CHARACTERIZATION OF THE SPOROZOITE AND ERYTHROCYTIC STAGES (SES) PROTEIN

A Dissertation
presented to
the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree

Doctor of Philosophy

by
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DECEMBER 2007

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CHARACTERIZATION OF THE SPOROZOITE AND ERYTHROCYTIC STAGES (SES) PROTEIN

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I dedicate this dissertation to my family members, living and deceased, who have always believed in and encouraged me throughout my graduate studies. I could not have done this without your love and support.

ACKNOWLEDGEMENTS

I thank my advisor Dr. Brenda T. Beerntsen for her guidance and encouragement and Drs. Cynthia Besch-Williford, Charles Carson, Robert Hall, and Qisheng Song for serving as members of my Ph.D. committee. I also thank Dr. Gerald Buening for accepting me into the Mentored Biomedical Research Training Initiative (MBRTI) program that partially funded this Ph.D. degree. To my lab colleagues, I thank you for always being available to assist with various projects and for making my time in the lab interesting and exciting.

To Ruguang Ou, who was there when I started my graduate work and there when I finished. I thank you for the tireless work you put in every day and all of the help you have given me throughout the years. I will truly miss you.

Finally, I thank my husband Joseph, my son Tynan, and my mother Wanda for their undying love and support and believing that I could accomplish anything I set my mind to. You are the most important people in my life and I could never have done this without you.

This study was supported by awards from the National Institutes of Health (NIH) (AI 064306, AI053156) and a NIH/ Mentored Biomedical Research Initiative Fellowship (MBRTI) (R25 GM56901) and a NSF/Missouri Alliance for Graduate Education and the Professoriate Fellowship (MAGEP).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table Page

CHARACTERIZATION OF THE *PLASMODIUM* SPOROZOITE AND ERYTHROCYTIC STAGES (SES) PROTEIN

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ABSTRACT

The *Plasmodium* sporozoite is infective for mosquito salivary glands and vertebrate host tissues. Although it is a key developmental stage of the malaria parasite, relatively few sporozoite surface or secreted proteins have been identified and characterized. A novel surface molecule, designated the Sporozoite and Erythrocytic Stage (SES) protein, is preferentially-expressed in salivary gland sporozoites versus oocyst and hemolymph sporozoites of *Plasmodium gallinaceum*. PgSES exhibits a spiral surface labeling pattern that overlays a known sporozoite surface antigen, the circumsporozoite protein, with only minor co-localization. It consists of 551 amino acids encoding a putative 63.2 kDa protein that has been shown to be expressed not only on particular sporozoite stages, but also during the erythrocytic stages. This novel protein has three conserved regions of unknown function that are present in eight *Plasmodium* spp. representing human, avian, non-human primate, and rodent malarias. Antibody blocking studies assessing the role of PgSES in sporozoite invasion of mosquito salivary glands show that anti-PgSES antibodies block invasion by 49-87%. The *Plasmodium falciparum* homolog, PfSES, also appears to have expression during the sporozoite and erythrocytic stages. Additional studies assessing the function of both PgSES and PfSES in the sporozoite and erythrocytic stages are being conducted. Ultimately, if the SES protein is found to be critical to parasite development and/or invasion of host tissues, it could be a target for novel malaria intervention effort

CHAPTER 1. INTRODUCTION

1. Background and Significance

Malaria is caused by a protozoan parasite from the genus *Plasmodium*. The four species that infect humans are *P. vivax*, *P. ovale*, *P. malariae*, and *P. falciparum* (the most pathogenic form). According to the World Health Organization (WHO), more than 500 million people are infected with *Plasmodium* throughout the world and every year over one million people, most of whom are children under the age of five in Africa, die from this devastating disease. The African continent accounts for 90% of the disease burden, followed by Southeastern Asia with 9% of the burden (Breman *et al*., 2004). Latin America, the Middle East and parts of Europe also are affected by this disease (WHO Fact Sheet, 2007). Malaria is exhibiting a resurgence and re-emergence, due in part to drug-resistant parasites, insecticide-resistant mosquitoes, civil conflict, and the transport of malaria from endemic countries to malaria-free regions by travelers (Porter, 2006, WHO, 2007).

