGGGaggagggttttac*
*c*ATGGACTGCCATACAAAGGAGACACTGGGGTCACACAATGGAGGCGATCAACGATG
ttcACACTATCAttcttcTACttcATCACTCCAGCG. Each primer contained a T7 promoter (upper case italicized) and a translational enhancer (lower case underlined) (Teilhet, Rashid et al. 1998). The coding region of VSG-117 starts at position 36 and ends at primer position 83 (upper case); it includes the n-region of VSG-117. This is followed by primer positions 84-128, which encodes for the h-region of the VSG-117 protein (upper case, italicized, and underlined). All VSG-117 h-region mutants contained mutations within the forward primer as exemplified above in the (Phe)₄-VSG-117₈₆ mutant (lower case, italicized, underlined nucleotide sections contained within the h-region). All VSG-117 proteins were truncated to 86 amino acids, using the reverse primer *cgaacaacgaagggttcTTATAGTGCGTAGATCGTAGCTTCGTTTC*, which has a stop codon (upper case underlined) after the 27 nucleotides priming for nucleotides 295-321 (upper case italicized) of the pVSG-117 sequence.

Generation of VSG-117 Mutant DNA

VSG-117 PCR products were generated in 100 µl reactions mixtures containing 224 ng pVSG-117 template, 0.5 µM of each forward and reverse primer, Innis buffer (10 mM Tris-HCl pH 8, 2.5 mM MgCl₂, 0.05% Tween-20, 0.05% nonidet P-40, 50 mM KCl) (Innis, Myambo et al. 1988), 250 µM dNTPs, and 5 units of Taq DNA polymerase. PCR reactions were carried out for 25 cycles as follows: 95°C for 90 seconds, 56°C for 90 seconds, and 74°C for 2 min. All PCR products were purified using a QIAquick PCR purification kit (Qiagen). Purified PCR products were quantitated at 260 nm.

***In Vitro* Transcription**

One µg of purified DNA was used as a template for transcription with an Ampliscribe™ T7 kit (Epicentre Technologies). One µl (1 MBU) of RNase free DNase I was added to the reaction (20 µl) and allowed to incubate at 37°C for 15 minutes. The mixture was extracted with an equal volume of TE-saturated phenol/chloroform, and the aqueous phase extracted with chloroform. To the aqueous phase, sodium acetate (27 mM final concentration) was added along with 2.5 times the total volume of 100% ethanol. This mixture was allowed to precipitate at -20°C overnight. The precipitate was recovered at 16,100 x g at 4°C for 15 minutes, rinsed with 70% ethanol, and pelleted at 16,100 x g. The pellet was allowed to air dry, and resuspended in 40 µl of nuclease free water. The RNA concentration was determined by measuring the absorbance at 260 nm.

Microsome Preparation from Bloodstream Form *Trypanosoma brucei*

Bloodstream form *T. brucei* lister 427 (1×10^{10} cells/ml) were resuspended in 5 ml of fresh homogenization buffer (HB) (250 mM sucrose, 50 mM HEPES-KOH, 50 mM KOAc, 6 mM Mg(OAc)₂, 1 mM EDTA, 1 mM DTT, 1 µg/ml TLCK, 5 µg/ml leupeptin, 0.5 mM PMSF).

In a clean, pre-chilled dounce homogenizer 2.5 ml of the resuspended cells were lysed on ice by 2 repetitions of 40 strokes with a tight-fitting pestle, with a one minute break after 40 strokes. The homogenates were pooled, aliquoted into microcentrifuge tubes (1 ml/tube), and centrifuged at 400 x g for 10 minutes at 4°C. The supernatants from all tubes were pooled, aliquoted into a new microcentrifuge tube, and centrifuged at 13,400 x g for 20 minutes at 4°C. The supernatants from the 13,400 x g centrifugation step were recovered and pooled. Pellets from the 13,400 x g centrifugation step were resuspended in 50 µl/pellet (total volume) of fresh rough microsome buffer (RMB) (250 mM sucrose, 50 mM HEPES-KOH, 50 mM KOAc, 1 mM DTT, 0.5 µg/ml TLCK, 2.5 µg/ml leupeptin). The microsome concentration was determined by measuring the absorbance at 260 nm, and the concentration was adjusted to an OD₂₆₀ of 50 (1 equivalent = OD₂₆₀ of 50 = 1 µl). The microsomes were aliquoted into 20 µl portions, quick frozen in liquid nitrogen, and stored at -80°C (pellets from the 400 x g centrifugation and the supernatant from the 13,400 x g centrifugation were also saved).

***In Vitro* Translation and Import of VSG Into *T. brucei* Microsomes (TbRM)**

RNA (250 – 500 ng) in DEPC-treated H₂O (7 µl) was incubated at 65°C for three minutes. After cooling on ice for one minute, 1.5 µl (75 µM final concentration) of an amino acid mixture lacking methionine and cysteine (Promega), 1.5 µl of ³⁵S-cysteine and methionine (2.5 mCi total activity, MP Biomedicals), and 10 µl of rabbit reticulocyte lysate (Promega) was added to the RNA mixture, bringing the final volume to 20 µl. This reaction mixture was incubated at 37°C for 1 hour. Translation was stopped with cycloheximide (1 mM final concentration).

The translation reaction was then divided into four separate portions of 5 µl each. Two of these portions were left untreated while the remaining two portions were supplemented with 1 µl

(1 μl = 1 equivalent, which has an OD_{260} of 50) of TbRM. The four reaction aliquots were further incubated at 37°C for 1.5 hours and then transferred to ice. Next, one of each sample (translation mixture with or without TbRM) was treated with a proteolysis mixture containing urea (3M) and proteinase K (0.5 mg/ml) (final concentrations) on ice for 1 hour. PMSF (34 mM final concentration) was added to quench the proteolysis reaction. Proteins were precipitated with 60% $(\text{NH}_4)_2\text{SO}_4$ on ice for 15 minutes, and centrifuged at 16,100 x g at 4°C for 7 minutes. The pellets were resuspended in 20 μl of 2.5x SDS sample buffer and resolved by SDS-PAGE in a Tris-Tricine gel system (16% - Resolving; 3% - Stacking) (reviewed in (Gallagher 2007)). Radioactive polypeptides were detected with a phosphorimager; bands were quantitated with Quantity-One software (version 4.6.5, Bio-Rad).

Quantitation of Percent VSG Import

Using Quantity-One software (version 4.6.5, Bio-Rad), phosphorimages were adjusted so that gel bands corresponding to proteinase K-protected VSG-117₈₆ were visible. Next, background noise signals were subtracted using the “filter wizard” application. A Tiff image preserving these adjustments to the gel image was acquired. From this Tiff image, gel bands in each lane were quantitated by measuring the volume (counts*mm²) of the band representing VSG-117₈₆. For background signal, the volume of an area in each lane of the gel image that best represented the average background pixel intensity was measured. In band and background intensity quantitation, the dimensions of the volume boxes were maintained. Next, a volume analysis report was performed to calculate the “adjusted volumes” (volume of VSG-117₈₆ band – volume of average background bands) of the VSG-117₈₆ bands. VSG import percentages were obtained as follows: the “adjusted volume” corresponding to a band detected in the presence of TbRM and challenged with proteinase K was divided by the “adjusted volume” corresponding to

a band detected in the presence of TbRM without proteinase K. This quotient was multiplied by 100 to obtain a percentage of VSG import.

Results

Factors Other Than h-Region Peak Hydrophobicity May Govern Protein Import into ER Microsomes

The hydrophobic core (h-region) of a signal peptide is necessary for protein import into the ER (reviewed in (Hegde and Bernstein 2006)). Surprisingly, h-region sequences are not conserved. It has been suggested that an h-region is composed of random hydrophobic amino acids, and that the overall hydrophobic nature of this region governs signal peptide activity (Ryan, Duncan et al. 1986; Kaiser, Preuss et al. 1987; Chou and Kendall 1990; Doud, Chou et al. 1993; Ryan, Robbins et al. 1993; Tomilo, Wilkinson et al. 1994; Ng, Brown et al. 1996). However, not all h-regions of similar hydrophobicity are equivalent in function (Abrahmsen, Moks et al. 1985; Bird, Gething et al. 1987; Laforet, Kaiser et al. 1989; Al-Qahtani, Teilhet et al. 1998; Zheng and Nicchitta 1999) (reviewed in (Zheng and Gierasch 1996; Hegde and Bernstein 2006)). Therefore, we have revisited the role of h-region hydrophobicity in protein import into the ER with the hope of offering new perspectives.

We chose to evaluate the role of h-region hydrophobicity in ER protein import in *T. brucei* using a truncated VSG-117 substrate (86 amino acids long) and a *T. brucei* cell-free translocation system. VSG-117 is imported into TbRM (Patham et al., submitted), but it is not imported into canine microsomes (Al-Qahtani, Teilhet et al. 1998), although VSG-117 possesses similar h-region hydrophobicity as other proteins that are imported into canine microsomes. From this, and other data (Abrahmsen, Moks et al. 1985; Bird, Gething et al. 1987; von Heijne and Abrahmsen 1989; Zheng and Nicchitta 1999), we formed a hypothesis that h-region

characteristics other than hydrophobicity may be responsible for ER protein import, and we sought to determine how an h-region directs import of VSG-117 into TbRM.

To examine the importance of h-region hydrophobicity in ER protein import we created h-region mutants from VSG-117 (VSG-117_h) where residues 4, 8, 9, and 11 were simultaneously mutated from Leu₄, Leu₈, Leu₉, Ala₁₁ to Gly₄, Gly₈, Gly₉, Gly₁₁ or Ser₄, Ser₈, Ser₉, Ser₁₁, creating the mutants (Gly)₄-VSG-117_h and (Ser)₄-VSG-117_h, respectively (Table 1). Unmutated VSG-117_h (VSG-117_h) contains seven hydrophobic amino acids (Table 1), and by mutating four of these hydrophobic amino acids to the non-hydrophobic amino acids glycine or serine the peak hydrophobicity was reduced by approximately 75% from 2.08 to 0.43 or 0.30, respectively (Fig. 1G). Because of the drop in peak hydrophobicity, VSG import into TbRM was expected to decrease in both (Gly)₄-VSG-117₈₆ and (Ser)₄-VSG-117₈₆. Curiously, while only 8% of (Gly)₄-VSG-117₈₆ was imported into TbRM, 44% of (Ser)₄-VSG-117₈₆ was imported into TbRM (Fig. 1A-C and 1G).

To test whether or not import of (Ser)₄-VSG-117₈₆ into TbRM was an anomaly, a new h-region mutant was created by mutating all seven hydrophobic amino acids in the VSG-117 h-region to alanine (*i.e.* seven alanine residues at positions 3, 4, 6, 8, 9, 11, & 12), creating (Ala)₇-VSG-117_h (Table 1). (Ala)₇-VSG-117_h has a peak hydrophobicity more than twice that of (Ser)₄-VSG-117_h (Fig. 1G), and was predicted to be imported into TbRM with greater efficiency than the former signal peptide. Surprisingly, (Ala)₇-VSG-117₈₆ was not imported into TbRM (1%) (Fig. 1D and Fig. 1G). This data is reminiscent of studies of *E. coli* signal peptides; a model h-region comprised of polyalanine is non-functional (Doud, Chou et al. 1993). However, activity was restored to this polyalanine signal peptide when more hydrophobic amino acids were introduced into the peptide. Using these data as precedent, amino acids at positions (*i*) 12

or (ii) 3, 6, and 12 from VSG-117_h were introduced back into (Ala)₇-VSG-117_h, making the new h-region mutants (Ala)₆-Ile12-VSG-117_h or (Ala)₄-VSG-117_h, respectively (Table 1). Changing Ala₁₂ in (Ala)₇-VSG-117_h to Ile₁₂ in (Ala)₆-Ile12-VSG-117_h raised the hydrophobicity from 0.89 in (Ala)₇-VSG-117_h to 1.21 in (Ala)₆-Ile12-VSG-117_h. Likewise, mutating Ala₃, Ala₆, and Ala₁₂ in (Ala)₇-VSG-117_h to Leu₃, Leu₆, and Ile₁₂ in (Ala)₄-VSG-117_h increased the hydrophobicity from 0.89 in (Ala)₇-VSG-117_h to 1.41 in (Ala)₄-VSG-117_h (Fig. 1G). Interestingly, 18% of (Ala)₆-Ile12-VSG-117₈₆ was imported into TbRM while 82% of (Ala)₄-VSG-117₈₆ was translocated into TbRM (Fig. 1E-F and 1G).

The data from translocation of (Gly)₄-VSG-117₈₆, (Ser)₄-VSG-117₈₆, (Ala)₇-VSG-117₈₆, (Ala)₆-Ile12-VSG-117₈₆, and (Ala)₄-VSG-117₈₆ showed that as peak hydrophobicity increases VSG import into TbRM did not increase correspondingly (Fig 1G). In fact, only (Ser)₄-VSG-117₈₆ and (Ala)₄-VSG-117₈₆ were imported into TbRM in significant amounts (44% and 82% respectively). The inability of (Gly)₄-VSG-117₈₆, (Ala)₇-VSG-117₈₆, and (Ala)₆-Ile12-VSG-117₈₆ to be imported into TbRM convincingly (Fig. 1B, 1D, 1E, and 1G) seems to be at odds with data suggesting that increases in h-region hydrophobicity increases ER protein import efficiency. (Gly)₄-VSG-117₈₆, (Ala)₇-VSG-117₈₆, and (Ala)₆-Ile12-VSG-117₈₆ all have h-region hydrophobicity values higher than that of (Ser)₄-VSG-117₈₆ (0.43, 0.89, and 1.21 vs. 0.3, respectively) (Fig. 1G). One would expect that h-regions with hydrophobicity values greater than 0.3 would be imported into TbRM; however, this was not observed. These data show how h-region hydrophobicity fails to predict the biological activity of a signal peptide, and do not support the idea that peak hydrophobicity is the major factor governing protein import into TbRM. Consequently, we investigated two factors that had potential to be responsible for VSG

import into microsomes; (a) kinetics of protein translocation into the ER, and (b) arrangement of amino acids within the h-region.

Kinetics of VSG-117₈₆ and (Ala)₄-VSG-117₈₆ Import into TbRM

We compared how hydrophobicity of VSG-117₈₆ (2.08 hydrophobicity units, Fig. 1G) and (Ala)₄-VSG-117₈₆ (1.41 hydrophobicity units, Fig. 1G) affected import of VSG into TbRM by evaluating the rate and extent of translocation of the two signal peptides.

A time-course of VSG import into TbRM showed that (Ala)₄-VSG-117₈₆ is translocated into TbRM at a slower rate than VSG-117₈₆. Whereas half of the total amount of VSG-117₈₆ is imported into TbRM within approximately 0.5 h (Fig 2A and 2C), it took 1 h for half of the total amount of (Ala)₄-VSG-117₈₆ to be imported into TbRM (Fig 2B-C). However, both VSG-117₈₆ and (Ala)₄-VSG-117₈₆ achieve maximum accumulation at 1.5 hours (81% and 75%, respectively) (Fig. 2A-C). From this data we infer that a hydrophobicity difference of 0.67 (approximately 30%) between the h-regions of VSG-117₈₆ and (Ala)₄-VSG-117₈₆ affected the rate of VSG import up to the 60 minute time-point. At 90 minutes and beyond, only differences in extent of VSG import into TbRM are discernable. Therefore, any reduction in import of a VSG-117 h-region mutant at the 90-minute time-point is not a consequence of slower import kinetics.

Arrangement of Amino Acids in h-Region Affects VSG Import into TbRM

If peak hydrophobicity could not explain the failure to import (Gly)₄-VSG-117₈₆, (Ala)₇-VSG-117₈₆, and (Ala)₆-Ile12-VSG-117₈₆ into TbRM (Fig. 1B, 1D, 1E, and 1G), what h-region characteristics influenced the process? One hypothesis to explain these data is that the arrangement of h-region amino acids is important for signal peptide activity. To test this hypothesis, new VSG-117 h-regions were designed by scrambling the sequences of (Ala)₄-VSG-

117_h and (Ser)₄-VSG-117_h, producing “scrambled (Ala)₄-VSG-117_h” and “scrambled (Ser)₄-VSG-117_h”, respectively (Table 2). Scrambling the (Ala)₄-VSG-117_h sequence reduced peak hydrophobicity by approximately 0.3 units (24% reduction), while “scrambled (Ser)₄-VSG-117_h” differed from (Ser)₄-VSG-117_h by only 0.01 units (3% reduction) (Fig. 3F). Also, a variant of (Ala)₄-VSG-117_h was created by exchanging tyrosine at position 10 for alanine at position 9, yielding (Ala)₄-Tyr9-VSG-117_h (Table 2). Because (Ala)₄-Tyr9-VSG-117_h was created from (Ala)₄-VSG-117_h by switching the positions of two amino acids, the peak hydrophobicity was not affected (Fig. 3G). Since the amino acid composition of each sequence was not altered, any change in translocation competence would be attributable to amino acid sequence alterations.

Microsomal import assays comparing “scrambled” h-region mutants to their unscrambled counterparts showed a drastic reduction in the import of the “scrambled” mutants. (Ala)₄-VSG-117₈₆ was imported into TbRM at approximately 86% while only 14% of “scrambled (Ala)₄-VSG-117₈₆” was imported into TbRM (Fig. 3B-C and 3G). Approximately 70% of (Ser)₄-VSG-117₈₆ was imported into TbRM while 27% of “scrambled (Ser)₄-VSG-117₈₆” was imported into the microsomes (Fig. 3D-E and 3G). Similarly, 64% of (Ala)₄-Tyr9-VSG-117₈₆ was imported into TbRM, whereas 84% of (Ala)₄-VSG-117₈₆ was translocated into TbRM (Fig. 3B and 3F-G). These data support the principle that arrangement of h-region amino acids is a crucial factor for translocation of VSG into TbRM.

Discovery of Peptide Motifs in *T. brucei* h-Regions

Our data indicated that h-region amino acid sequence was crucial to VSG import into TbRM (Fig. 3A-G). However, an alignment of trypanosome signal peptides fails to unearth a consensus sequence. To resolve this conundrum, we hypothesized that h-region amino acids formed peptide motifs that could not be discovered with sequence alignments. In an initial test

of this hypothesis, we analyzed 50 h-regions of secretory proteins from *T. brucei*. Through computational peptide pattern searches we identified motifs (Table 3) (Patham et al., in preparation).

An h-region motif (h-motif) has three “identity components” and a “wild card region”. There are two types of “identity components”; (i) a “fixed identity component” is a unique amino acid, and (ii) a “variable identity component”, which is a position that can be filled by one of a defined set of amino acids. The “wild card region” separates “identity components”; it can be one or multiple amino acids. For example, the h-motif L-x(1,2)-L-[AILV] possesses three “identity components”. The first two are “fixed identity components”, namely leucine (L), where the first leucine is separated from the second leucine by one or two “wild card” residues. The last “identity component” is a “variable identity component” that can be alanine (A) or isoleucine (I) or leucine (L) or valine (V) (*i.e.* [AILV]).

Three h-motifs were discovered in *T. brucei* (L-L-x-[AILV], L-x(1,2)-L-[AILV], and L-x(2,3)-L-[AILPV]) (Table 3). Of the 50 signal peptides, 37 (74%) contained at least one *T. brucei* h-motif. Most of the signal peptides contain multiple *T. brucei* h-motifs. This is exemplified by VSG-117, whose h-region contains one of each h-motif (Table 3). These h-motifs are different in organization and “identity component” composition from those h-motifs identified in yeast, *E. coli*, and humans (Patham et al., in preparation).

Experimental Data Can Be Used to Expand “Identity Components” of Bioinformatically Identified h-Motifs

Existence of h-motifs does not prove their biological relevance. Therefore, we hypothesized that h-motifs are important for signal peptide activity. To evaluate this hypothesis, we mutated the four positions necessary to form every motif in the h-region of VSG-117.