In sub-Saharan Africa, about 60% of the population lives in malaria endemic areas where immunity is acquired at approximately the age of five and the disease burden is concentrated in young children and pregnant women (Goodman and Mills, 1999). The remaining 40% of the population lives in areas of seasonal transmission or unstable transmission, where immunity is acquired at approximately the age of 10 and when epidemics occur there is morbidity and mortality in all age groups (Goodman and Mills, 1999).

The increase in malaria cases has had a devastating effect to the economy in developing countries (WHO, 2007). According to the WHO (2007), in countries with intense transmission, malaria causes an average loss of 1.3% annual economic growth and as a result, traps families and communities with limited access to health care in a downward spiral of poverty. In some countries, the disease may account for as much as 30-50% of inpatient admissions, 40% of public health expenditure, and up to 60% of outpatient visits (WHO, 2007). For example, malaria in pregnancy poses an economic burden due to the cost of preventative treatments and the costs associated with low birth weight babies, such as hospitalization, tests, drugs, and other treatments (Worrall *et al*., 2007). In a poor household these costs can consume a large amount of income.

The lifelong effects of malaria include increased poverty, impaired learning, and a decrease in attendance in school and the workplace (WHO, 2007). All of these devastating effects have made it necessary to develop more cost effective and novel ways (*i.e.*, new drugs, vaccines, and vector control strategies) to complement or replace existing malaria control methods.

2. Life-cycle

The *Plasmodium* parasite requires both the mosquito vector and the vertebrate host to complete its life cycle (Figure 1.1). When a female mosquito takes a blood meal from an infected host, she ingests the infective gametocytes that develop into male and female gametes within the mosquito midgut. Fertilization then occurs resulting in the formation of a diploid zygote that elongates into a motile ookinete. The ookinete migrates through the mosquito midgut epithelial cells and lodges between the cell and the basal lamina, where it develops into an oocyst. Within the oocyst, sporozoites develop that later break

out of the oocyst, enter the hemocoel, and migrate to the salivary glands. Once in the salivary glands, the sporozoites then can be injected into a new host at the next blood feeding. With the human malaria parasites, the sporozoites initiate an infection in the liver that results in the development of schizonts which contain thousands of merozoites that are eventually released into the circulatory system and infect red blood cells (RBCs). Within RBCs, the merozoites undergo erythrocytic schizogony or develop into gametocytes, via a process known as gametocytogenesis, and they are infective to the mosquito (Figure 1.2).

3. Gametogenesis

Within the mosquito midgut, male and female gametocytes transform into mature gametes in a process known as gametogenesis (Vlachou *et al*., 2006). Depending on the species, maturation *in vivo* can be triggered by various factors such as a drop in temperature from 37°C by 5°C, exposure to xanthurenic acid, an increase in pH, and other mosquito factors (Micks *et al*., 1948; Bishop *et al*., 1956; Sinden *et al*., 1983; Carter and Graves, 1988; Billker *et al*., 1998; Garcia *et al*., 1998). As shown in *P. berghei*-infected *An. stephensi*, the environmental and mosquito-specific factors trigger a cascade of biochemical events that include the following: 1) calcium is released into the gametocyte cytoplasm, 2) gametocytes emerge from the red blood cell and begin to develop, 3) CDPK4, a calcium-dependent protein kinase, is activated and stimulates exflagellation in male gametocytes that produces eight motile gametes (Billker *et al*., 2004), and 4) Pbmap2, a mitogen-activated kinase, stimulates the release of the flagellated gametes (Khan *et al*., 2005; Tewari *et al*., 2005). Fertilization occurs and the result is a diploid zygote that elongates into a motile ookinete.