Residues at h-region positions 4, 8, 9, and 11 (Leu₄, Leu₈, Leu₉, and Ala₁₁, respectively) were all changed to phenylalanine or valine (residues that were not discovered as “fixed identity components” in our data set), resulting in the new h-region mutants (Phe)₄-VSG-117_h and (Val)₄-VSG-117_h, respectively (Table 4). Although the mutants lack h-motifs (Table 3), their hydrophobicities were similar to that of VSG-117_h (Fig. 4D). Therefore, any reduction in protein import would be due principally to the loss of the h-motif. Both (Phe)₄-VSG-117₈₆ and (Val)₄-VSG-117₈₆ were imported into TbRM with similar efficiencies (78% and 83%, respectively) as compared to VSG-117₈₆ (99%) (Fig. 4A-D). These data were compared with earlier ones (Figs. 1B-G and 4A-D) to obtain a general picture of the contributions of h-motifs to signal peptide activity.

The signal peptides of (Ala)₄-VSG-117₈₆ and (Ser)₄-VSG-117₈₆ were efficient at directing protein import into TbRM (Fig. 3B, 3D, and 3G). However, scrambling the h-region sequences of (Ala)₄-VSG-117₈₆ and (Ser)₄-VSG-117₈₆ caused a drastic reduction in the ability of these signal peptides to direct import into TbRM (Fig. 3C, 3E, and 3G). This indicated that the arrangement of h-region amino acids is crucial for signal peptide activity. Bioinformatic analysis of *T. brucei* signal peptides suggests that h-region amino acids are arranged into h-motifs where specific hydrophobic amino acids serve as “identity components” (Table 3). Data from “scrambled (Ala)₄-VSG-117₈₆” and “scrambled (Ser)₄-VSG-117₈₆” combined with bioinformatic data suggests that the rearrangement of h-region amino acids in “scrambled (Ala)₄-VSG-117₈₆” and “scrambled (Ser)₄-VSG-117₈₆” disrupted the arrangement of h-region amino acids serving as “identity components” in these h-motifs. This, in turn, affected the ability of these signal peptides to direct import into TbRM. Like (Ser)₄-VSG-117₈₆ and (Ala)₄-VSG-117₈₆, (Phe)₄-VSG-117₈₆ and (Val)₄-VSG-117₈₆, were imported into TbRM efficiently (Fig. 1C, 1F, 1G, and

4A-D) while (Gly)₄-VSG-117₈₆, (Ala)₇-VSG-117₈₆, and (Ala)₆-Ile12-VSG-117₈₆ were barely imported into TbRM (Fig. 1B, 1D, 1E, and 1G). However, all VSG-117_h mutants with functional signal peptides or non-functional signal peptides lack *T. brucei* h-motifs identified by bioinformatics (compare sequences in Table 1 and 4 with motifs in Table 3). To reconcile this dilemma the bioinformatic and experimental data were combined. We conclude that “identity components” are important for efficient protein import into TbRM, and only a select set of amino acids can serve as “identity components” for a signal peptide to function. However, our data indicate that *T. brucei* h-motifs allow hydrophobic residues (or serine residues) other than those found by bioinformatics to serve as “identity components”.

VSG-117 h-Regions do Not Require Leucines

Mammalian h-regions are leucine-rich (von Heijne 1981; von Heijne and Abrahmsen 1989; Izard and Kendall 1994). Experimental demonstration of a requirement for a leucine in a mammalian h-region was obtained in a study of *E. coli* proLamB. proLamB was non-functional at the canine microsome, but gained translocation activity into CfRM when leucine was substituted for alanine residues in its h-region (Zheng and Nicchitta 1999). In an earlier study, replacement of glycine with leucine in the h-region of *S. cerevisiae* carboxypeptidase Y allowed the yeast protein to be imported into canine microsomes (Bird, Gething et al. 1987). The importance of leucine in trypanosome signal peptides has not been tested directly.

The majority of VSG-117 signal peptide variants that were imported into TbRM possessed at least one leucine in their h-regions. However, data from (Gly)₄-VSG-117₈₆, “scrambled (Ala)₄-VSG-117₈₆”, and “scrambled (Ser)₄-VSG-117₈₆” showed that leucine is not sufficient for efficient VSG import into TbRM because each of these h-regions contained leucine, yet they were imported into TbRM in negligible amounts (Fig. 1B, 1G, 3C, 3E, and 3G).

Nevertheless, leucine is found in all h-motifs identified in *T. brucei* (Table 3). From these facts we hypothesized that while leucine is not sufficient for signal peptide activity, it could facilitate efficiency of h-region activity at TbRM. To investigate this idea we made new variants of (Ala)₄-VSG-117_h where its sole leucine at position six was mutated to other hydrophobic amino acids, namely, phenylalanine, valine, or isoleucine, creating the mutants (Ala)₄-Phe6-VSG-117_h, (Ala)₄-Val6-VSG-117_h, and (Ala)₄-Ile6-VSG-117_h, respectively (Table 5). Since (Ala)₄-VSG-117_h, (Ala)₄-Phe6-VSG-117_h, (Ala)₄-Val6-VSG-117_h, and (Ala)₄-Ile6-VSG-117_h only differed by one amino acid at position six their h-region peak hydrophobicity values were similar (Fig. 5F). Therefore, any difference in the extent of VSG import among these h-regions is not attributable to peak hydrophobicity.

The h-regions were imported into TbRM with different efficiencies; import efficiencies for (Ala)₄-Phe6-VSG-117₈₆, (Ala)₄-Val6-VSG-117₈₆, and (Ala)₄-Ile6-VSG-117₈₆ were 71%, 54%, and 40%, respectively (Fig. 5A-F). Therefore, while mutating leucine to phenylalanine altered VSG import efficiency into TbRM by 6%, substitution of leucine with valine or isoleucine caused significant reduction (14% or 28%, respectively) in the amount of VSG imported into TbRM (Fig. 5F).

We conclude that leucine in the VSG-117 h-region is not essential for signal peptide activity; other hydrophobic residues can be used in the signal sequence. Further, as the hydrophobicity of the amino acid occupying position six of (Ala)₄-VSG-117_h increased the efficiency of import decreased (Fig. 5A-F), suggesting a hierarchy of hydrophobic residues ($L \geq F > V > I$) in that position of the h-region.

Discussion

Arrangement of h-Region Amino Acids Is Important For Signal Peptide Function

Cell surface protein expression is essential for cell viability in all biological kingdoms. Instrumental to entry into the secretory pathway is protein translocation into the ER (Crowley, Liao et al. 1994; Mothes, Prehn et al. 1994) (reviewed in (Shimizu and Hendershot 2007)), a process that requires an ER signal peptide (Blobel and Dobberstein 1975; Lingappa and Blobel 1980) (reviewed in (Schatz and Dobberstein 1996; Hegde and Bernstein 2006)). To date the best model available to explain signal peptide function relies heavily on h-region peak hydrophobicity as the determinant of activity (Ryan, Duncan et al. 1986; Chou and Kendall 1990; Doud, Chou et al. 1993; Ryan, Robbins et al. 1993; Tomilo, Wilkinson et al. 1994; Ng, Brown et al. 1996). Although many experiments support this paradigm, there are numerous exceptions to this dogma (Abrahmsen, Moks et al. 1985; Bird, Gething et al. 1987; Laforet, Kaiser et al. 1989; von Heijne and Abrahmsen 1989; Ryan and Edwards 1995; Al-Qahtani, Teilhet et al. 1998; Matoba and Ogrydziak 1998; Ramirez, Boscardin et al. 1999). These observations bolster two ideas; *(i)* h-region hydrophobicity alone is not sufficient to impart signal peptide function, and *(ii)* the h-region sequence contains cryptic information that is crucial for signal peptide activity.

Three sets of our data show that h-region peak hydrophobicity cannot predict import of VSG-117 h-region variants into TbRM (Fig 1A-G) (also see (Al-Qahtani, Teilhet et al. 1998)). First, introduction of four serine residues into specified positions of the VSG-117 h-region (Table 1) produced an h-region with one of the lowest peak hydrophobicity values in this study (Fig. 1G), yet it was imported into TbRM (Fig. 1C and 1G). In contrast, (Gly)₄-VSG-117₈₆ was imported into TbRM in negligible amounts even though the peak hydrophobicity of this h-region was very similar to that of (Ser)₄-VSG-117₈₆ (Fig. 1G). Further, (Ala)₇-VSG-117₈₆ and (Ala)₆-

Ile12-VSG-117₈₆ possessed even higher h-region peak hydrophobicities, but were not translocated convincingly (1% and 18%, respectively) (Fig. 1D-E and 1G). Second, import of “scrambled (Ser)₄-VSG-117₈₆” (Fig. 3D-E and 3G) was the most striking example of how h-region peak hydrophobicity does not solely govern signal peptide function. Peak hydrophobicity of “scrambled (Ser)₄-VSG-117_h” differed by only 0.01 from (Ser)₄-VSG-117_h, yet 27% of “scrambled (Ser)₄-VSG-117₈₆” was imported into TbRM while 70% of (Ser)₄-VSG-117₈₆ was imported into TbRM (Fig. 3D-E and 3G). Third, exchanging amino acids at positions Ala₉ and Tyr₁₀ in (Ala)₄-VSG-117₈₆ for Tyr₉ and Ala₁₀ in (Ala)₄-Tyr9-VSG-117₈₆ (Table 2) did not alter the peak hydrophobicity of (Ala)₄-VSG-117₈₆ and (Ala)₄-Tyr9-VSG-117₈₆, but caused a significant reduction in import (22%) of (Ala)₄-Tyr9-VSG-117₈₆ into TbRM (Fig. 3B and 3F-G). These data make a compelling case that peak hydrophobicity of the h-region is not the sole determinant of signal peptide activity.

h-Regions Contain Amino Acid Motifs That Are Important for Biological Function of Signal Peptides

To discover what other information, apart from hydrophobicity, that might be present in h-regions we used bioinformatic approaches (Jonassen, Collins et al. 1995) to analyze h-regions from *T. brucei*, and discovered three motifs (Table 3). This observation supports the idea that h-region sequences are not random, as previously believed (Kaiser, Preuss et al. 1987) (reviewed in (Zheng and Gierasch 1996)).

The functional significance of h-motifs was evaluated experimentally by mutating “identity component” positions 4, 6, 8, 9, and 11 (Tables 1, 4, and 5). The data showed that hydrophobic amino acids at positions 4, 8, 9, and 11 could be replaced with other hydrophobic amino acids and also with serine (Fig. 1B, 1C, 1F, 1G, and 4B-D). Further, leucine at h-region

position six could be functionally replaced with phenylalanine, valine, or isoleucine (Fig. 5A-F). However, hydrophobicity of the amino acid occupying this position appeared to be inversely proportional to efficiency of VSG import for that h-region. This begs the question that if other hydrophobic amino acids and serine can serve as identity components why were they not found in the bioinformatic analysis?

One possible explanation as to why h-motifs containing phenylalanine, valine, isoleucine, or serine were not identified (Table 3) is that proteins containing them were not a significant proportion of the *T. brucei* proteome. Our bioinformatic analysis would not score motifs from small sets because they did not comprise 52% (PRATT minimum percentage (C%)) of the total input data set.

Natural selection and signal peptide competition for ER entry could also explain why some h-motifs direct VSG import *in vitro*, but are not detected in trypanosome signal peptides. Mutations could have created h-region sequences that were preferred by the translocation machinery over other h-regions. Thus, while they still directed import *in vivo* these less efficient h-region sequences might have been out-competed by the more efficient signal peptides. Similarly, an example of signal peptide competition in *E. coli* showed that advantageous mutations in a signal peptide corresponded with preferential use of this more efficient signal peptide over its non-mutated counterpart to direct protein translocation (Chen, Kim et al. 1996). Alternatively, h-region sequences that direct import *in vitro* might be too efficient *in vivo*, out-competing signal peptides that direct import of proteins essential for viability, possibly causing cells to be non-viable. As such, these signal peptides would not be preserved during evolution.

Collectively, our data supports the idea that arrangement of hydrophobic amino acid within an h-region is crucial for the biological activity of a signal peptide. We propose that h-

region amino acids are arranged into motifs, and it is the presence of these h-motifs that direct protein translocation into the ER. In this scenario, we argue that h-regions are hydrophobic because they contain h-motifs that possess predominantly hydrophobic “identity components”. The elucidation of h-motifs in signal peptides indicates that primary sequence has a role in signal peptide activity.

Hypothesis: h-Motifs Interact With TM2b and/or TM7 of Sec61 α

Signal peptides are proposed to have a high propensity to form α -helices (Bruch and Gierasch 1990; Izard, Doughty et al. 1995). Further, we have insight about how the ER protein translocation pore, Sec61 α , may recognize a signal peptide. From chemical cross-linking studies in *S. cerevisiae*, and X-ray crystal structure of Sec61 α , it has been proposed that transmembrane domains (TMs) 2b and 7 of Sec61 α are in proximity to signal peptides (Plath, Mothes et al. 1998; Van den Berg, Clemons et al. 2004). However, it is not known what areas of signal peptide h-regions are contacted by Sec61 α . Because of this data, helical-wheel modeling of the VSG-117 h-region was used to offer a structure-based explanation of how h-motifs might interact with the Sec61 translocon and confer biological function to a signal peptide.

T. brucei h-motifs Tb1-3 (Table 3) specify positions 4, 6, 8, 9, and 11 of the VSG-117 h-region as “identity components”. When the VSG-117 h-region is arranged into an α -helix, the “identity components” at h-region positions 4, 8, and 11 are placed on one side of an α -helix. Interestingly, the “identity components” at h-region positions 6 and 9 are positioned on the other half of the α -helix of the h-region (Fig. 6). Such positioning of h-motif “identity components” could allow amino acids at those positions to make concurrent contacts with transmembrane helices TM2b and/or TM7 of Sec61 α (Fig. 6 and 7A-D) (Van den Berg, Clemons et al. 2004). Chemical cross-linking studies and a Sec61-signal peptide model from yeast (Plath, Mothes et al.

1998) suggests that h-region amino acids on opposite sides of the signal peptide α -helix could make three contacts with TM2b of Sec61 α , and two contacts with TM7 of Sec61 α . Using this as a model, we propose that the three h-region “identity components” in positions 4, 8, and 11 contact one half of the signal peptide-binding site of Sec61 α (possibly at TM2b) while the two h-region “identity components” in positions 6 and 9 contact the other half of the Sec61 α signal peptide-binding site (possibly at TM7) (Fig. 7A-D).

The data presented here sheds new light on a basic but unresolved problem in cell biology: what is an ER signal peptide? What was once thought to be a random set of hydrophobic residues turns out to be unexpectedly more complex than originally anticipated: Hydrophobic cores are non-charged “scaffolds” in which h-motifs, important for signal peptide function, are embedded.

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Table 1 – Amino Acid Sequences of VSG-117_h, (Gly)₄-VSG-117_h, (Ser)₄-VSG-117_h, (Ala)₇-VSG-117_h, (Ala)₆-Ile12-VSG-117_h, and (Ala)₄-VSG-117_h

h-Region sequences of VSG-117_h, (Gly)₄-VSG-117_h, (Ser)₄-VSG-117_h, (Ala)₇-VSG-117_h, (Ala)₆-Ile12-VSG-117_h, and (Ala)₄-VSG-117_h are aligned. Position 1 denotes the beginning of the h-region and position 14 marks its end. Mutations to VSG-117_h are listed in bold.

Table 1

<u>VSG</u>	<u>Position of Amino Acids in h-region Sequence</u>													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
VSG-117 _h	S	T	M	L	T	L	S	L	L	Y	A	I	T	P
(Gly) ₄ -VSG-117 _h	S	T	M	G	T	L	S	G	G	Y	G	I	T	P
(Ser) ₄ -VSG-117 _h	S	T	M	S	T	L	S	S	S	Y	S	I	T	P
(Ala) ₇ -VSG-117 _h	S	T	A	A	T	A	S	A	A	Y	A	A	T	P
(Ala) ₆ -Ile12-VSG-117 _h	S	T	A	A	T	A	S	A	A	Y	A	I	T	P
(Ala) ₄ -VSG-117 _h	S	T	M	A	T	L	S	A	A	Y	A	I	T	P

Figure 1 –Import into *T. brucei* Microsomes and Hydrophobicity Properties of VSG-117₈₆, (Gly)₄-VSG-117₈₆, (Ser)₄-VSG-117₈₆, (Ala)₇-VSG-117₈₆, (Ala)₆-Ile12-VSG-117₈₆, and (Ala)₄-VSG-117₈₆ h-Regions

Import of (A) VSG-117₈₆, (B) (Gly)₄-VSG-117₈₆, (C) (Ser)₄-VSG-117₈₆, (D) (Ala)₇-VSG-117₈₆, (E) (Ala)₆-Ile12-VSG-117₈₆, and (F) (Ala)₄-VSG-117₈₆ into *T. brucei* Microsomes. mRNAs of VSG-117 variants truncated to 86 amino acids were translated in rabbit reticulocyte lysate.

Translation was stopped with cycloheximide, and the translation mixture was supplemented with *T. brucei* microsomes (TbRM), allowing translocation to occur for 1.5 hours at 37°C.

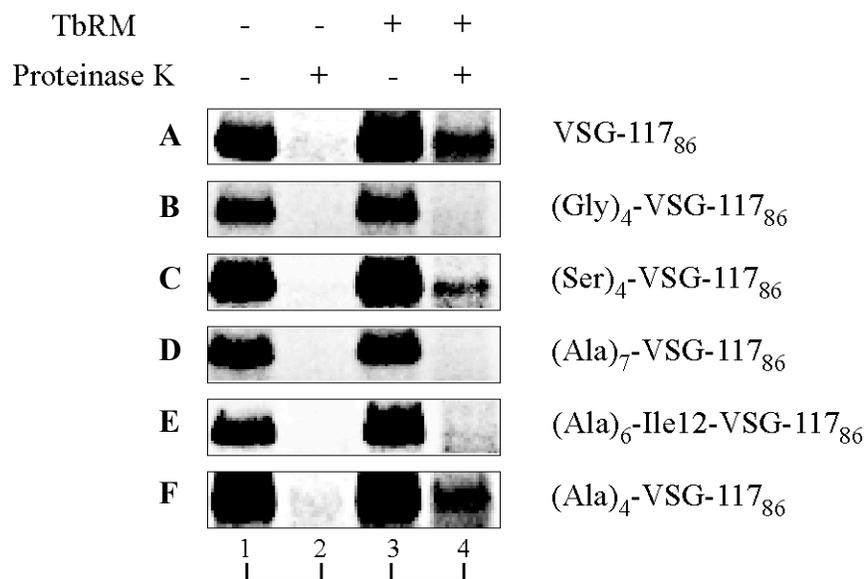
Translocation was stopped by incubation of the sample on ice, and import of the protein was tested with proteinase K (PK) digestion on ice for 1 hour. The samples were precipitated with ammonium sulfate, resolved on Tris-Tricine gels, and analyzed with a phosphorimager and Quantity One software. Gels pictured are representative results. (A-F) Lane 1: no TbRM, no PK; lane 2: no TbRM, with PK; lane 3: with TbRM, no PK; lane 4: with TbRM, with PK.

Brackets denote lanes that were compared to obtain percentage of VSG imported into TbRM.

(G) Peak hydrophobicity values (black bars) were obtained by Kyte-Doolittle hydrophobicity analysis (window = 7 amino acids), using LASERGENE (DNASTAR) (version 4.0.3).

Percentage of VSG imported (gray bars) from data in panels A-F was calculated by dividing the amount of the residual VSG detected after protease treatment (lane 4) by the amount of the VSG detected after the addition of TbRM with no protease treatment (lane 3) and multiplying the quotient by 100. Quantitation of percent VSG import may not correspond to gel image because control lanes (VSG detected in the presence of TbRM without proteinase K) were adjusted to pixel saturation.

Figure 1



G

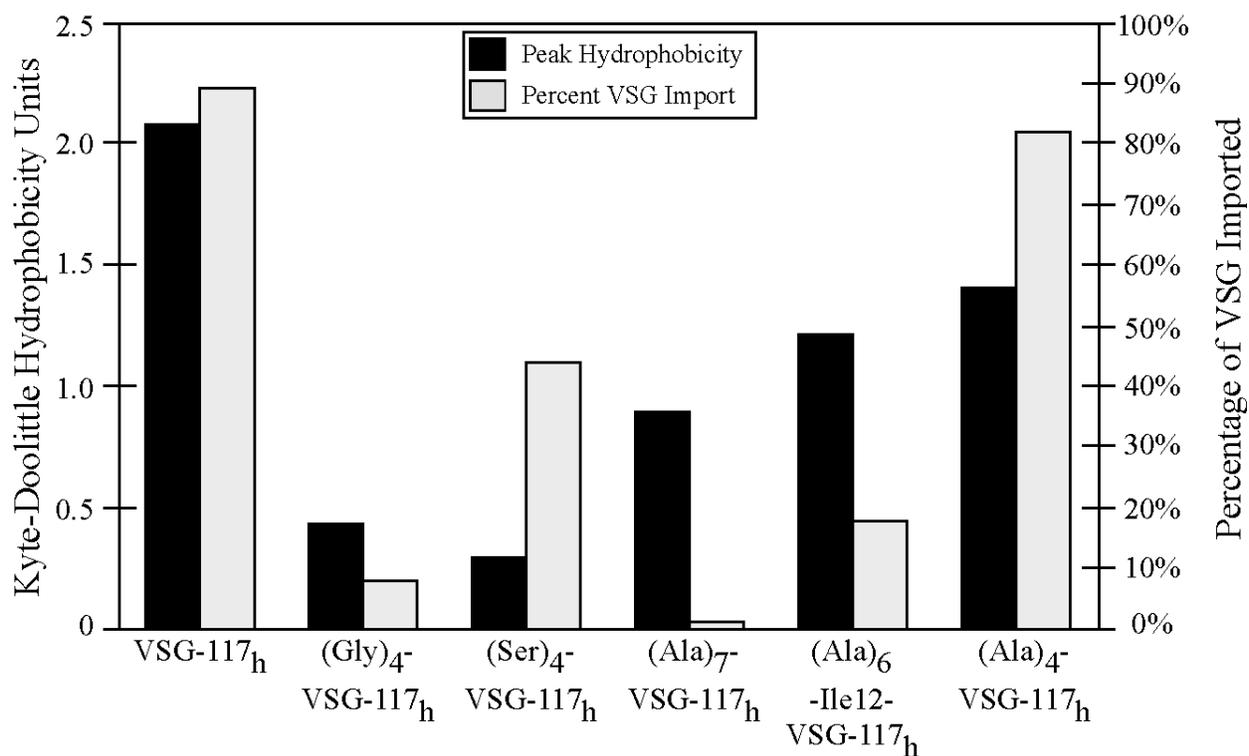


Figure 2 – Translocation Time Course of VSG-117₈₆ and (Ala)₄-VSG-117₈₆ into *T. brucei* Microsomes

(A) Time course of importing VSG-117₈₆ into TbRM. (B) Time course of importing (Ala)₄-VSG-117₈₆ into TbRM. mRNAs of VSG-117's truncated to 86 amino acids were translated in rabbit reticulocyte lysate. Translation was stopped with cycloheximide, and the translation mixture was supplemented with TbRM and incubated at 37°C. Translocation was allowed to occur for 0.25 hours (*lanes 1 & 2*), 0.5 hours (*lanes 3 & 4*), 1.0 hour (*lanes 5 & 6*), 1.5 hours (*lanes 7 & 8*), or 2.0 hours (*lanes 9 & 10*). Translocation was stopped by incubating the sample on ice. VSG import into TbRM was tested with proteinase K (PK) digestion on ice for 1 hour. Reaction mixtures were precipitated with ammonium sulfate, resolved on Tris-Tricine gels, and analyzed with a phosphorimager and Quantity One software. Gels pictured are representative results. *Lanes 1, 3, 5, 7, and 9* – TbRM present, no PK treatment; *lanes 2, 4, 6, 8, and 10* – TbRM present, PK present. Brackets denote lanes that were compared to obtain percentage of VSG imported into TbRM. (C) Graphical representation of data in panels A and B. Percentage of VSG imported was calculated by dividing the amount of the residual VSG detected after protease treatment by the amount of the VSG detected after the addition of TbRM without protease treatment, and multiplying the quotient by 100.

Figure 2

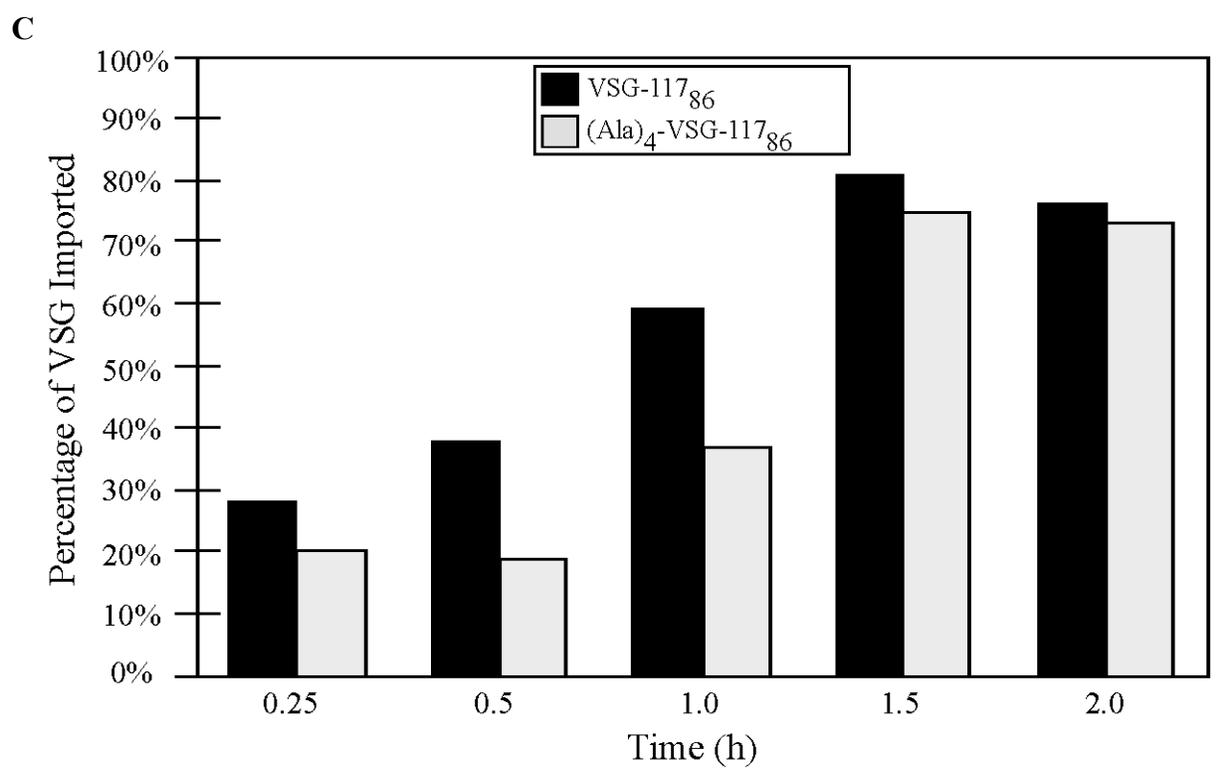
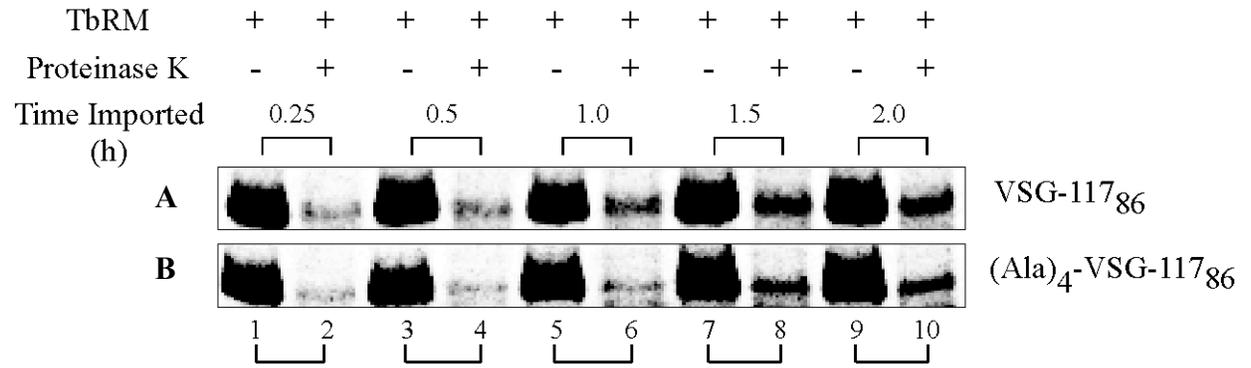


Table 2 - Amino Acid Sequences of VSG-117_h, Scrambled (Ala)₄-VSG-117_h, Scrambled (Ser)₄-VSG-117_h, (Ala)₄-VSG-117_h, and (Ala)₄-Tyr9-VSG-117_h

VSG-117_h, scrambled (Ala)₄-VSG-117_h, scrambled (Ser)₄-VSG-117_h, (Ala)₄-VSG-117_h, and (Ala)₄-Tyr9-VSG-117_h mutants aligned by h-region sequences. Position 1 denotes the beginning of the h-region and position 14 marks its end. h-Region sequence differences in (Ala)₄-VSG-117_h and (Ala)₄-Tyr9-VSG-117_h are listed in bold.

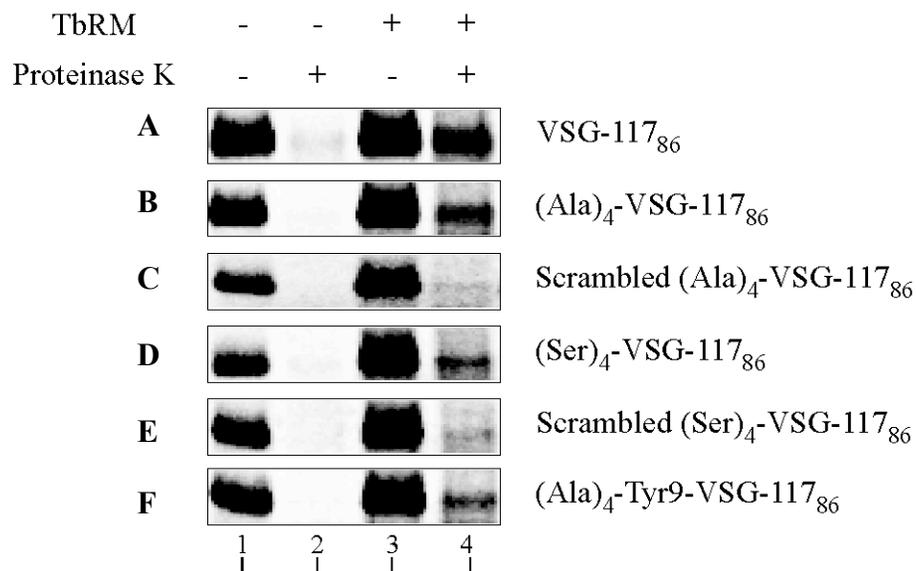
Table 2

<u>VSG</u>	<u>Position of Amino Acids in h-region Sequence</u>													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
VSG-117 _h	S	T	M	L	T	L	S	L	L	Y	A	I	T	P
Scrambled (Ala) ₄ -VSG-117 _h	A	S	L	T	A	M	A	T	S	A	Y	I	T	P
Scrambled (Ser) ₄ -VSG-117 _h	S	S	L	T	S	M	S	T	S	S	Y	I	T	P
(Ala) ₄ -VSG-117 _h	S	T	M	A	T	L	S	A	A	Y	A	I	T	P
(Ala) ₄ -Tyr ⁹ -VSG-117 _h	S	T	M	A	T	L	S	A	Y	A	A	I	T	P

Figure 3 – Rearrangement of h-Region Sequences Affects VSG Translocation into TbRM

mRNAs of VSGs were translated in rabbit reticulocyte lysate. Translation was stopped with cycloheximide. TbRM was added. The mixtures were incubated at 37°C for 1.5 hours. Import of VSG-117₈₆ into TbRM was tested by proteinase K (PK) digestion on ice for 1 hour. Proteins in the reaction mixture were precipitated with ammonium sulfate, resolved on Tris-Tricine gels, and analyzed with a phosphorimager and Quantity One software. Gels pictured are representative results. (A-F) Lane 1: no TbRM, no PK; lane 2: no TbRM, with PK; lane 3: with TbRM, no PK; lane 4: with TbRM, with PK. Brackets denote lanes that were compared to obtain percentage of VSG imported into TbRM for each study. h-Regions studied were (A) VSG-117₈₆, (B) (Ala)₄-VSG-117₈₆, (C) scrambled (Ala)₄-VSG-117₈₆, (D) (Ser)₄-VSG-117₈₆, (E) scrambled (Ser)₄-VSG-117₈₆, and (F) (Ala)₄-Tyr9-VSG-117₈₆. (G) Peak hydrophobicity values (black bars) were obtained by Kyte-Doolittle hydrophobicity analysis (window = 7 amino acids), using LASERGENE (DNASTAR) (version 4.0.3). Percentage of VSG imported into TbRM (gray bars) are from data in panels A-F; it was calculated by dividing the amount of the residual VSG detected after protease treatment by the amount of the VSG detected after addition of TbRM without protease treatment, and multiplying the quotient by 100.

Figure 3



G

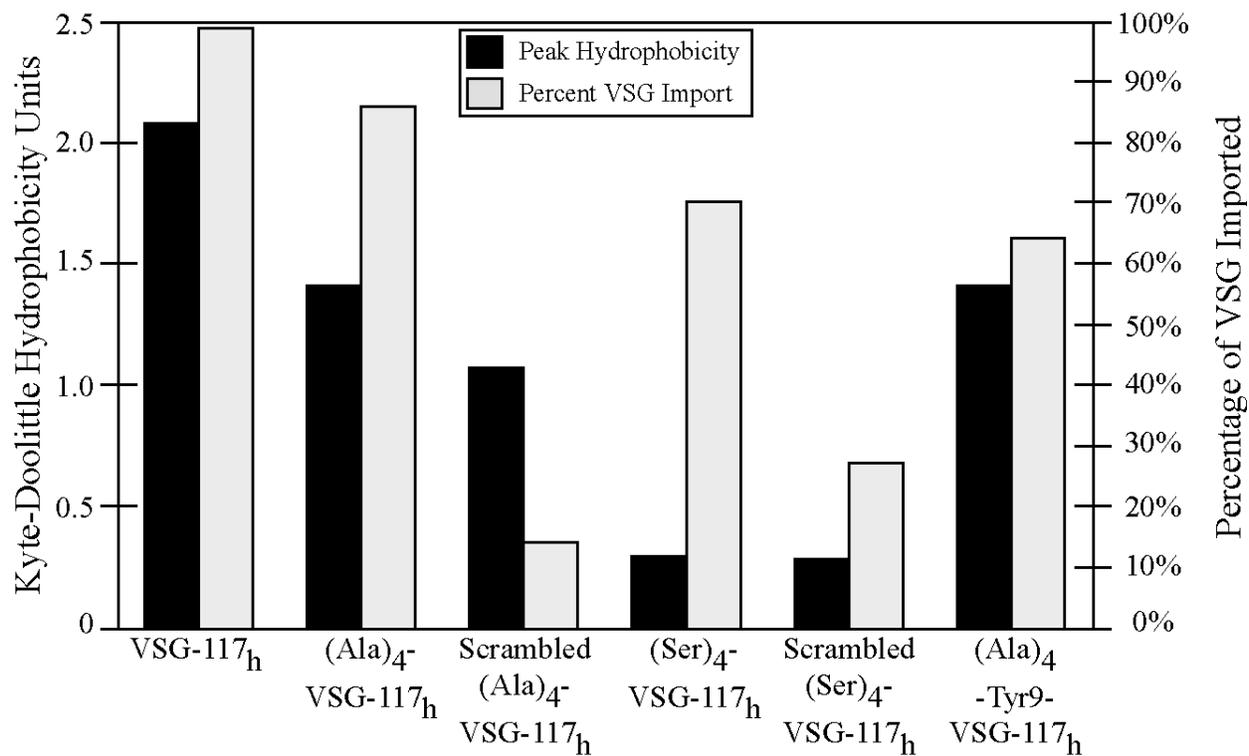


Table 3 - h-Region Peptide Motifs in VSG-117

Peptide motifs in the VSG-117 h-region are shown. Motifs are displayed in PROSITE syntax.

Peptide patterns were obtained from analysis of 50 *T. brucei* h-regions, using PRATT pattern matching tool (Jonassen, Collins et al. 1995). The PRATT parameters were determined empirically to identify patterns with three or more “identity components”. The parameters were: C% (52); PL (50); PN (50); PX (5); FN (5); FL (2); FP (20); E (3) (Patham et al., in preparation).

Table 3

<u>Name</u>	<u>h-Motif</u>	<u>Location of Amino Acids in h-Motifs of VSG-117_h</u>													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		S	T	M	L	T	L	S	L	L	Y	A	I	T	P
Tb1	L-L-x-[AILV]									L	L	x	A		
Tb2	L-x(1,2)-L-[AILV]								L	x	L	L			
Tb3	L-x(2,3)-L-[AILPV]						L	x	x	x	L	L			

Table 4 – Sequences of VSG-117_h, (Phe)₄-VSG-117_h, and (Val)₄-VSG-117_h h-Regions

Amino acids in VSG-117_h, (Phe)₄-VSG-117_h, and (Val)₄-VSG-117_h are aligned by h-region sequences. Position 1 is the beginning of the h-region and position 14 marks its end. Mutations in the VSG h-regions are listed in bold.

Table 4

<u>VSG</u>	<u>Position of Amino Acids in h-region Sequence</u>													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
VSG-117 _h	S	T	M	L	T	L	S	L	L	Y	A	I	T	P
(Phe) ₄ -VSG-117 _h	S	T	M	F	T	L	S	F	F	Y	F	I	T	P
(Val) ₄ -VSG-117 _h	S	T	M	V	T	L	S	V	V	Y	V	I	T	P

Figure 4 – Microsomal Import and Hydrophobicity Properties of VSG-117₈₆, (Phe)₄-VSG-117₈₆, and (Val)₄-VSG-117₈₆

Import of (A) VSG-117₈₆, (B) (Phe)₄-VSG-117₈₆, and (C) (Val)₄-VSG-117₈₆ into *T. brucei* microsomes. mRNAs of VSG-117 variants were translated in rabbit reticulocyte lysate.

Translation was stopped with cycloheximide. The reaction mixtures were supplemented with TbRM, and incubated at 37°C for 1.5 hours. Translocation into TbRM was stopped by incubation of the samples on ice, and import of VSG was tested by proteinase K (PK) digestion on ice for 1 hour. The samples were precipitated with ammonium sulfate, proteins were resolved on Tris-Tricine gels, and analyzed with a phosphorimager and Quantity One software. Gels pictured are representative results. (A-C) Lane 1: no TbRM, no PK; lane 2: no TbRM, with PK; lane 3: with TbRM, no PK; lane 4: with TbRM, with PK. Brackets denote pairs of lanes that were compared to obtain percentage of VSG imported into TbRM in each set. (D) Peak hydrophobicity values (black bars) were obtained by Kyte-Doolittle hydrophobicity analysis (window = 7 amino acids), using LASERGENE (DNASTAR) (version 4.0.3). Percentage of VSG imported (gray bars) was calculated from data in panels A-C by dividing the amount of the residual VSG detected after protease treatment by the amount of the VSG detected after addition of TbRM without protease treatment, and multiplying the quotient by 100.