4. Oocyst formation

Plasmodium oocyst formation begins when the ookinete penetrates the peritrophic matrix of the mosquito midgut and migrates through the epithelium where it becomes lodged between the epithelium and the basal lamina. Laminin and collagen are two major components of the basal lamina that the ookinete has been shown to bind to via *in vitro* studies (Hynes and Zhao, 2000). In *P. berghei* and *P. gallinaceum*, several ookinete proteins such as the secreted ookinete adhesive protein (SOAP) (Dessens *et al*., 2003), P25/P28 (Adini and Warburg, 1999; Vlachou *et al*., 2001), and the circumsporozoite and TRAP-related protein (CTRP) (Mahairaki *et al*., 2005) appear to mediate the interaction between the ookinete and laminin (Figure 1.1). Studies by Adini and Warburg (1999) suggest that these receptor-ligand interactions may trigger the ookinete to develop into an oocyst. Other proteins such as the membrane-attack ookinete protein (MAOP) and the *Plasmodium* chitinase are also involved in oocyst formation (Dessens *et al*., 2001, Kadota *et al*., 2004).

4.1 SOAP

The *P. berghei* SOAP was identified via a differential screening of a substracted cDNA library (Dessens *et al*., 2003). It is 921 bp in length and encodes an 18.2 kDa protein that localizes to the ookinete micronemes. Dessens *et al.* (2003) used a yeast-two-hybrid system to show that SOAP strongly interacts with domain V of vector-competent *Anopheles spp*. laminin γ1. In a related study, SOAP-knockout (KO) ookinetes resulted in reduced oocyst numbers by 15-40% and sporozoites developed normally and were infective for the mosquito salivary glands and vertebrate host tissues. These data

suggested that the main role for SOAP is in transformation of the ookinete to the oocyst and not sporozoite development or infectivity.

4.2 P25 and P28

The ookinete surface molecules, P25 and P28, also are involved in the ookinete-oocyst transformation. Both proteins are structurally similar (Duffy *et al*., 1993; Paton *et al*., 1993; Fried *et al*., 1994), have been shown to bind to laminin (Adini and Warburg, 1999; Vlachou *et al*., 2001) and studies by Tomas *et al.* (2001) have shown that they have redundant functions in *P. berghei*. In those studies, single (Sko) and double (Dko) knockouts were performed to determine the function of P25 and P28. The P25 and P28 Sko parasites produced similar numbers of ookinetes *in vivo* and *in vitro* when compared to the wild-type but oocyst production was significantly reduced by 32-43%. The P25/P28 double-KO parasites reduced ookinete production by 92% and when equal numbers of wild-type and Dko parasites were used to infect mosquitoes, oocyst formation was reduced by 99% (Tomas *et al*., 2001). These data suggested that P25 and P28 have redundant functions in ookinete development and transformation to oocysts that cannot be compensated for by other genes (Tomas *et al*., 2001).

In *P. vivax,* P25 interacts with calreticulin (crt), a calcium-dependent lectin, in the microvilli region of *An. albimanus* midgut cells (Rodriguez *et al*., 2007). The role of crt in the midgut epithelial cells is still unclear; however, it has been suggested that crt is expressed as part of the immune defense against invading pathogens and that the ookinete interacts with crt to evade the mosquito immune response. It is also possible that crt acts as a recognition signal for midgut invasion by the ookinete (Rodriguez *et al*., 2007).

4.3 CTRP

CTRP is an ookinete micronemal protein that contains extra-cellular von Willebrand factor A (vWA) and thrombospondin type 1 domains and a cytoplasmic domain that interacts with actomyosin machinery (Templeton *et al*., 2000; Kaneko *et al*., 2006). Antibodies against CTRP, which were acquired via membrane feeding, reduced oocyst formation by 85-94% in *P. falciparum*-infected *An. gambiae* and 92-100% in *An. stephensi* (Li *et al*., 2004). Dessens *et al*. (1999) showed that CTRP is essential for ookinete infectivity via gene disruption studies. In those studies, *P. berghei* ookinetes with a disrupted CTRP gene had reduced motility, did not trigger an immune response in *An. stephensi*, and failed to develop into oocysts. The lack of oocyst development was most likely due to the mutant parasites failure to invade the midgut epithelium and bind to the basal lamina which is a trigger for oocyst formation (Weathersby, 1952; Dessens *et al*., 1999).

4.4 MAOP and Plasmodium chitinase

The MAOP and *Plasmodium* chitinase also have been shown to be involved in ookinete invasion of host cells and oocyst development (Dessens *et al*., 2001; Kadota *et al*., 2004). *Plasmodium berghei* ookinetes with a disrupted MAOP gene were unable to invade *An. stephensi* midgut epithelial cells and establish an infection in the mosquito (Kadota *et al*., 2004). *Plasmodium* ookinetes secrete a chitinase to breakdown the chitin containing peritrophic membrane that surrounds a blood meal so that they can invade the mosquito midgut epithelium (Huber *et al*., 1991). In *P. berghei*, targeted gene disruption of *PbCHT1* (chitinase 1) significantly reduced oocyst formation in *An. stephensi* by 30-90%; however, the oocysts formed normal numbers of sporozoites that were infective for

the mosquito salivary glands (Dessens *et al*., 2001). These studies suggested *PbCHT1* is important for the ookinete stage, but not essential for sporozoite invasion of the mosquito salivary glands. The data also suggested that *PbCHT1* may function in ookinete egress from the epithelial cells and microvilli, but further studies are still needed (Dessens *et al*., 2001).

5. Sporozoite formation

The formation of sporozoites involves a series of nuclear divisions that occur within the oocyst, resulting in haploid nuclei (Kappe *et al*., 2004). At the same time, the endoplasmic reticulum cisternal space expands and segregates the cytoplasm into sporoblasts that give rise to sporozoites (Terzakis *et al*., 1967). In *P. falciparum*, 9-10 days following an infective blood meal, the mature oocyst will be 40-50 μm in diameter and contain thousands of sporozoites (Gills and Warrell, 1993).

The sporozoite consists of an elongate nucleus, mitochondria, endoplasmic reticulum, free ribosomes, and a Golgi apparatus. Also present are specialized secretory vesicles, the rhoptries, micronemes and dense granules, which define the apical complex (Matuschewski, 2006) (Figure 1.3). The sporozoite is surrounded by a three-layered pellicle supported by microtubules. The pellicle consists of an outer plasma membrane and a bilayered inner membrane complex (IMC). The actin-myosin motor, which is important for sporozoite motility, is located between the outermost layer of the IMC and the outer plasma membrane (Kappe *et al*., 2004).

5.1 The circumsporozoite (CS) protein

The circumsporozoite (CS) protein, the most abundant sporozoite surface protein, has been shown to play a role in sporozoite development (Yoshida *et al*., 1981;

Nussenzweig and Nussenzweig, 1985). A study investigating the role of CS in the development of *P. berghei* sporozoites in *An. stephensi* found that sporozoites were not able to develop within CS^- mutant oocysts (Menard *et al*., 1997). Another *P. berghei* knockout study examining the role of the CS protein in oocyst differentiation has shown that the CS protein organizes the formation of the sporozoite cytoskeleton which is necessary for sporozoite budding inside the oocyst (Thathy *et al*., 2002).

5.2 Plasmodium multidomain PxSR

Another molecule that has been implicated in oocyst sporozoite development is the *Plasmodium* multidomain scavenger receptor-like protein, PxSR (Claudianos *et al*., 2002). PxSR is composed of multiple domains such as the scavenger receptor cysteine rich (SRCR), pentraxin (PTX), polycystine-1, lipoxygenase, alpha toxin (LH2/PLAT) and Limulus clotting factor C, Coch-5b2 and Lgl1 (LCCL) domains (Claudianos *et al*., 2002). Gene-disruption studies showed that mutant *P. berghei* parasites developed oocyst numbers comparable to wild-type parasites, but sporozoites failed to develop within the oocysts of infected *An. stephensi*. Also the oocysts were much larger in size than the wild-type. It is possible that because PxSR encodes a complex multidomain protein that its disruption had multiple effects during oocyst development (Claudianos *et al*., 2002).