Figure 4

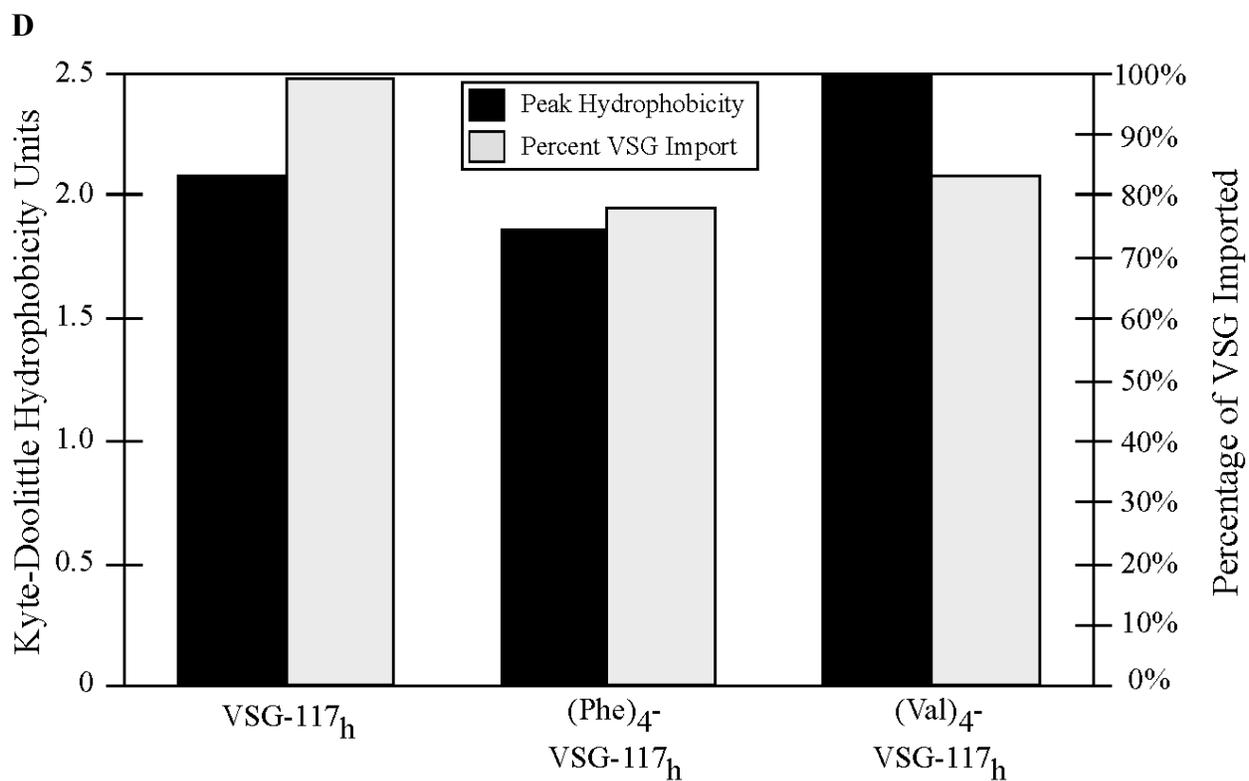
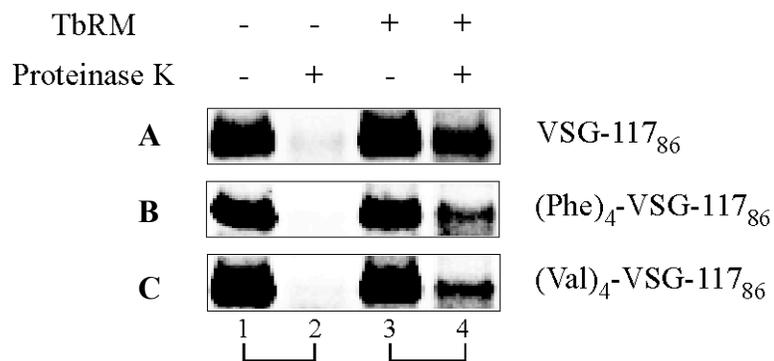


Table 5 – Amino Acid Sequences of (Ala)₄-VSG-117_h, (Ala)₄-Phe6-VSG-117_h, (Ala)₄-Val6-VSG-117_h, and (Ala)₄-Ile6-VSG-117_h

h-Region sequences of (Ala)₄-VSG-117_h, (Ala)₄-Phe6-VSG-117_h, (Ala)₄-Val6-VSG-117_h, and (Ala)₄-Ile6-VSG-117_h are aligned. Position 1 marks the beginning of the h-region and position 14 is its end. Mutations to the VSG h-regions are listed in bold.

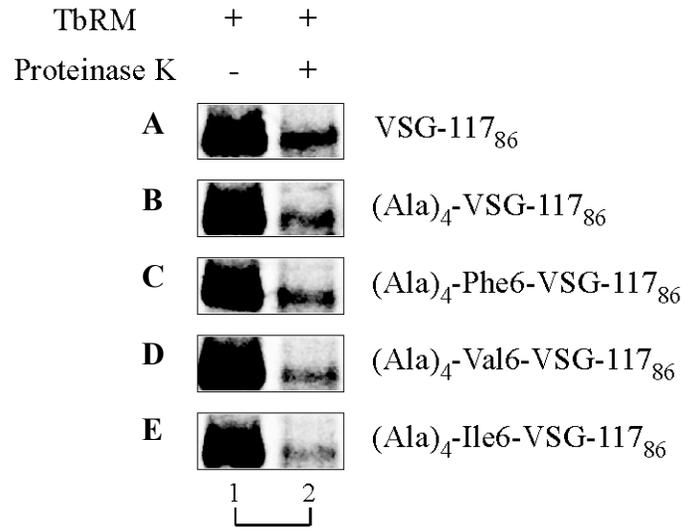
Table 5

<u>VSG</u>	<u>Position of Amino Acids in h-region Sequence</u>													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
(Ala) ₄ -VSG-117 _h	S	T	M	A	T	L	S	A	A	Y	A	I	T	P
(Ala) ₄ -Phe6-VSG-117 _h	S	T	M	A	T	F	S	A	A	Y	A	I	T	P
(Ala) ₄ -Val6-VSG-117 _h	S	T	M	A	T	V	S	A	A	Y	A	I	T	P
(Ala) ₄ -Ile6-VSG-117 _h	S	T	M	A	T	I	S	A	A	Y	A	I	T	P

Figure 5 – Effect of Replacing Leucine at h-Region Position Six with Phenylalanine, Valine, or Isoleucine on VSG Translocation into TbRM

Import of (A) VSG-117₈₆, (B) (Ala)₄-VSG-117₈₆, (C) (Ala)₄-Phe6-VSG-117₈₆, (D) (Ala)₄-Val6-VSG-117₈₆, and (E) (Ala)₄-Ile6-VSG-117₈₆ into *T. brucei* Microsomes. mRNAs of VSG-117 were translated in rabbit reticulocyte lysate. Translation was stopped with cycloheximide, and the translation mixture was supplemented with TbRM, followed by incubation at 37°C for 1.5 hours. Translocation was stopped by incubation of the reaction mixture on ice, and import of proteins was tested by proteinase K (PK) digestion on ice for 1 hour. Proteins were precipitated with ammonium sulfate, resolved on Tris-Tricine gels, and analyzed with a phosphorimager and Quantity One software. Gels pictured are representative results. (A-E) Lane 1: with TbRM, no PK; lane 2: with TbRM, with PK. Brackets denote pairs of lanes that were compared in order to obtain percentage of VSG imported into TbRM. (D) Peak hydrophobicity values (black bars) were obtained by Kyte-Doolittle hydrophobicity analysis (window = 7 amino acids), using LASERGENE (DNASTAR) (version 4.0.3). Percentage of VSG imported (gray bars) was calculated from data in panels A-E by dividing the amount of the residual VSG detected after protease treatment by the amount of the VSG detected after addition of TbRM without protease treatment, and multiplying the quotient by 100.

Figure 5



F

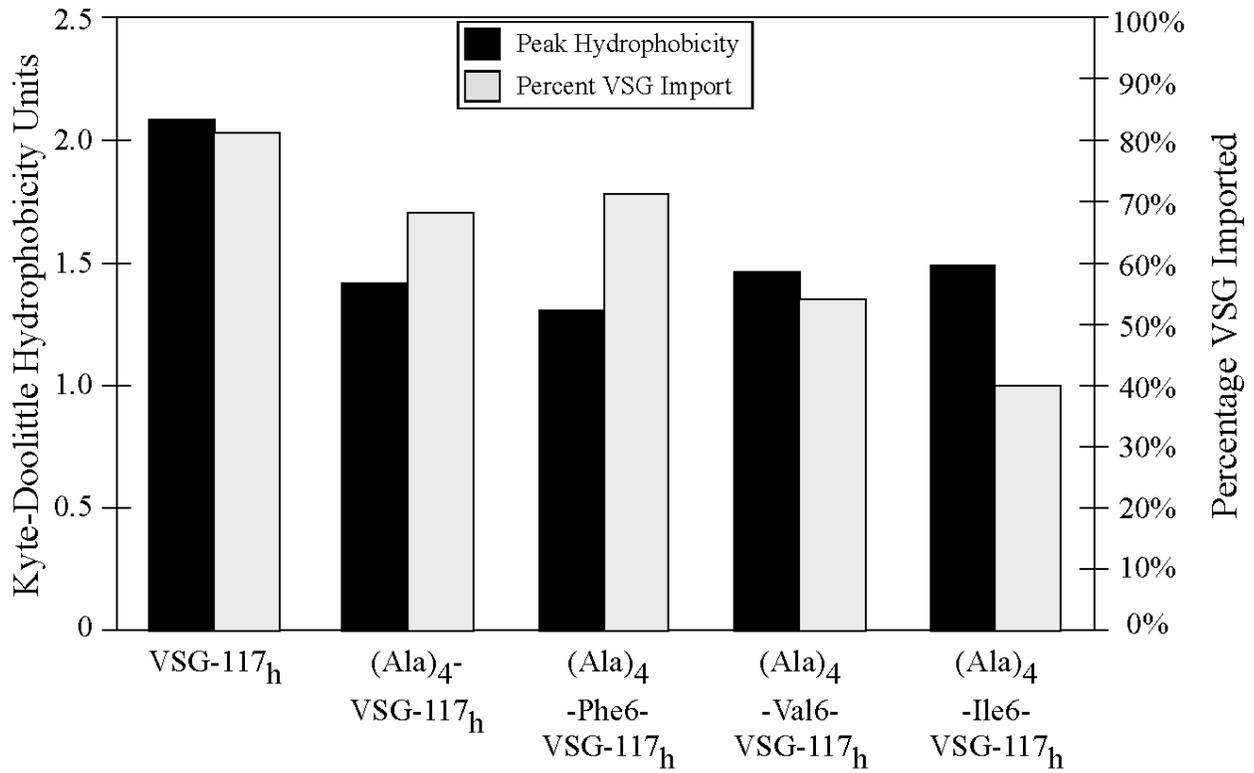


Figure 6 – Helical Wheel Analysis of VSG-117 h-Region

Helical wheel model of VSG-117 h-region was generated with LASERGENE (DNASTAR) (Protean Module, version 4.0.3). Arrows indicate h-region “identity components” (IC₁, IC₂, IC₃, IC₄, and IC₅) in the VSG-117 h-region (see Table 3).

Figure 6

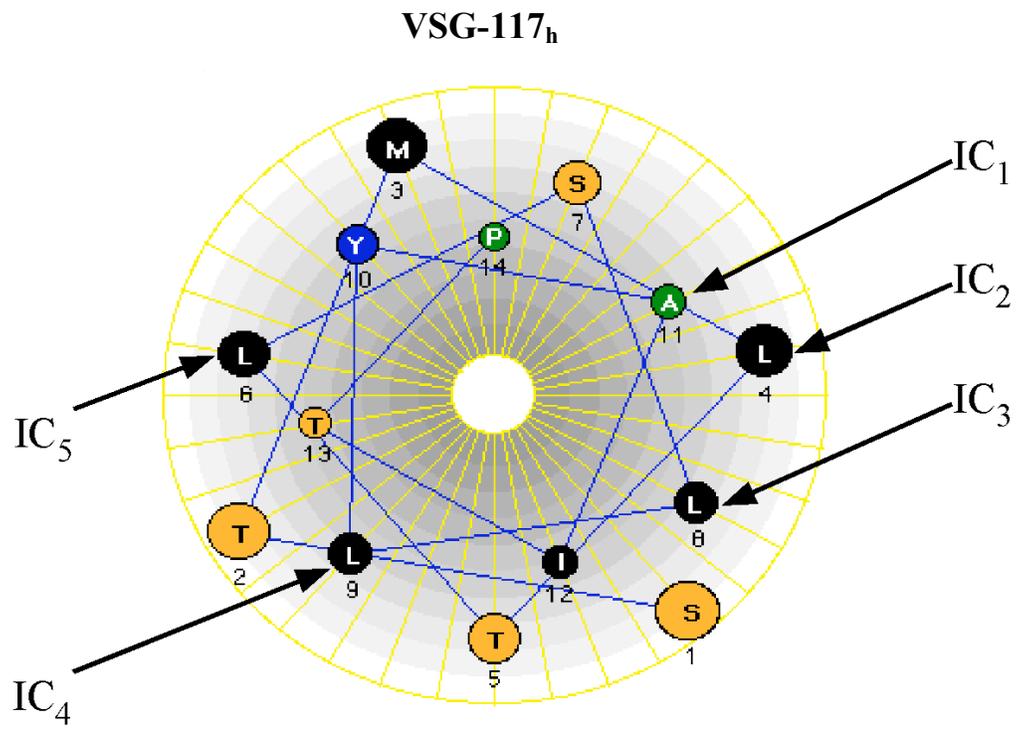
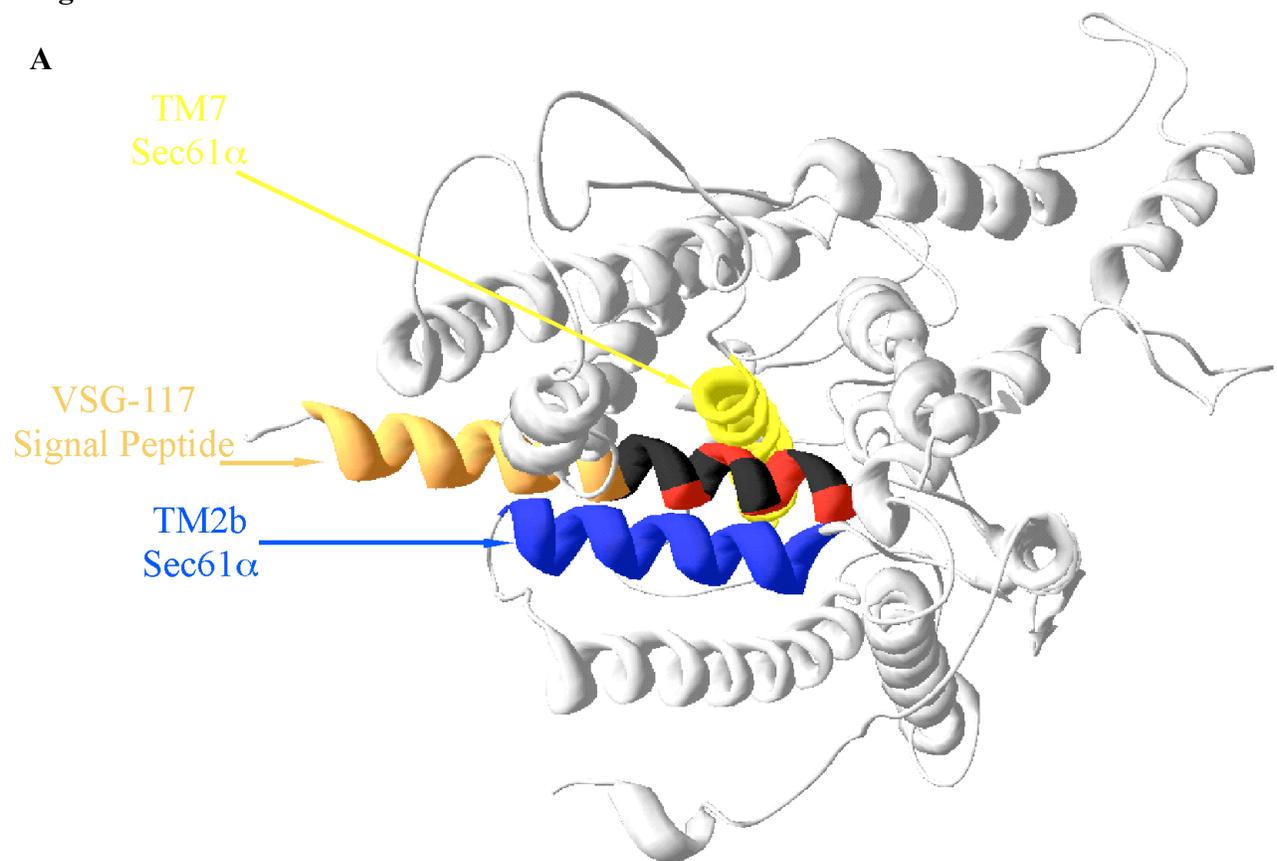


Figure 7 – Hypothesis for Sec61 and Signal Peptide h-Region Interactions

A model of interaction between a signal peptide and Sec61 has been offered (Plath, Mothes et al. 1998; Van den Berg, Clemons et al. 2004). In that model, h-region interactions with Sec61p were not addressed. We have extended that model by using our data on functional relevance of h-motifs. (A) Top view of a VSG-117 signal peptide embedded within a *T. brucei* Sec61 translocon model (white) (C. Hardin, unpublished). (B) Top view (C) Side view of a VSG-117 signal peptide placed between TM2b and TM7 of *T. brucei* Sec61 α (TbSec61 α). (D) *T. brucei* VSG-117 h-region α -helix interacting with TM2b and TM7 of *T. brucei* Sec61 α (not drawn to scale). It is predicted that the h-region (black) of a VSG signal peptide (orange) forms an α -helix, and uses h-motif identity components (red) to interact with TM2b (blue) and TM7 (yellow) of TbSec61 α . Side-chains of “identity components” of the h-motif are positioned to interact with TM2b and/or TM7 of TbSec61 α . The h-motif “identity components” displayed here are from VSG-117 (L₄, L₆, L₈, L₉, and A₁₁), and the TbSec61 translocon is a “homology model” of the *T. brucei* Sec61 translocon based on the X-Ray crystal structure from *Methanococcus jannaschii* (Van den Berg, Clemons et al. 2004).

Figure 7

A



B

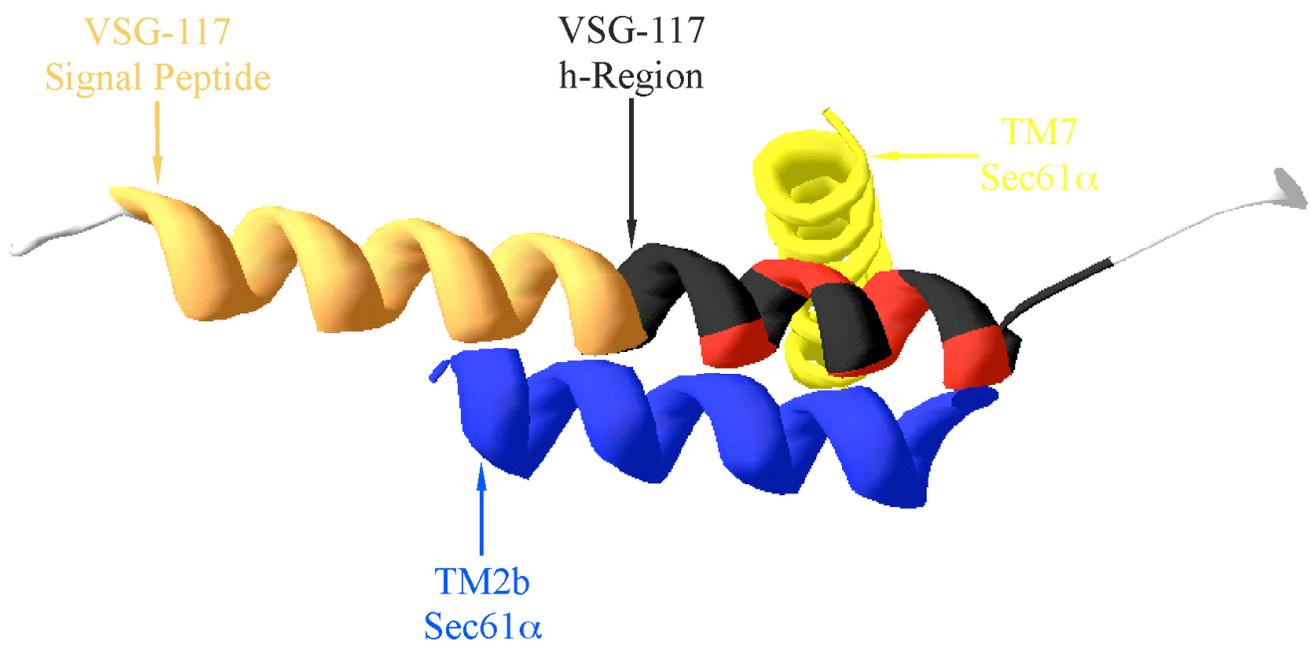
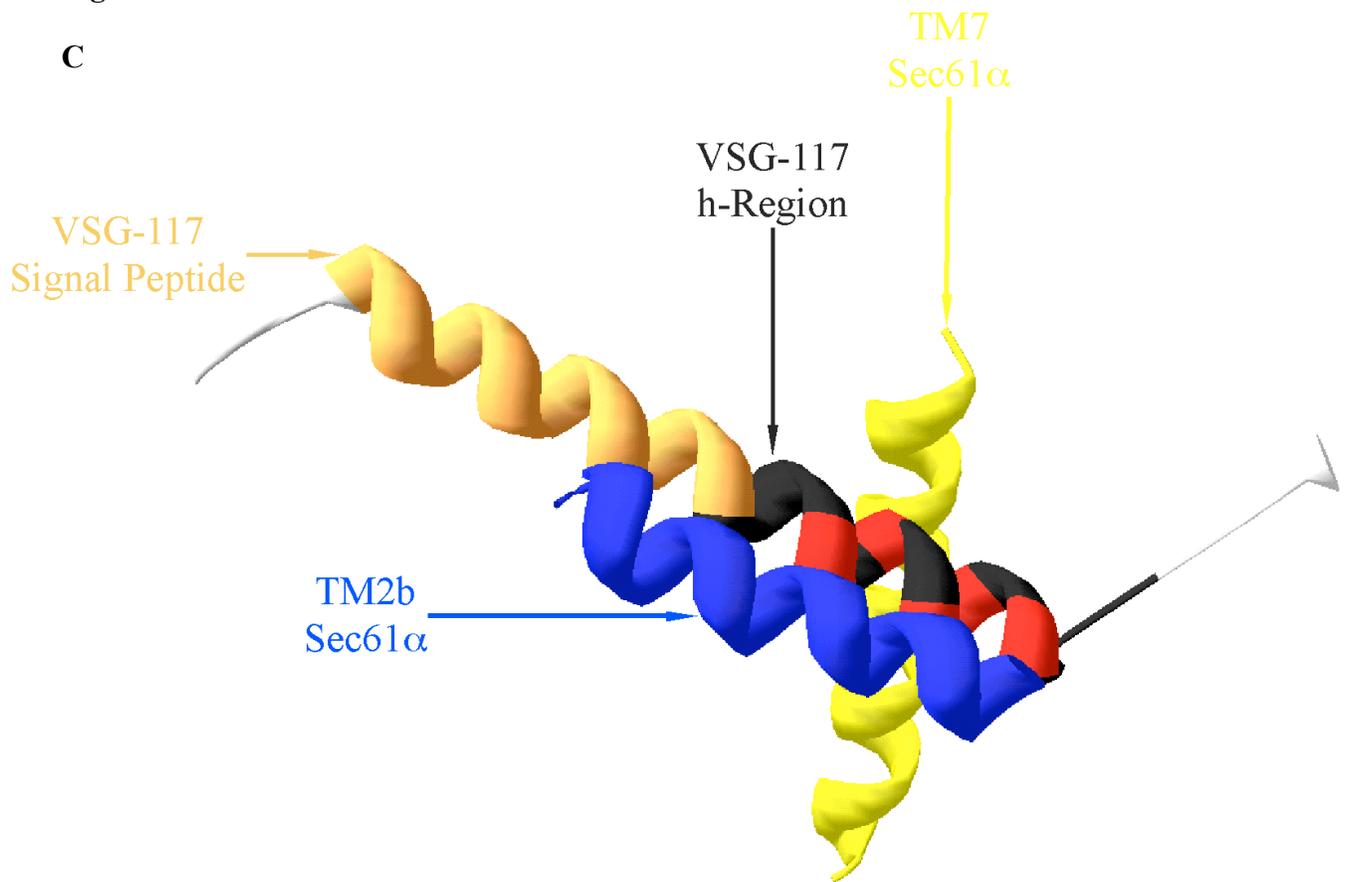
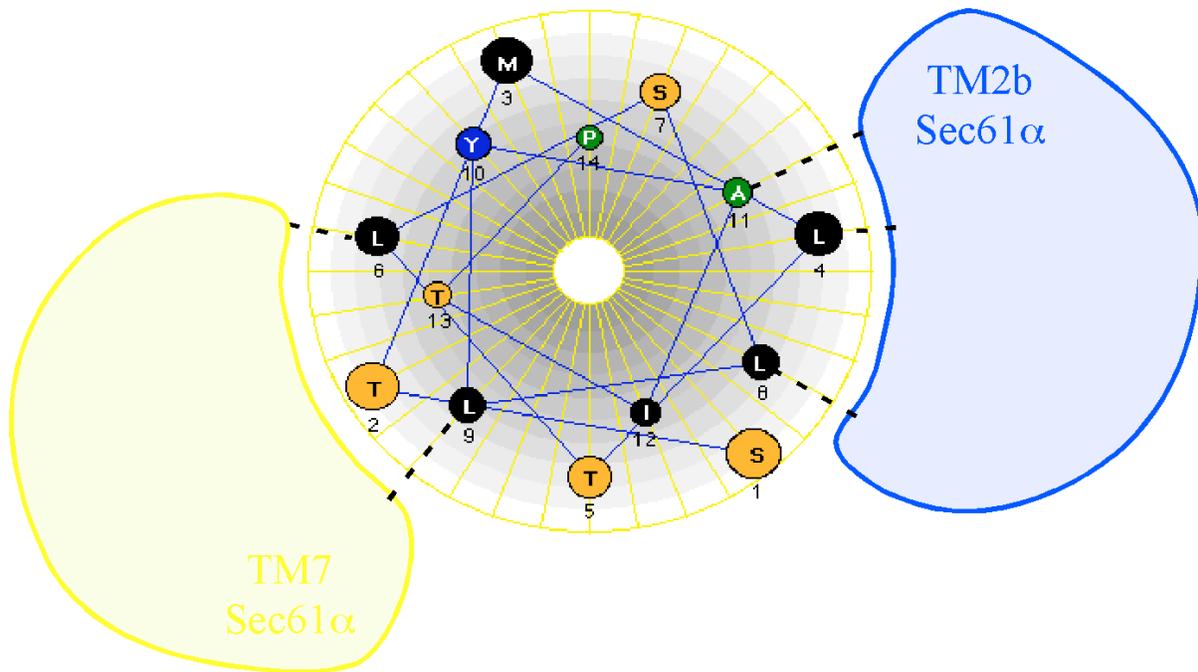


Figure 7

C



D



CHAPTER III

SMALL MOLECULE INHIBITORS OF *IN VITRO* PROTEIN IMPORT INTO THE TRYPANOSOME ENDOPLASMIC RETICULUM¹

¹ Joshua Duffy, Ariel Lane, Peter Wipf, Jeffrey Brodsky, Hanna Harant, and Kojo Mensa-Wilmot. To be submitted to *Biochemical Journal*.

Abstract

Trypanosoma brucei causes human African trypanosomiasis (HAT), a disease against which new drugs are needed. To maintain an infection *in vivo*, *T. brucei* relies on its cell surface proteins, including variant surface glycoproteins (VSGs). For VSG to be targeted to the exoplasmic side of the plasma membrane it must first translocate into the endoplasmic reticulum. Thus, protein import into the ER is a good focus for the discovery of novel anti-trypanosome lead compounds. To contribute to this effort, we sought to identify compounds that inhibited protein translocation into ER microsomes, using an *in vitro* *T. brucei* microsomal system (TbRM) and a model VSG substrate (VSG-117₈₆). Post-translational import of VSG-117₈₆ into TbRM was inhibited by sodium azide, but not by valinomycin. Sodium azide is an inhibitor of F-type ATPases and SecA: we infer that an azide-sensitive chaperone is important for protein translocation into TbRM. Pyrimidinone-peptoid derivatives MAL3-101 and MAL3-51 inhibited post-translational import of VSG-117₈₆ into microsomes: compounds of this class modulate Hsp70 ATPase activity, and we surmise that MAL3-101 and MAL3-51 affect ER protein import by inhibiting Hsp70 interaction with VSG-117₈₆. Against cultured bloodstream *T. brucei*, MAL3-101 was trypanocidal with an IG₅₀ of 1.5 μM; however, MAL3-51 did not kill the parasite. A cyclopeptolide, NFI028, inhibited import of VSG-117₈₆ into microsomes while a similar compound, CAM741, did not. Cyclopeptolides affect signal peptide recognition at the Sec61 translocon in a signal peptide-dependent manner. Thus, the signal peptide of VSG-117₈₆ is inhibited at the Sec61 translocon by NFI028, but not by CAM741. Both NFI028 and CAM741 were trypanocidal with IG₅₀'s of 1.5 μM and 2.0 μM, respectively. Therefore, MAL3-101, NFI028, and CAM741 are lead compounds for the discovery of pyrimidinone-peptoid and cyclopeptolide derivatives as new anti-trypanosome drugs.

Introduction

Trypanosoma brucei causes human African trypanosomiasis (HAT). Without chemotherapeutic treatment, *T. brucei* kills infected humans. Currently, there are only four drugs approved for the treatment of HAT. All are quite toxic and cause serious side effects and in some cases death (reviewed in (Fairlamb 2003; Barrett, Boykin et al. 2007)). Consequently, there is a strong need for better chemotherapeutic options for the treatment of HAT.

Central to *T. brucei* survival in a vertebrate is variant surface glycoprotein (VSG) (Shedder, Vaughan et al. 2005). Proper VSG expression on the exoplasmic leaflet of the plasma membrane is crucial for *T. brucei* viability (Cross 1975; Shedder, Vaughan et al. 2005) (reviewed in (Taylor and Rudenko 2006)). VSGs are delivered to the plasma membrane by way of the secretory pathway. The committed step for protein entry into the secretory pathway is translocation into the endoplasmic reticulum (reviewed in (Shimizu and Hendershot 2007)); a process directed by ER signal peptides. ER signal peptides occur at the N-terminus of secretory precursor proteins (reviewed in (Izard and Kendall 1994; Hegde and Bernstein 2006)), and must be recognized by a Sec61 translocon complex at the ER membrane (Simon and Blobel 1991; Crowley, Liao et al. 1994; Jungnickel and Rapoport 1995).

Signal peptides can be targeted to the ER by two pathways. Co-translational import is an ER import pathway that occurs as translation of a polypeptide on the ribosome is ongoing; it depends on a signal recognition particle (SRP) and an SRP-receptor (SR) at the ER membrane. In an alternative pathway, post-translational import, protein translocation into the ER occurs after full translation of the secretory precursor in the cytoplasm; it does not depend on SRP. Protein import into the ER occurs at the Sec61 translocon in both pathways (reviewed in (Rapoport 2007)). In yeast, co-translational and post-translational import is mediated by the Sec62/63

complex and an ER luminal Hsp70, Kar2p (BiP) (Brodsky, Goeckeler et al. 1995; Panzner, Dreier et al. 1995) (reviewed in (Corsi and Schekman 1996; Rapoport 2007)). Further, post-translational protein import is dependent on cytoplasmic Hsp70 chaperones, which bind nascent precursors and render them “import-competent” (Caplan, Cyr et al. 1992; McClellan, Endres et al. 1998; McClellan and Brodsky 2000) in an ATP-dependent reaction (Misselwitz, Staeck et al. 1998). Hsp40s, co-chaperones of Hsp70s, contain a J-domain that interacts with the Hsp70 ATPase (Cheetham and Caplan 1998), stimulating the intrinsic Hsp70 ATPase. This Hsp40/Hsp70 interaction enhances the Hsp70 chaperone effect by stabilizing the association of an Hsp70 with its bound cargo (Nicoll, Botha et al. 2007) (reviewed in (Hennessy, Nicoll et al. 2005; Qiu, Shao et al. 2006)).

In *T. brucei*, protein import into the ER occurs both co-translationally and post-translationally (Liu, Liang et al. 2002; Lustig, Vagima et al. 2007; Goldshmidt, Sheiner et al. 2008). We have evaluated the effects of small molecule compounds on post-translational import of VSG-117₈₆ into *T. brucei* microsomes (TbRM).

Because Hsp70s in the cytoplasm and ER lumen are instrumental in protein translocation into the ER, compounds that inhibit their function could potentially block VSG entry into the secretory pathway. Similarly, compounds that selectively inhibit *T. brucei* Sec61 translocon function could block VSG expression at the cell surface, and could be anti-trypanosome lead compounds.

The pyrimidinone-peptoid MAL3-101 (Fig. 1A) inhibits large T antigen (TAg, a J-domain containing protein) stimulation of Hsp70 ATPase activity, and inhibits post-translational translocation of pre-pro-alpha mating factor (pp α MF) into *S. cerevisiae* microsomes (Fewell,

Smith et al. 2004). Based on this data we decided to test the effects of MAL3-101 and a related compound, MAL3-51 (Fig. 1B), on the post-translational import of VSG-117₈₆ into TbRM.

Cyclopeptolides CAM741 (Fig. 1C) and NFI028 (Fig. 1D) inhibit co-translational translocation of certain secretory proteins in a signal peptide-dependent fashion. Both CAM741 and NFI028 inhibit secretion of alkaline phosphatase possessing a signal peptide from vascular cell adhesion molecule 1 (VCAM1) by interfering with signal peptide binding to the Sec61 translocon (Besemer, Harant et al. 2005; Harant, Lettner et al. 2006; Harant, Wolff et al. 2007). However, CAM741, but not NFI028, inhibited protein import directed by a vascular endothelial growth factor (VEGF) signal peptide. Because both CAM741 and NFI028 inhibit ER protein import with non-identical selectivity of signal peptides, we tested both compounds on VSG-117₈₆ import into TbRM.

Azide interferes with a variety of cellular processes across biological kingdoms. Growth of bloodstream *T. brucei* is inhibited by azide (IG₅₀ of 100 μM); however, it is not known how azide affects *T. brucei* (Steverding and Scory 2004). In *E. coli* and other prokaryotes, azide blocks protein translocation *in vivo* and *in vitro* by inhibiting the ATPase of SecA, a protein translocation chaperone (Oliver, Cabelli et al. 1990; van der Wolk, de Wit et al. 1997) (reviewed in (Schmidt and Kiser 1999)). Based on data showing *T. brucei* azide sensitivity and the ability of azide to inhibit post-translational protein translocation in prokaryotes we decided to test whether sodium azide affected post-translational translocation of VSG-117₈₆ into TbRM. From the data presented herein, we identify pyrimidinone-peptoids and cyclopeptolides as two classes of compounds that inhibit *T. brucei* ER protein import. Further, some of the compounds are trypanocidal. Therefore, pyrimidinone-peptoids and cyclopeptolides are lead compounds for discovery of new anti-trypanosomal compounds.

Materials and Methods

Materials

Taq DNA polymerase, amino acid mixture minus methionine and cysteine, and nuclease treated rabbit reticulocyte lysate were obtained from Promega (Madison, Wisconsin). dNTPs were obtained from Sigma (St. Louis, Missouri). DEPC-treated water was from USB Corporation (Cleveland, Ohio); QIAquick PCR purification kit was from Qiagen (Valencia, California); Ampliscribe T7 *in vitro* transcription kit was from Epicentre (Madison, Wisconsin); TRAN35S - LABEL™ No-thaw metabolic labeling reagent was from MP Biomedicals (Solon, Ohio); cycloheximide was from Calbiochem (La Jolla, California); proteinase K and leupeptin were from Roche (Indianapolis, Indiana); PMSF was from Boehringer Mannheim (Mannheim, Germany); ethanol and urea were from Fisher Scientific (Norcross, Georgia); Acrylagel and Bis-Acrylagel were from National Diagnostics (Atlanta, Georgia); TEMED and ammonium persulfate, were from Bio-Rad (Hercules, California); DE52 was from Whatman (Hillsboro, Ohio); MAL3-101 and MAL3-51 were gifts from Dr. Jeffrey L. Brodsky and Dr. Peter Wipf (University of Pittsburgh); AEE788, CAM741 and NFI028 were gifts from Novartis (Vienna, Austria); sodium azide was from J.T. Baker (Phillipsburg, New Jersey); valinomycin and all other chemicals and reagents were obtained from Sigma (St. Louis, Missouri).

Plasmids

VSG-117₈₆ DNA was made through PCR mutagenesis (Innis, Myambo et al. 1988; Bangs, Brouch et al. 1996) of a plasmid template, pVSG-117, provided by Dr. James D. Bangs (University of Wisconsin).

Cell Strain and Isolation

Monomorphic *Trypanosoma brucei* strain 427 were grown in and purified from Sprague Dawley® rats, using chromatography on DE52 (Cross 1975).

Primer Construction For VSG-117₈₆

PCR mutagenesis was used to create VSG-117₈₆ from the template pVSG-117 using variations of the forward primer *TAATACGACTCACTATAGGGaggagggttttaccATGGACTGCC ATACAAAGGAG*. Each primer contained a T7 promoter (upper case italicized) and a translational enhancer (lower case underlined) (Teilhet, Rashid et al. 1998). The coding region of VSG-117 starts at position 36 and ends at primer position 56 (upper case). The VSG-117 protein was truncated to 86 amino acids, using the reverse primer *cgaacaacgaaggggttcTTATAGT GCGTAGATCGTAGCTTCGTTTC*, which has a stop codon (upper case underlined) after the 27 nucleotides priming for nucleotides 295-321 (upper case italicized) of the pVSG-117 sequence.

Generation of VSG-117₈₆ DNA

A VSG-117₈₆ PCR product was generated in a 100 µl reaction mixture containing 224 ng pVSG-117 template, 0.5 µM of each forward and reverse primer, Innis buffer (10 mM Tris-HCl pH 8, 2.5 mM MgCl₂, 0.05% Tween-20, 0.05% nonidet P-40, 50 mM KCl) (Innis, Myambo et al. 1988), 250 µM dNTPs, and 5 units of Taq DNA polymerase. The PCR reaction was carried out for 25 cycles as follows: 95°C for 90 seconds, 56°C for 90 seconds, and 74°C for 2 min. The PCR product was purified using a QIAquick PCR purification kit (Qiagen). Purified PCR products were quantitated at 260 nm.

In Vitro Transcription

One µg of purified DNA was used as a template for transcription with an Ampliscribe™ T7 kit (Epicentre Technologies). One µl (1 MBU) of RNase free DNase I was added to the

reaction (20 μ l) and allowed to incubate at 37°C for 15 minutes. The mixture was extracted with an equal volume of TE-saturated phenol/chloroform, and the aqueous phase extracted with chloroform. To the aqueous phase, sodium acetate (27 mM final concentration) was added along with 2.5 times the total volume of 100% ethanol. This mixture was allowed to precipitate at -20°C overnight. The precipitate was recovered at 16,100 x g at 4°C for 15 minutes, rinsed with 70% ethanol, and pelleted at 16,100 x g. The pellet was allowed to air dry, and resuspended in 40 μ l of nuclease free water. The RNA concentration was determined by measuring the absorbance at 260 nm.

Microsome Preparation from Bloodstream *Trypanosoma brucei*

Bloodstream *T. brucei* lister 427 (1×10^{10} cells/ml) were resuspended in 5 ml of fresh homogenization buffer (HB) (250 mM sucrose, 50 mM HEPES-KOH, 50 mM KOAc, 6 mM Mg(OAc)₂, 1 mM EDTA, 1 mM DTT, 1 μ g/ml TLCK, 5 μ g/ml leupeptin, 0.5 mM PMSF). In a clean, pre-chilled dounce homogenizer 2.5 ml of the resuspended cells were lysed on ice by 2 repetitions of 40 strokes with a tight-fitting pestle, with a one minute break after 40 strokes. The homogenates were pooled, aliquoted into microcentrifuge tubes (1 ml/tube), and centrifuged at 400 x g for 10 minutes at 4°C. The supernatants from all tubes were pooled, aliquoted into a new microcentrifuge tube, and centrifuged at 13,400 x g for 20 minutes at 4°C. The supernatants from the 13,400 x g centrifugation step were recovered and pooled. Pellets from the 13,400 x g centrifugation step were resuspended in 50 μ l/pellet (total volume) of fresh rough microsome buffer (RMB) (250 mM sucrose, 50 mM HEPES-KOH, 50 mM KOAc, 1 mM DTT, 0.5 μ g/ml TLCK, 2.5 μ g/ml leupeptin). The microsome concentration was determined by measuring the absorbance at 260 nm, and the concentration was adjusted to an OD₂₆₀ of 50 (1 equivalent with an OD₂₆₀ of 50 = 1 μ l). The microsomes were aliquoted into 20 μ l portions, quick frozen in

liquid nitrogen, and stored at -80°C (Pellets from the 400 x g centrifugation and the supernatant from the 13,400 x g centrifugation were also saved).