6. Sporozoite egress

Sporozoites exit the oocyst via small fenestrae (Sinden, 1974) and recent studies by Aly and Matuschewski (2005) suggest that this is an active process that is mediated by a papain-like cysteine protease referred to as egress cysteine protease 1 (*ECP1*). In those studies, *P. berghei* sporozoites with a disrupted *ECP1* gene were viable, but unable to exit the oocyst. Additionally, the mutant parasites continued to move in a circular pattern

within the oocyst for several days, suggesting that motility precedes oocyst rupture and that parasite locomotion might be a prerequisite for sporozoite egress. In that same study, when the mutant oocyst sporozoites were mechanically liberated and injected into the mammalian host, they failed to produce infections, suggesting a further role for *ECP1* beyond sporozoite egress (Aly and Matuschewski, 2005). It is still unclear as to the mechanisms by which *ECP1* assists with sporozoite egress.

7. Sporozoite motility

When sporozoites are released from the oocyst, they make their way through the mosquito hemolymph to the salivary glands where they can be transmitted during the next blood meal. Like other apicomplexans, the sporozoites do not have cilia or flagella for locomotion, but instead move by gliding in a circular pattern followed by flexing, turning, and twisting (Hakansson *et al*., 1999) (Figure 1.4). These movements are driven by an actin-myosin motor (Kappe *et al*., 2004). The motor consists of a myosin tail interacting protein (MTIP) that is anchored to the IMC via its interaction with the myosin-A (MyoA) docking protein (MADP) (Herm-Gotz *et al*., 2002; Bergman *et al*., 2003). MTIP binds to the tail domain of MyoA which interacts with F-actin (Figure 1.4). Proteins from the thrombospondin-related adhesive protein (TRAP)/sporozoite surface protein 2 (SSP2) are linked to F-actin via aldolase, a glycolytic enzyme that localizes to the cytoplasm and surface of micronemes in resting parasites (Robson *et al*., 1988; Rogers *et al*., 1992; Buscaglia *et al*., 2003). When parasites are activated, the micronemes, which contain TRAP/SSP2, fuse with the sporozoite membrane at its apical end giving aldolase access to the cortical space where the motor is engaged. Aldolase is then translocated posteriorly along with TRAP and the molecular motor pulls

TRAP/SSP2 backward, resulting in a forward movement of the parasite (Figure 1.4). As the sporozoite glides, large amounts of CS are released at the posterior end of the parasite (Vanderberg, 1974; Cochrane *et al*., 1976; Stewart *et al*., 1986). It still remains to be determined as to whether CS plays a role in sporozoite gliding (Kappe *et al*., 2004).

Sultan *et al*. (1997) showed that *P. berghei* TRAP-KO sporozoites had impaired gliding motility and failed to invade *An. stephensi* salivary glands, suggesting a role for TRAP in sporozoite binding and traversal of the mosquito salivary glands. Also, mutant salivary gland sporozoites were 10,000 times less infectious than wild-type parasites for mammalian liver cells, possibly caused by the sporozoites inability to bind to receptors on the liver cells due to impaired gliding motility (Sultan *et al*., 1997).

According to Hillyer *et al.* (2007), *P. berghei* oocyst sporozoites migrate with the flow of the hemolymph to all parts of the mosquito body. The sporozoites can be found not only in the salivary glands, but also in the legs, wings, heart, cuticle, antennae, halteres and ovaries. In this study, naïve *Aedes aegypti* were injected with 10,000 purified *P. gallinaceum* oocyst sporozoites, body tissues were collected, and RNA was isolated and used for RT-PCR analysis. They found that after 8 hours 19% of the sporozoites released into the hemolymph actually entered the salivary glands, 39% were still in the hemocoel, and the remaining 42% were degraded by the mosquito immune system. The number of sporozoites in the salivary glands did not increase after 8 hours and by day 7 there were very few (~1%) sporozoites detected in the hemocoel. These studies suggested that sporozoites have a narrow window in which to invade the salivary glands before the host response (*i.e*., immune effector molecules, pathogen recognition molecules, and haemocytes) occurs against sporozoites released from the oocyst.