Cytosol Preparation from Bloodstream *Trypanosoma brucei*

The supernatant saved from the 13,400 x g centrifugation step (above) during the microsome preparation was centrifuged at 179,500 x g at 4°C for 1 hour in a Beckman Coulter Optima TLX ultracentrifuge. The supernatant was recovered, pooled (approximately 4 ml total), and concentrated using a Centricon-10 filter by centrifuging at 5000 x g at 4°C in 30 minute intervals until approximately 250 μl of concentrated cytosol remained. The cytosol was adjusted to an OD_{280} of 50 (1 equivalent = 50 OD units/ μl), aliquoted, quick-frozen in liquid nitrogen, and stored at -80°C .

***In Vitro* Translation and Import of VSG Into *T. brucei* Microsomes (TbRM)**

RNA (250 – 500 ng) in DEPC-treated H_2O (7 μl) was incubated at 65°C for three minutes. After cooling on ice for one minute, 1.5 μl (75 μM final concentration) of an amino acid mixture lacking methionine and cysteine (Promega), 1.5 μl of ^{35}S -cysteine and methionine (2.5 mCi total activity, MP Biomedicals), 10 μl of rabbit reticulocyte lysate (Promega), and 1 μl of *T. brucei* cytosol was added to the RNA mixture, bringing the final volume to 21 μl . This reaction mixture was incubated at 37°C for 1 hour. Translation was stopped with cycloheximide (1 mM final concentration).

The translation reaction was then divided into four separate portions of 5 μl each. Two of these portions were left untreated while the remaining two portions were supplemented with 1 μl (1 μl = 1 equivalent, which has an OD_{260} of 50) of TbRM. For inhibitor studies, one of the following was then added: MAL3-101 (10 μM final concentration), MAL3-51 (10 μM final concentration), CAM741 (1 μM final concentration), NFI028 (1 μM final concentration), sodium

azide (8 μ M final concentration), or AEE788 (8 μ M final concentration). The four reaction aliquots were further incubated at 37°C for 1.5 hours and then transferred to ice. Next, one of each sample (translation mixture with or without TbRM) was treated with a proteolysis mixture containing urea (3M) and proteinase K (0.5 mg/ml) (final concentrations) on ice for 1 hour. PMSF (34 mM final concentration) was added to quench the proteolysis reaction. Proteins were precipitated with 60% $(\text{NH}_4)_2\text{SO}_4$ on ice for 15 minutes, and centrifuged at 16,100 x g at 4°C for 7 minutes. The pellets were resuspended in 20 μ l of 2.5x SDS sample buffer and resolved by SDS-PAGE in a Tris-Tricine gel system (16% - Resolving; 3% - Stacking) (reviewed in (Gallagher 2007)). Radioactive polypeptides were detected with a phosphorimager; bands were quantitated with Quantity-One software (version 4.6.5, Bio-Rad).

Quantitation of Percent VSG Import

Using Quantity-One software (version 4.6.5, Bio-Rad), phosphorimages were adjusted so that gel bands corresponding to proteinase K-protected VSG-117₈₆ were visible. Next, background noise signals were subtracted using the “filter wizard” application. A Tiff image preserving these adjustments to the gel image was acquired. From this Tiff image, gel bands in each lane were quantitated by measuring the volume (counts*mm²) of the band representing VSG-117₈₆. For background signal, the volume of an area in each lane of the gel image that best represented the average background pixel intensity was measured. In band and background intensity quantitation, the dimensions of the volume boxes were maintained. Next, a volume analysis report was performed to calculate the “adjusted volumes” (volume of VSG-117₈₆ band – volume of average background bands) of the VSG-117₈₆ bands. VSG import percentages were obtained as follows: the “adjusted volume” corresponding to a band detected in the presence of TbRM and challenged with proteinase K was divided by the “adjusted volume” corresponding to

a band detected in the presence of TbRM without proteinase K. This quotient was multiplied by 100 to obtain a percentage of VSG import.

Cell Culture

Bloodstream *T. brucei* cell culture and drug susceptibility studies were performed as previously described (Patham and Mensa-Wilmot, submitted). Bloodstream *T. brucei* CA427 were cultured in HMI-9 media (Hirumi and Hirumi 1994) to a density of 10^6 cells/ml. Cells were seeded (500 μ l of 4×10^4 cells/ml) to a 24-well plate and exposed to MAL3-101, CAM741, and NFI028 at listed concentrations (see figure legends) or an equal volume of DMSO. Cells were incubated at 37°C and counted after 24 and 48h, using a hemocytometer.

Results

MAL3-101 and MAL3-51 Inhibit Post-Translational Import of VSG-117 into *T. brucei*

Microsomes

Cytosolic chaperones are essential for post-translational protein import into the ER (McClellan, Endres et al. 1998; McClellan and Brodsky 2000; Ngosuwan, Wang et al. 2003; Wickner and Schekman 2005). Specifically, homologues of Hsp70 and its co-chaperone Hsp40 facilitate import in yeast by rendering secretory precursors “import competent” (Caplan, Cyr et al. 1992; McClellan and Brodsky 2000; Ngosuwan, Wang et al. 2003). MAL3-101 inhibits Hsp40 stimulation of Hsp70 ATPase, and inhibits post-translational translocation of pp α MF into yeast microsomes (Fewell, Smith et al. 2004). In *T. brucei*, VSG can enter ER microsomes (TbRM) post-translationally (Patham and Mensa-Wilmot, submitted). Since post-translational VSG translocation into TbRM may depend on cytosolic chaperones, we tested the effects of MAL3-101 and MAL3-51 on import of the protein into TbRM. As a model substrate we used VSG-117 that is truncated to 86 amino acids because unlike longer substrates it appears to have

less stringent requirements for efficient import into TbRM (Patham and Mensa-Wilmot, unpublished).

MAL3-101 (10 μ M) reduced VSG-117₈₆ import into TbRM by 54% compared to a DMSO control (Fig. 2A-B). Thus, MAL3-101 inhibits post-translational import of VSG-117₈₆ into TbRM. In *T. brucei*, cytosol is crucial for post-translational import of full-length VSG into TbRM (Patham and Mensa-Wilmot, submitted). Therefore, we tested the effect of *T. brucei* cytosol on VSG-117₈₆ import into TbRM in the presence of MAL3-101. *T. brucei* cytosol suppressed MAL3-101 inhibition of VSG-117₈₆ import into TbRM; 90% of VSG-117₈₆ was imported into TbRM in the presence of MAL3-101 (10 μ M) (Fig. 2A-B).

MAL3-51 (10 μ M) also reduced import of VSG-117₈₆ into TbRM by 41% compared to the control (Fig. 2A-B). However, *T. brucei* cytosol failed to rescue the MAL3-51 inhibition of VSG-117₈₆ import into TbRM; 45% of VSG-117₈₆ was imported into TbRM in the presence of MAL3-51 (10 μ M) (Fig. 2A-B).

From this data we conclude that the target of MAL3-101 is most likely a cytosolic protein (or factor); excess quantities of it from *T. brucei* cytosol bypass inhibition by the compound. On the other hand, excess amounts of the target of MAL3-51 are not present in cytosol from *T. brucei*.

The effects of MAL3-101 and MAL3-51 on VSG import into TbRM suggested that these compounds might inhibit translocation of other proteins *in vivo*, and compromise viability of *T. brucei*. Therefore, we tested whether MAL3-101 and MAL3-51 were trypanocidal. MAL3-101 inhibited cell growth (IG₅₀ of 1.5 μ M), killing all parasites at 3 μ M (Fig. 2C). However, MAL3-51 did not kill *T. brucei* at concentrations up to 500 μ M (data not presented).

NFI028 Inhibits VSG-117₈₆ Import into TbRM

CAM741 and NFI028 (Fig. 1C-D) are cyclopeptolides, both of which inhibit mammalian co-translational protein import into the ER in a signal peptide-dependent fashion (Besemer, Harant et al. 2005; Harant, Lettner et al. 2006; Harant, Wolff et al. 2007). The effects of NFI028 and CAM741 on post-translational import have not been tested. In general however, *T. brucei* signal peptides are different from most mammalian signal peptides and are not translocated into canine microsomes (Al-Qahtani, Teilhet et al. 1998). Based on these facts, we tested the effects of NFI028 and CAM741 on import of VSG-117₈₆ into TbRM to evaluate their possible effects on *T. brucei* VSG-117.

NFI028 (1 μ M) inhibited VSG-117₈₆ translocation into TbRM by 49%, compared to a DMSO control (Fig. 3A-B). Higher concentrations of NFI028 (5 and 10 μ M) inhibited VSG-117₈₆ import into TbRM to the same degree (data not presented). In contrast, CAM741 (1 μ M) did not affect import; VSG-117₈₆ import into TbRM was 73% and 74% in the presence of CAM741 or DMSO (solvent), respectively (Fig. 3A-B). Similar results were obtained when import of VSG-117₈₆ into TbRM was tested with 10 μ M CAM741 (data not presented).

NFI028 and CAM741 are Trypanocidal

Based on the observation that NFI028 inhibited post-translational import of VSG-117₈₆ into TbRM, we sought to determine if NFI028 was trypanocidal. Our hypothesis was that if NFI028 inhibited ER protein import of VSG-117₈₆ in our cell-free system it might also block translocation of other proteins *in vivo*. CAM741 was studied as a control compound.

Upon exposure of bloodstream *T. brucei* to NFI028, the cells were killed (IG₅₀ of 1.5 μ M) (Fig. 3C). Surprisingly, cells exposed to CAM741 were also killed with an IG₅₀ of 2 μ M (Fig. 3D).

Sodium Azide Inhibits Post-Translational Import of VSG-117₈₆ into TbRM

Azide is a metabolic poison (Detimary, Gilon et al. 1994; Van de Castele, Kefas et al. 2003; Bowler, Montgomery et al. 2006) that inhibits growth of bloodstream *T. brucei* (Steверding and Scory 2004). Azide inhibits F-type ATPases (Bowler, Montgomery et al. 2006) and SecA, blocking protein secretion in prokaryotes (Oliver, Cabelli et al. 1990; van der Wolk, de Wit et al. 1997; Miller, Wang et al. 2002). To test the nature of (chaperone) ATPases that might influence post-translational translocation of VSG-117₈₆ into TbRM, we evaluated the effects of azide and AEE788, an ATP mimic which inhibits kinase activities of endothelial growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) (Traxler, Allegrini et al. 2004; Park, Younes et al. 2005; Yazici, Kim et al. 2005).

When VSG-117₈₆ import into TbRM was tested in the presence of sodium azide (8 μ M), post-translational import of the protein into TbRM was inhibited by 54% (Fig. 4A-B). Similarly, VSG-117₈₆ import into TbRM was inhibited by 20% with 4 μ M sodium azide (data not presented). However, AEE788 (8 μ M) failed to inhibit post-translational import of VSG-117₈₆ into TbRM (Fig. 4A-B).

Next, we sought to determine if sodium azide was inhibiting ER protein import by acting on a membrane component of TbRM. This was accomplished by testing import of VSG-117₈₆ into TbRM that had been pre-treated with sodium azide (8 μ M); TbRM pre-treated with NFI028 (1 μ M) was used as a control. TbRM were treated with concentrations of sodium azide or NFI028 that were previously shown to inhibit ER protein import (Fig. 4A-B and 3A-B, respectively). Addition of pre-treated microsomes to the reaction mixture reduced the effective concentrations of sodium azide and NFI028 from 8 μ M and 1 μ M to 1.4 μ M and 0.2 μ M, respectively. Protein import of VSG-117₈₆ into azide-treated microsomes was not inhibited (Fig.

4C-D), suggesting that azide does not act on TbRM membranes. As a control, TbRM were pre-treated with NFI028 (1 μ M) and tested for protein import. Import of VSG-117₈₆ into these NFI028-treated microsomes was inhibited by 10% (Fig. 4C-D), indicating that, unlike sodium azide, NFI028 could act on TbRM membranes, or NFI028 inhibits protein import into TbRM when present at low concentrations in the reaction mixture.

Azide inhibits cytochrome c oxidase leading to a reduction of mitochondrial membrane potential, and blocks protein translocation into mitochondria (Bennett, Diamond et al. 1992). However, bloodstream form *T. brucei* do not possess a functional mitochondria and lack cytochromes (Vickerman 1985; Gull 2002; Steverding and Scory 2004). Therefore, azide is unlikely to reduce mitochondrial membrane potential in bloodstream *T. brucei*. Nevertheless, to ensure that the inhibitory effect of azide on VSG-117₈₆ import was occurring at ER-derived TbRM and not indirectly through inhibition of mitochondrial functions, we tested the effect of valinomycin on VSG-117₈₆ post-translational import. Valinomycin is a potassium ionophore that lowers membrane potential and inhibits mitochondrial protein import (at 0.5 μ M) in *T. brucei* (Yermovsky-Kammerer and Hajduk 1999; Priest and Hajduk 2003). The efficiency of VSG-117₈₆ import into TbRM was not affected by 0.5 μ M valinomycin (Fig. 4C-D), a concentration shown to inhibit mitochondrial protein import (Priest and Hajduk 2003).

From this data we conclude that sodium azide reduces post-translational import of VSG-117₈₆ into TbRM. However, azide does not have a direct effect on the *T. brucei* ER membrane.

Discussion

MAL3-101 and MAL3-51 Inhibit TbRM Protein Import But Only MAL3-101 Kills *T.*

brucei

MAL3-101 inhibits Hsp40-stimulated Hsp70 activity (Fewell, Smith et al. 2004). In *T. brucei*, MAL3-101 inhibited post-translational VSG-117₈₆ import into TbRM (Fig. 2A-B). However, cytosol from the parasite suppressed MAL3-101 inhibition of protein import (Fig. 2A-B). *T. brucei* cytosol contains proteins that could function as chaperones necessary for post-translational protein import. Cytosol may reduce MAL3-101 inhibition for a variety of reasons: one possibility is that the addition of cytosol raised the concentration of Hsp40, excess amounts of which overcame the effect of the inhibitor. There is precedent for this phenomenon: MAL3-101 inhibition of T-antigen (TAg) stimulated Hsp70 ATPase activity could be suppressed with excess amounts of TAg (Fewell, Smith et al. 2004).

MAL3-51 inhibited import of VSG-117₈₆ into TbRM in the absence of *T. brucei* cytosol, but unlike MAL3-101, cytosol did not block MAL3-51 effects on VSG-117₈₆ import into TbRM (Fig. 2A-B). We propose that MAL3-51 and MAL3-101 inhibit post-translational protein import into TbRM by different mechanisms. First, MAL3-51 could inhibit Hsp70 chaperoning of VSG-117₈₆, but not by inhibiting Hsp40/Hsp70 ATPase stimulation. Second, MAL3-51 could interact with different types of Hsps than those bound by MAL3-101. As an example of such phenomenon, MAL3-101 inhibits TAg stimulation of Hsp70 ATPase, but does not inhibit Ydj1 (Hsp40) stimulation of Hsp70 ATPase activity (Fewell, Smith et al. 2004).

MAL3-101 is trypanocidal, inhibiting bloodstream *T. brucei* growth *in vitro* (IG₅₀ of 1.5 μM) (Fig. 2C). This data suggests that MAL3-101 can cross the plasma membrane of the parasite to exert its effects. Given the diverse roles of Hsp40/Hsp70 chaperones *in vivo* we

predict that MAL3-101 affects ER protein import as well as a variety of other cellular processes (e.g. protein folding, protein import into the nucleus, protein import into glycosomes, signal transduction pathways) (reviewed in (Brodsky and Chiosis 2006)).

MAL3-51 did not kill bloodstream *T. brucei* cultured *in vitro* (data not presented). When the structures of MAL3-101 and MAL3-51 are compared (Fig. 1A-B), it is evident that the difference in abilities of MAL3-101 and MAL3-51 to inhibit protein import into TbRM and kill *T. brucei* may be a direct result of their structural differences. MAL3-101 possesses a pyrimidinone linked to a peptoid group by a hexylamino group (Fig. 1A, grey boxes) (Wright, Chovatiya et al. 2008). MAL3-51 has a peptoid group, but does not possess a pyrimidinone group (Fig. 1B) (Wright, Chovatiya et al. 2008). Also, the peptoid scaffolds of MAL3-101 and MAL3-51 have different substituents. Studies in mammalian tumor cells suggest that substituents off of the pyrimidinone group (R1) and peptoid group (R3) (Fig. 1A) are crucial for MAL3-101's ability to inhibit tumor growth (Wright, Chovatiya et al. 2008). MAL3-51 does not have a pyrimidinone group as seen in MAL3-101, and thus lacks a chemical group at the R1 position (compare Figs. 1A and 1B). Ultimately, these chemical differences may affect their cell permeability properties, causing MAL3-101 to be more effective than MAL3-51 *in vivo* (Wright, Chovatiya et al. 2008).

Two Related Cyclopeptolides have Different Effects on VSG Import into TbRM

NFI028, but not CAM741, inhibited VSG-117₈₆ import into TbRM (Fig. 3A-B). This is the first demonstration that NFI028 inhibits signal peptide function in a post-translational system. In a mammalian system, CAM741 and NFI028 inhibit ER protein import of a select group of signal peptides (Besemer, Harant et al. 2005; Harant, Lettner et al. 2006; Harant, Wolff et al. 2007). Specifically, CAM741 inhibits ER import of proteins whose signal peptides are

relatively inefficient at directing import, and those with “helix-breaking” residues (glycine and proline) in their signal peptides (Harant, Lettner et al. 2006; Harant, Wolff et al. 2007). It is suggested that CAM741 prevents proper association of the signal peptide with the Sec61 translocon by binding directly to the signal peptide and/or by competitively associating with the signal peptide-binding site at the translocon (Harant, Wolff et al. 2007).

Both NFI028 and CAM741 inhibited *T. brucei* growth, with similar IG_{50} concentrations (1.5 μ M and 2.0 μ M, respectively) (Fig. 3C-D). This data suggests that CAM741, along with NFI028, could inhibit other signal peptides *in vivo* thereby compromising viability of *T. brucei*. Also, the data indicates that CAM741 and NFI028 are transported into *T. brucei*, and thus are candidates for possible anti-trypanosome lead compounds. Furthermore, NFI028 and CAM741 have differences in their structures (Fig. 1C-D, light grey box); NFI028 possesses a piperidine-1-carbaldehyde substitution while CAM741 is substituted with a propyl propionate group in that position (Fig. 1C-D, dark grey box) (Harant, Wolff et al. 2007). We propose that these differences in structure produce unique effects on the VSG-117 signal peptide. CAM741 could inhibit other signal peptides *in vivo*, but not VSG-117. This indicates that derivatives of cyclopeptolides would be worth exploring for possible anti-trypanosomal properties.

Azide Inhibits Post-Translational Import of VSG-117₈₆ Into TbRM

Post-translational import of VSG-117₈₆ into TbRM was inhibited by sodium azide (Fig. 4A-B), but protein translocation was not inhibited when TbRM was pre-incubated with sodium azide (Fig. 4C-D). AEE788, a competitive inhibitor of ATP for protein tyrosine kinases, had no effect on protein translocation (Fig. 4A-B). These data indicated that azide did not inhibit all ATP-binding proteins, but blocked a select group of ATP-binding proteins. Further, valinomycin, an inhibitor of mitochondrial protein import, did not alter VSG-117₈₆ import into

TbRM (Fig. 4C-D) at a concentration shown to inhibit mitochondrial protein import (Priest and Hajduk 2003), verifying that azide inhibited protein import at ER-derived membranes and not some mitochondrial contaminant of TbRM. From these data we infer that azide inhibits a cytosolic chaperone that is important for VSG import into TbRM. Azide inhibits F-ATPases and SecA ATPase activity by forming a tight complex with bound-ADP in the ATPase catalytic site (Bowler, Montgomery et al. 2006), preventing the exchange of ADP for ATP. Since ATP hydrolysis is needed to drive SecA-dependent protein translocation, prokaryotic protein secretion is blocked in the presence of azide because the site of ATP hydrolysis is competitively inhibited by the ADP-azide complex (Oliver, Cabelli et al. 1990; van der Wolk, de Wit et al. 1997; Miller, Wang et al. 2002; Bowler, Montgomery et al. 2006). One possible explanation for azide sensitive ER protein import observed in *T. brucei* is that *T. brucei* could possess an azide-sensitive SecA-like cytosolic chaperone that is crucial for post-translational protein translocation into the ER. Alternatively, azide might inhibit chaperones with F-ATPase domains, halting protein translocation through the Sec61 channel in TbRM.