8. Salivary gland invasion

Studies by Rosenberg (1985) suggested that sporozoite recognition and invasion of the salivary glands is receptor-ligand mediated and species specific. For example, most primate malarias can complete their life-cycle in *An. freeborni* with the exception of the Asian monkey parasite *P. knowlesi*. To understand this phenomena, Rosenberg (1985) transplanted naïve salivary glands from *An. freeborni* into the abdomen of *An. dirus* that were heavily infected with mature *P. knowlesi* oocysts and vice versa. Interestingly, sporozoites from *P. knowlesi* oocysts that developed in *An. freeborni* were unable to invade the native glands, but they were able to invade the *An. dirus* transplanted salivary glands. However, oocyst sporozoites that developed in *An. dirus* successfully invaded the native glands, but did not invade the transplanted *An. freeborni* salivary glands. Thus, in both cases, *P. knowlesi* sporozoites failed to invade *An. freeborni* salivary glands. In a related study, *P. knowlesi* oocyst sporozoites isolated from infected *An. freeborni* were able to establish an infection in monkeys (Rosenberg, 1985). Combined, these studies suggested that sporozoites use different antigens for identifying and invading the salivary glands and liver.

Using the *P. gallinaceum-Ae. aegypti* model system, Pimenta *et al.* (1994) showed that there are several steps that take place during sporozoite invasion of salivary glands. Initially, the sporozoite 1) adheres to and breaches the basal lamina, 2) penetrates the basal plasma membrane of the salivary gland cells (*i.e.*, acinar cells), 3) traverses the apical plasma membrane and enters the secretory cavity, 4) arranges into bundles, and 5) enters the secretory duct and can be transmitted to the vertebrate host at the next blood meal (Figure 1.5). Several parasite-derived molecules such as the circumsporozoite

protein, the merozoite associated erythrocyte binding protein (MAEBL), and PfCCp1 (Sidjanski *et al*., 1997; Kariu *et al*., 2002; Pradel *et al*., 2004) and host salivary gland molecules (Brennan *et al*., 2000; Korochkina *et al*., 2006) have been shown to play a role in sporozoite invasion of host tissues.

8.1 The circumsporozoite (CS) protein

In addition to sporozoite development, the CS protein also plays a role in host tissue invasion. Sidjanski *et al.* (1997) showed that the CS protein in *P. yoelii* has binding sites on the *An. stephensi* salivary glands, suggesting that CS plays a role in host tissue invasion. Monoclonal antibodies directed against CS from *P. berghei* and *P. gallinaceum* have been shown to block sporozoite invasion of *An. stephensi* and *Ae. aegypti* salivary glands respectively as well as vertebrate host tissue (Yoshida *et al*., 1981; Warburg *et al*., 1992; Ramirez *et al*., 1995). A more recent study by Coppi *et al.* (2005) showed that cleavage of the *P. berghei*, *P. falciparum*, and *P. yoelii* CS protein by a cysteine protease of parasite origin is required for sporozoite invasion of hepatocytes *in vitro* for all three species and for establishing an infection in mice *in vivo* for the rodent malarias.

8.2 MAEBL and PfCCp1

Other less characterized molecules such as MAEBL and PfCCp1 also have been shown to be involved in sporozoite binding and invasion of the salivary glands. Kariu *et al.* (2002) showed that *P. berghei* sporozoites with a disrupted MAEBL gene were unable to bind to the mosquito salivary glands and as a result invasion was hindered. Another molecule that has been implicated in sporozoite invasion of salivary glands is the PfCCp1, 2, and 3 multidomain adhesion proteins (Pradel *et al*., 2004). Members of this

family contain a Limulus coagulation factor C (LCCL) domain and putative polysaccharide-binding domains (Pradel *et al*., 2004). *P. falciparum* sporozoites with a disrupted PfCCp2 or PfCCp3 gene formed oocysts comparable in number to wild-type; however, the sporozoites were unable to invade the salivary glands possibly due to a disruption of interactions with salivary gland glycoproteins (Pradel *et al*., 2004).