Protein translocation chaperones (*e.g.* Hsp40/Hsp70) and ER signal peptides are essential to post-translational protein import into the ER. Using a *T. brucei in vitro* translocation system we have shown that protein translocation into TbRM can be blocked by small molecule inhibitors that target these components. Thus, the properties of these inhibitors should be further explored for the discovery of new anti-trypanosome lead compounds.

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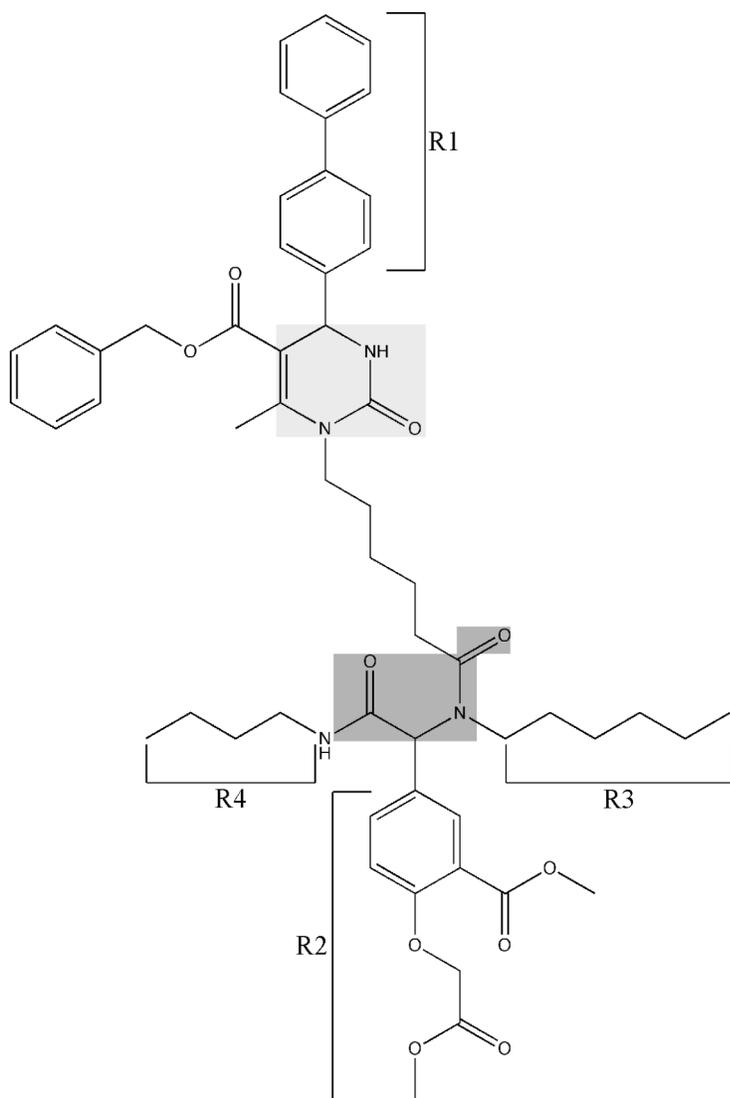
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Figure 1 – Structures of MAL3-101, MAL3-51, CAM741, and NFI028

The chemical structures for (A) MAL3-101 and (B) MAL3-51 were reproduced from (Wright, Chovatiya et al. 2008), using ChemBioDraw Ultra (version 11.0.1 CambridgeSoft). The pyrimidinone group is highlighted by a light grey box. The peptoid group is highlighted by a dark grey box, and substitutions (R1-R4) are marked by brackets. (C) CAM741 and (D) NFI028 chemical structures were reproduced from (Harant, Wolff et al. 2007), using ChemBioDraw Ultra (version 11.0.1 CambridgeSoft). Different spatial orientations between CAM741 and NFI028 are highlighted by light grey boxes, and different chemical substituents are denoted by dark grey boxes.

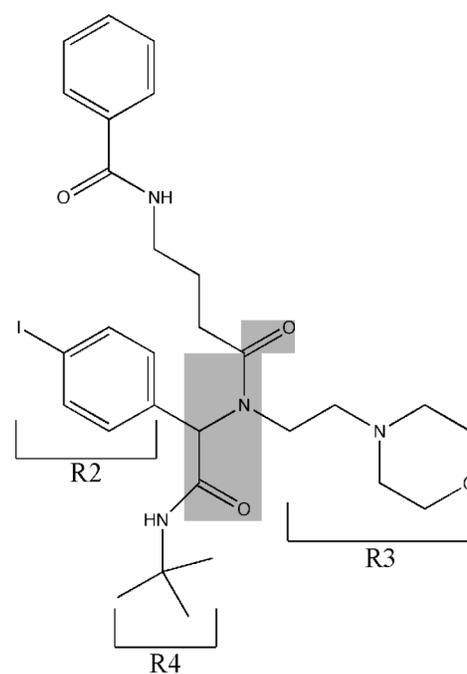
Figure 1

A



MAL3-101

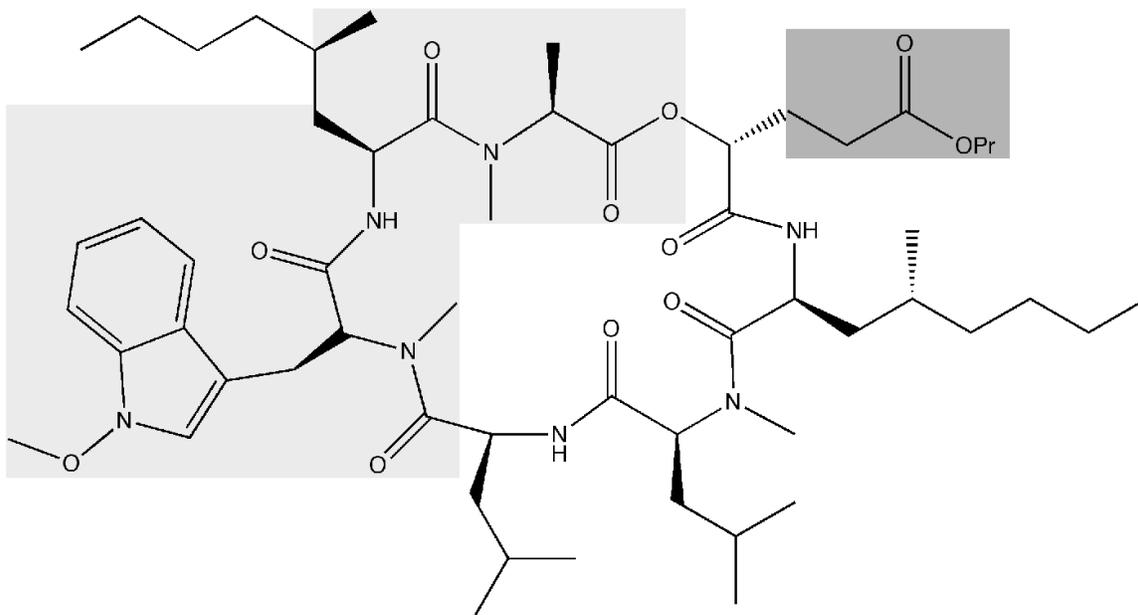
B



MAL3-51

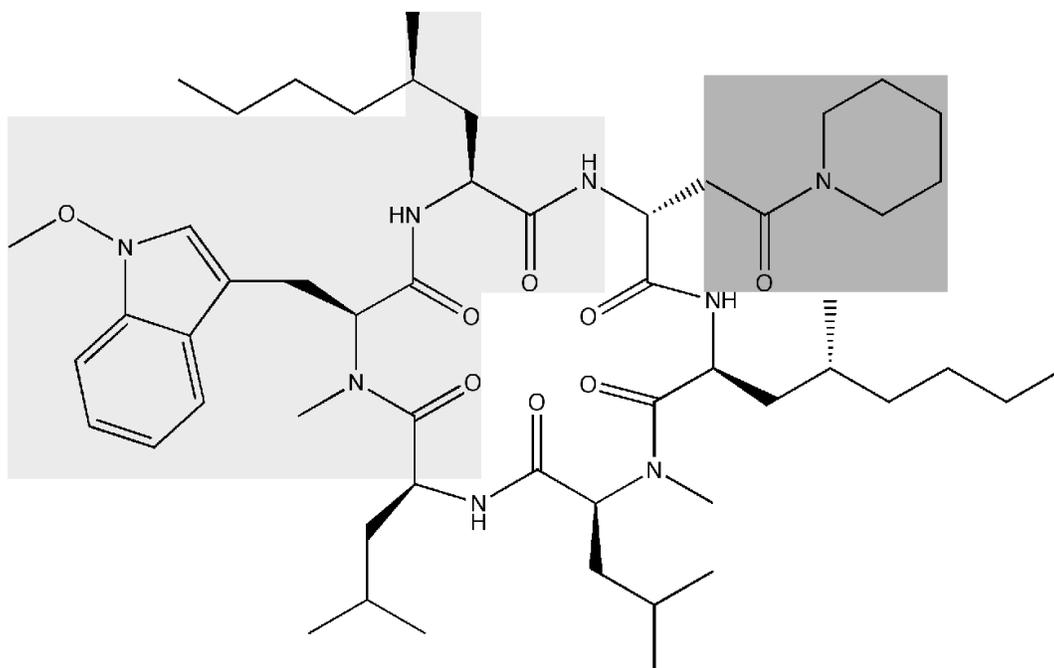
Figure 1

C



CAM741

D



NFI028

Figure 2 – MAL3-101 and MAL3-51 Affect Import of VSG-117₈₆ into *T. brucei* Microsomes

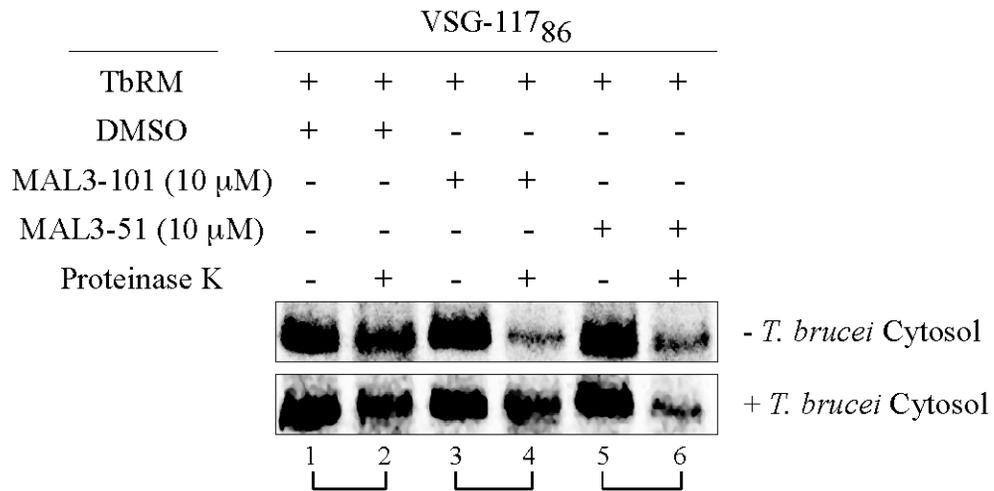
(A) Effects of MAL3-101 and MAL3-51 (10 μM) on VSG-117₈₆ import into *T. brucei*

microsomes in the absence or presence of *T. brucei* cytosol. VSG-117₈₆ mRNA was translated in rabbit reticulocyte lysate in the absence or presence of *T. brucei* cytosol for 1 hour at 37°C, and then cycloheximide was added to stop protein synthesis. TbRM (1 equivalent) and MAL3-101 (10 μM), MAL3-51 (10 μM), or an equal volume of DMSO was added to the samples on ice, and the mixture was incubated at 37°C for 1.5 hours for translocation of VSG-117₈₆ into the microsomes. Reaction mixtures were treated with proteinase K (PK) for 1 hour on ice.

Proteolysis was terminated with PMSF. Proteins were precipitated with ammonium sulfate, pelleted by centrifugation, resuspended in SDS-PAGE sample buffer, and resolved by Tris-Tricine SDS-PAGE. Gel images were obtained with a phosphorimager and analyzed with Quantity-One software. Reaction mixtures for *odd numbered lanes* contained TbRM, but not proteinase K; *even numbered lanes* contained TbRM and proteinase K. Brackets denote lanes that were compared to obtain percentage of VSG-117₈₆ import values. (B) Graphical representation of data from panel A. Quantitation of percent VSG import may not correspond to gel image because control lanes (VSG detected in the presence of TbRM without proteinase K) were adjusted to pixel saturation. (C) The effect of MAL3-101 on *T. brucei* growth.

Figure 2

A



B

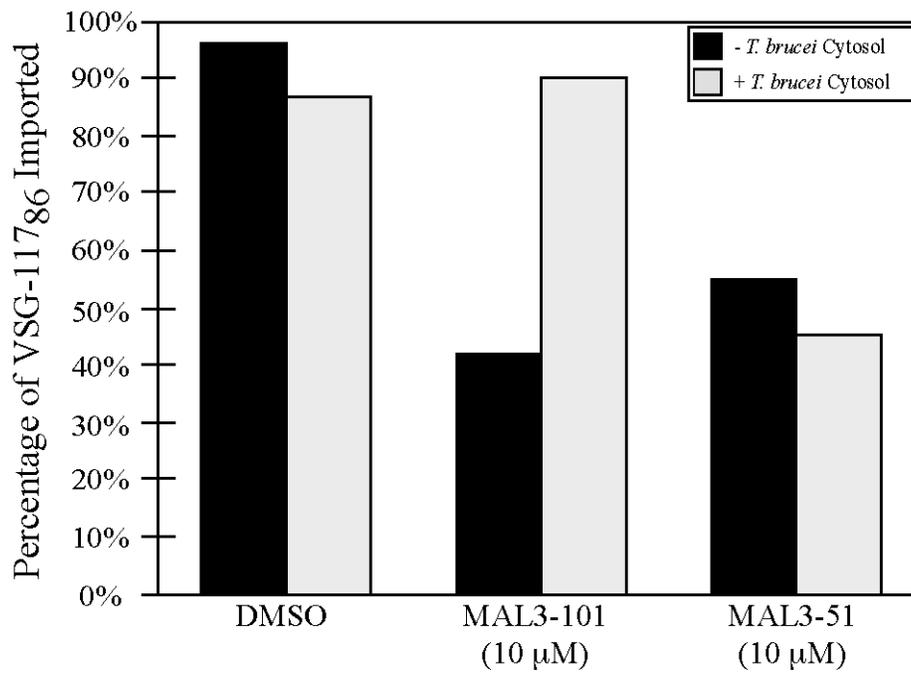


Figure 2

C

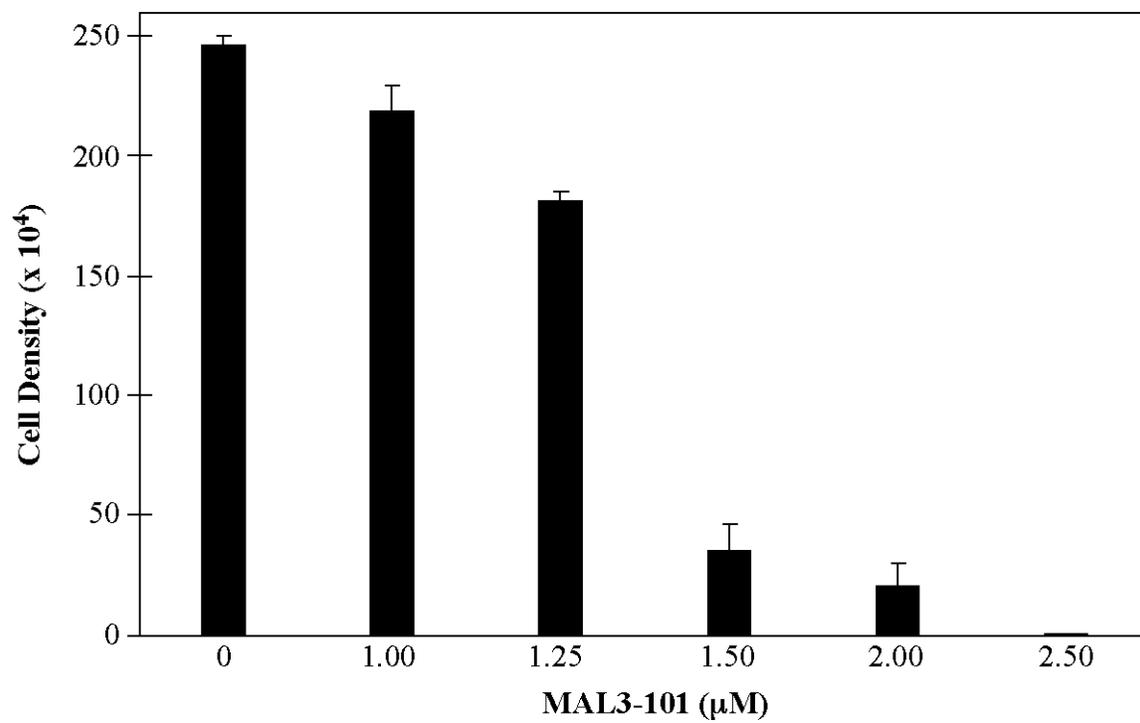


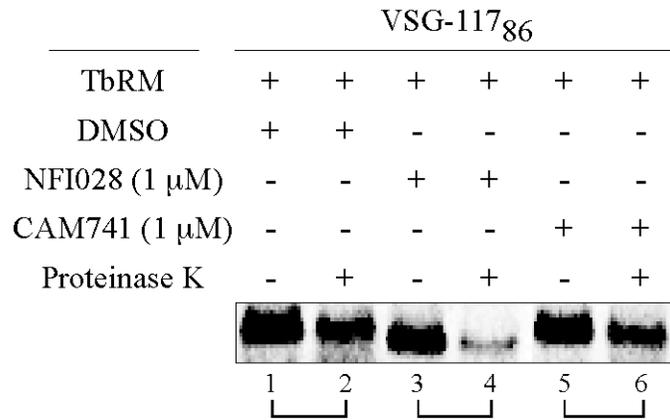
Figure 3 – Effects of NFI028 and CAM741 on Import of VSG-117₈₆ into *T. brucei*

Microsomes

(A) Import of VSG-117₈₆ into *T. brucei* microsomes in the presence of NFI028 or CAM741 (1 μM). VSG-117₈₆ mRNA was translated in rabbit reticulocyte lysate in the presence of *T. brucei* cytosol for 1 hour at 37°C, and then protein synthesis was stopped by the addition of cycloheximide. TbRM (1 equivalent) and NFI028 or CAM741 (1 μM) or an equal volume of DMSO was added to the translation mixture on ice, and incubated at 37°C for 1.5 hours. Reaction mixtures were treated with proteinase K (PK) on ice for 1 hour. Proteolysis was terminated with PMSF. Proteins were precipitated with ammonium sulfate, pelleted, resuspended in SDS-PAGE sample buffer, and resolved by Tris-Tricine SDS-PAGE. Images of radiolabeled proteins were obtained with a phosphorimager and quantitated with Quantity-One software. Samples depicted in *odd numbered lanes* contained TbRM, but were not treated proteinase K. Reaction mixtures shown in *even numbered lanes* contained TbRM and were treated with proteinase K. Brackets denote lanes that were compared to each other in order to obtain percentage of VSG-117₈₆ import values. (B) Graphical representation of data from panel A. (C) Effect of NFI028 on growth of *T. brucei*. (D) The effect of CAM741 on *T. brucei*.

Figure 3

A



B

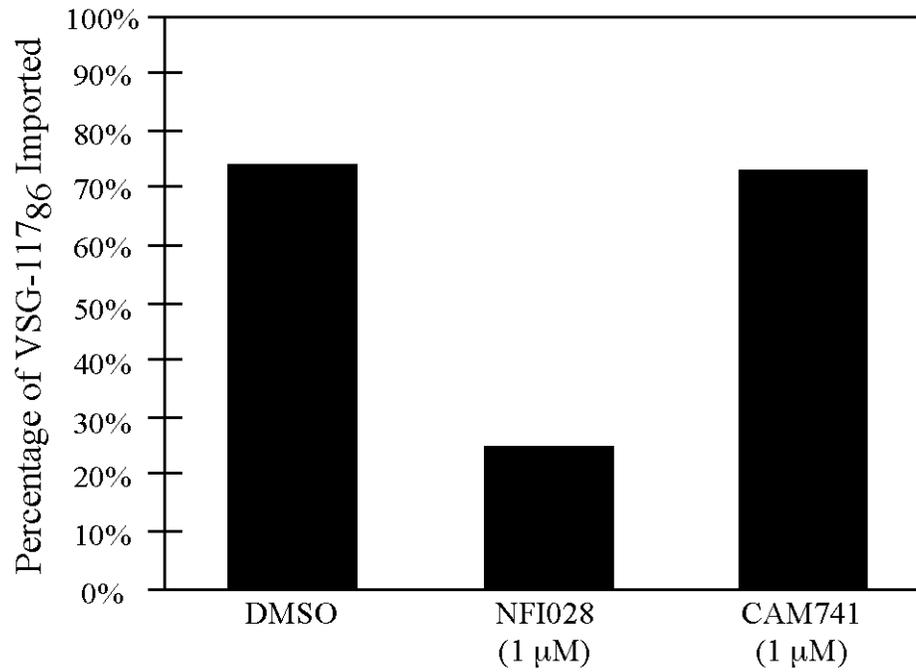
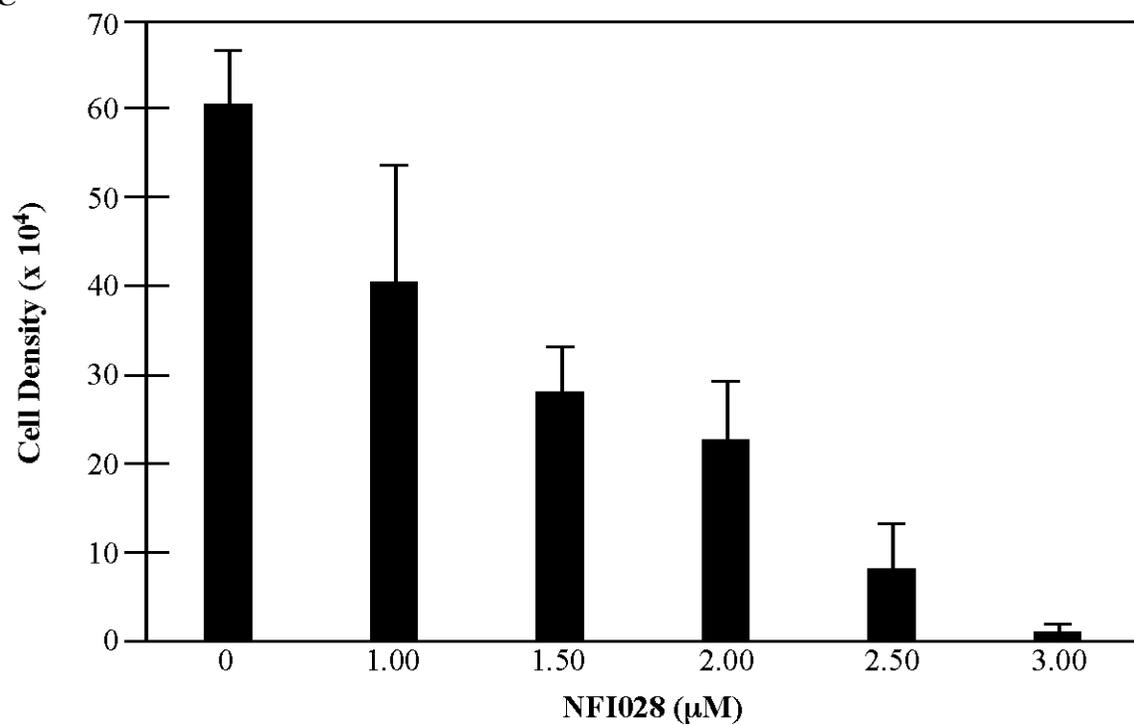


Figure 3

C



D

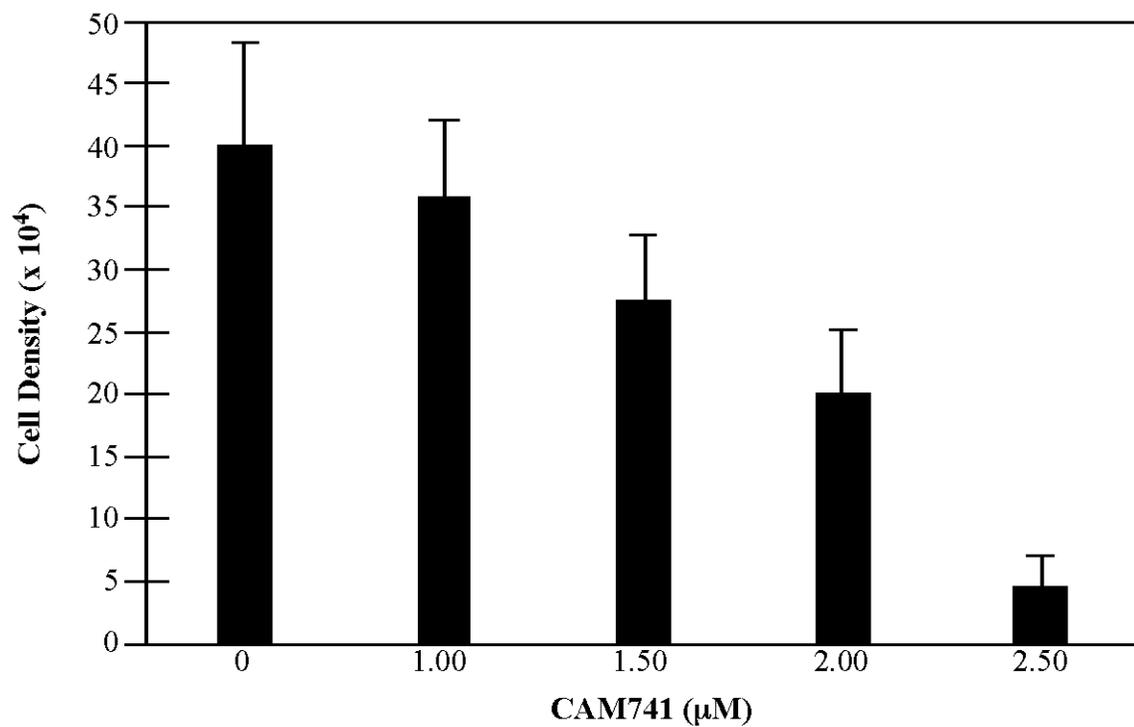
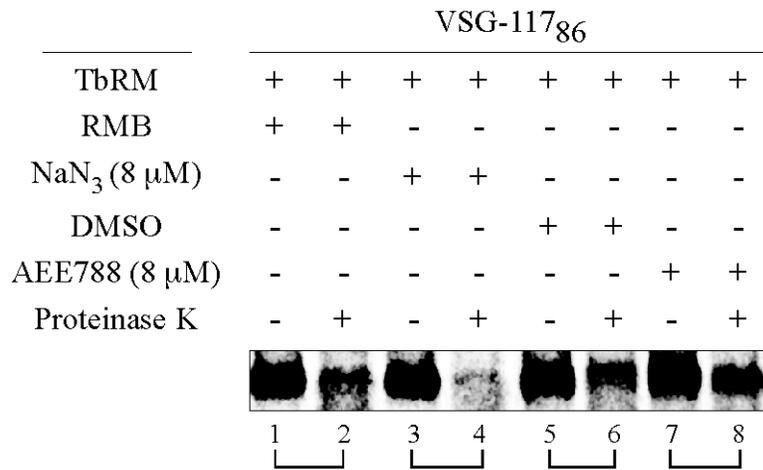


Figure 4 – Effects of Azide, AEE788, and Valinomycin on VSG-117₈₆ Import into *T. brucei* Microsomes

(A) Import of VSG-117₈₆ into TbRM in the presence of sodium azide or AEE788. VSG-117₈₆ mRNA was translated in rabbit reticulocyte lysate in the presence of *T. brucei* cytosol for 1 hour at 37°C. Cycloheximide was added to stop proteins synthesis. TbRM (1 equivalent) and sodium azide (8 µM), AEE788 (8 µM), an equal volume of microsome buffer (RMB) or DMSO was added to the samples on ice, and the mixtures were incubated at 37°C for 1.5 hours. Reaction mixtures were treated with proteinase K (PK) on ice for 1 hour. Proteolysis was terminated with PMSF. Proteins were precipitated with ammonium sulfate, pelleted, resuspended in SDS-PAGE sample buffer, and resolved by Tris-Tricine SDS-PAGE. Images of radiolabeled proteins were obtained with a phosphorimager and quantitated with Quantity-One software. Brackets show lanes that were compared to each other in order to obtain percentage of VSG-117₈₆ imported into TbRM. *Lanes 1, 3, 5, & 7* were treated with TbRM, but not proteinase K. *Lanes 2, 4, 6, & 8* were treated with TbRM and proteinase K. (B) Graphical representation of data from panel A. (C) VSG-117₈₆ import into TbRM pre-treated with sodium azide, NFI028, or valinomycin was tested using a protocol similar to that described in panel A, but with the following changes. TbRM that had been pre-incubated for 1 hour on ice with control buffer (RMB) (*lanes 1 & 2*), sodium azide (8 µM) (*lanes 3 & 4*), an equal volume of DMSO (*lanes 5 & 6*), NFI028 (1 µM) (*lanes 7 & 8*), valinomycin (0.5 µM) (*lanes 9 & 10*), or an equal volume of 100% ethanol (*lanes 11 & 12*) was added to the reaction mixtures and incubated at 37°C for 1.5 hours. Reaction mixtures depicted in *odd lanes* contained TbRM, but were not treated with proteinase K; *even lanes* contained TbRM and were treated with proteinase K. (D) Graphical representation of data from panel C.

Figure 4

A



B

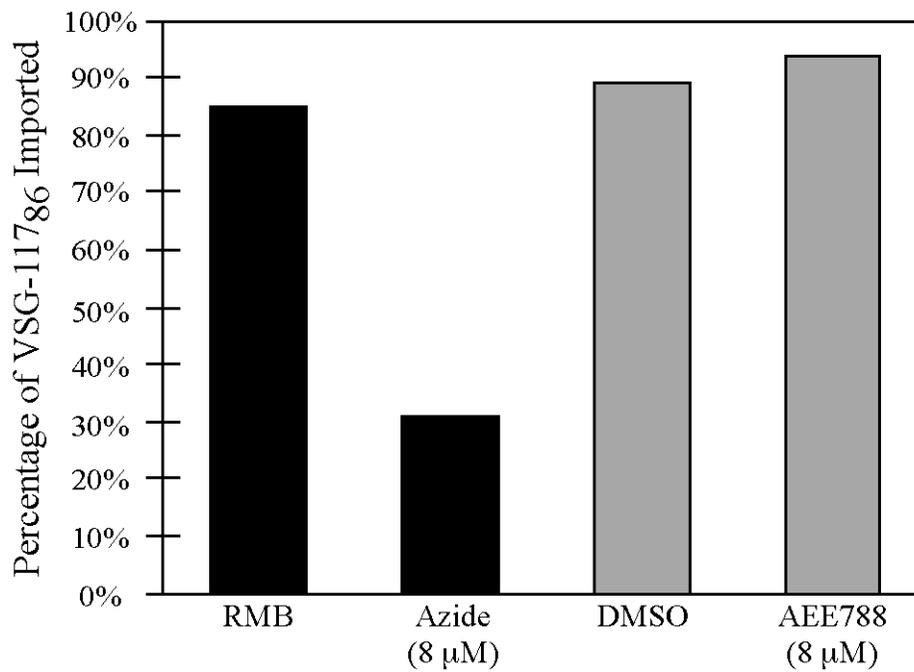
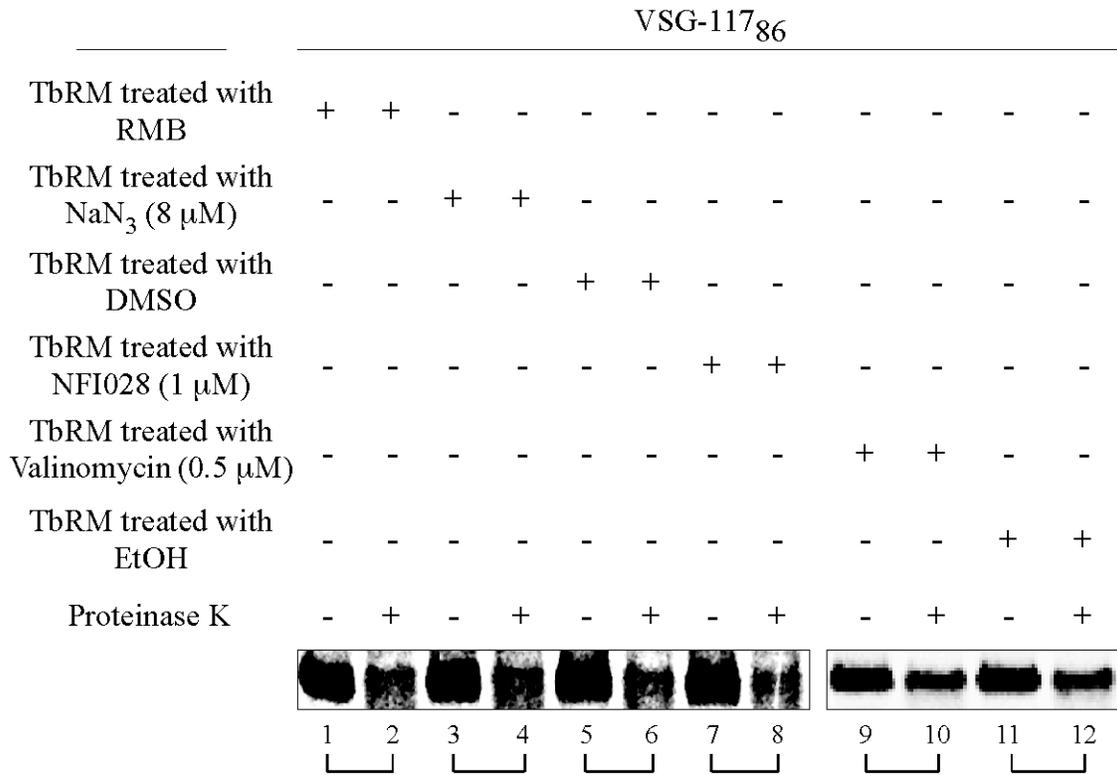
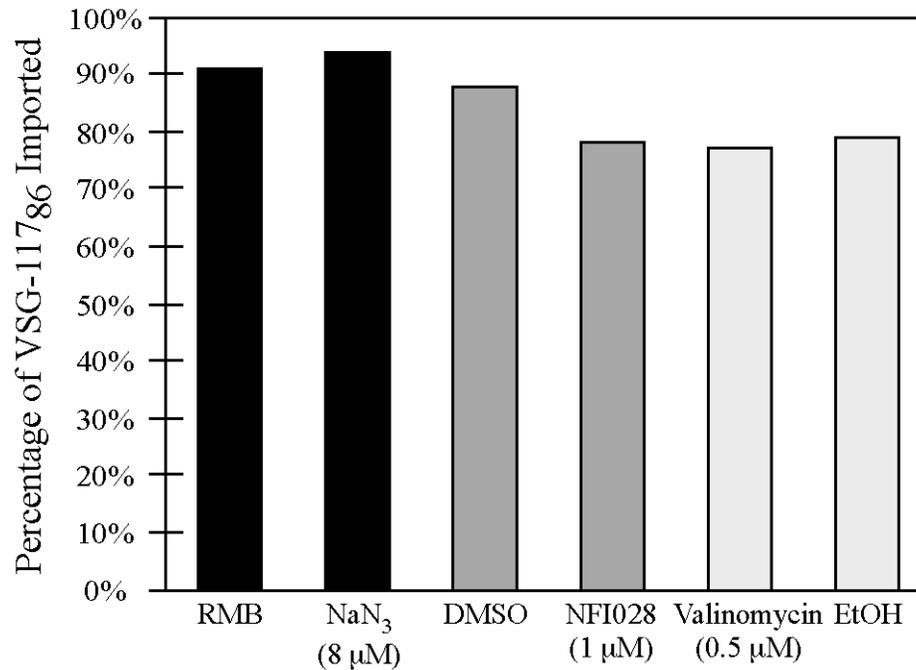


Figure 4

C



D



CHAPTER IV

DISCUSSIONS

Conclusions

Peak hydrophobicity of endoplasmic reticulum signal peptide h-regions has long been thought to govern signal peptide activity. However, h-region peak hydrophobicity failed to predict whether or not VSG-117₈₆ was imported into *T. brucei* microsomes (TbRM). Sequence alignments of signal peptides have failed to find any h-region sequence conservation. Moreover, h-regions are thought to be composed of random hydrophobic amino acids, where the arrangement of h-region amino acids does not affect biological function of a signal peptide. Further, when the sequences of VSG-117 h-regions were rearranged, signal peptide activity was dramatically reduced. From bioinformatic analysis, a set of conserved tri-component h-motifs (e.g. L-L-x-[AILV], L-x(1,2)-L-[AILV], and L-x(2,3)-L-[AILPV]) was found in the h-regions of *T. brucei*. Functional analysis showed that h-motifs have flexible amino acid requirements, and that the order of amino acids in h-motifs is key to signal peptide activity. We speculate that h-regions form α -helices with h-motif “identity components” arranged on opposite sides of the α -helix. This arrangement enables side chains of “identity component” amino acids to contact transmembrane domains (TMs) 2b and 7 of Sec61 α , the suggested signal peptide-binding site.

A *T. brucei* microsomal protein import system was used to identify small molecule compounds that blocked translocation of VSG-117₈₆ into TbRM. Sodium azide, but not valinomycin or AEE788, inhibited post-translational import of VSG-117₈₆ into TbRM. However, ER import of VSG-117₈₆ was not inhibited when TbRM were pre-incubated with sodium azide. This data indicates that the factor inhibited by azide is not specific to the ER membrane. Also, pyrimidinone-peptoid derivatives MAL3-101 and MAL3-51 and the cyclopeptolide NFI028 inhibited post-translational import of VSG-117₈₆ into TbRM. Further, MAL3-101, NFI028, and CAM741 were trypanocidal (IG₅₀'s of 1.5 μ M, 1.5 μ M, and 2.0 μ M,

respectively). Thus, pyrimidinone-peptoid and cyclopeptolide derivatives are lead classes of compounds for discovering new anti-trypanosome drugs.

New Quantitation Method for Percent VSG Import

Gel images were adjusted with Quantity One so that the relatively weak band intensities representing protease-protected VSG proteins could be visualized. VSG import percentages were calculated from a Tiff image that preserved these adjustments (see Methods and Materials). These adjustments to the gel images to visualize the protease-protected bands resulted in a visual saturation of the controls bands (*i.e.* with TbRM but no proteinase K). This pixel saturation produced bands of equivalent intensities among the control bands. Thus, calculations of percent VSG import from this adjusted Tiff image were in some cases overestimated. To correct this problem we devised a new method for calculating percent VSG import.

In this new method, gel images were adjusted in the same manner as previously described, so that protease-protected VSG bands were visible. However, instead of calculating import percentages from this Tiff image that preserved these visual adjustments and thus altering the band intensities, we calculated band intensities from the original gel scan, which preserved the “raw data”. Band intensities were calculated from this unaltered image as previously described (see Methods and Materials). Next, these lane-specific VSG import percentages were compared to the VSG import percentage of a VSG-117₈₆ internal control present in each gel. As a result, VSG import percentages of VSG-117₈₆ h-region variants and reactions performed in the presence of small molecule inhibitors were expressed as a percentage of import of VSG-117₈₆. By this calculation, the control VSG-117₈₆ import percentage represents 100% import, and all other VSG import is expressed as a fraction of VSG-117₈₆ import and multiplied by 100.