8.3 Salivary gland proteins

Barreau *et al*. (1995) showed that anti-salivary gland antibodies and lectins can block *P. gallinaceum* sporozoite invasion of *Ae. aegypti* salivary glands. A subsequent study by Barreau *et al.* (1999) used monoclonal antibodies to identify surface molecules on regions of *Ae. aegypti* salivary glands that are preferential sites for invasion by sporozoites. Brennan *et al.* (2000) showed that antibodies directed against a 29 kDa protein and a 100 kDa protein from *An. gambiae* could block *P. yoelii* sporozoite invasion of the mosquito salivary glands by ~75%. Studies by Korochkina *et al.* (2006) showed that aaSGS1, a female *Ae. aegypti* specific salivary gland protein, binds to the mosquito salivary glands and blocks *P. gallinaceum* sporozoite invasion by ~65%. All of these studies suggest that the sporozoite must interact with receptors on the salivary glands in order to invade and that the mosquito salivary glands could be potential targets for blocking the transmission of malaria by the mosquito.

9. Sporozoite transmission to the vertebrate host

The female mosquito takes a blood meal either by feeding from a large blood vessel or small pools of blood generated from damaged capillaries (Figure 1.6) (Frevert *et al*., 2004). During the blood meal, 15 to 200 sporozoites are deposited into the skin of the host (Vanderberg, 1977; Ponnudurai *et al*., 1991; Kappe *et al*., 2003) and then

migrate to a blood vessel, circulate to the liver, cross the sinusoidal cellular layer, and enter the hepatocytes (Mota and Rodriguez, 2004). Studies by Sidjanski and Vanderberg (1997) suggested that *P. yoelii* sporozoites remain in the dermal tissue of BALB/C mice for 10 minutes before making their way through the blood vessels to the liver. However, more recent studies by Amino *et al.* (2006) suggest that 50% of *P. berghei* sporozoites actually remain in the dermis of mice for up to seven hours after injection. The other sporozoites either enter the blood vessels (~35%) or the lymphatic vessels (~15%). The most surprising result from this study was that instead of being destroyed by dendritic leucocytes in the lymph node, some sporozoites partially differentiate into exoerythrocytic stages. The fate of these parasites still remains to be determined.

Sporozoites that enter the bloodstream travel to the liver sinusoids through the portal venule or hepatic arteriole and glide along the endothelia (Pradel *et al*., 2002). Studies suggest that the *P. berghei* sporozoite surface molecules, CSP and TRAP, recognize proteoglycans on the surface of mouse Kupffer cells that allow the sporozoite to safely invade and traverse the cells in a vacuole without being phagocytosed (Meis *et al*., 1985; Pradel *et al*., 2002). The sporozoite destroys hepatocyte membranes as it continuously traverses through them in search of a hepatocyte to develop in (Mota *et al*., 2001). Within the hepatocyte, the sporozoite lies close to the nucleus and begins to go through a series of nuclear divisions after which time merozoites form that are released into the bloodstream to commence the erythrocytic cycle.

Bhanot *et al.* (2005) found that a phospholipase may play a role in sporozoite invasion of hepatocytes. Ninety percent of *P. berghei* sporozoites containing a disrupted phospholipase gene were unable to invade hepatocytes of young Sprague-Dawley (SD)

rats. Other less characterized sporozoite molecules thought to be involved in sporozoite invasion of the liver are the *P. falciparum* sporozoite antigen Pfs16, sporozoite and liver stage antigen (SALSA), sporozoite threonine-and asparagine-rich protein (STARP), and sporozoite micronemal protein essential for cell transversal (SPECT), (Moelans *et al*., 1991; Fidock *et al*., 1994; Bottius *et al*., 1996; Matuschewski *et al*., 2002; Ishino *et al.*, 2004).

10. Vaccines

To date there are still very few *Plasmodium* molecules that are considered to be possible vaccine candidates. This is partially due to the size of the *Plasmodium* genome (≥ 5200 genes) and the large percentage of proteins with unknown functions (~60%) which makes the identification of potential candidates difficult (Gardner *et al*., 2002; Aguiar *et al*., 2004; Duffy *et al*., 2005; Girard *et al*., 2007). For this reason, efforts have been focused on three well characterized antigens, the CSP, merozoite surface protein (MSP), and apical membrane antigen-1 (AMA-1) (Reed *et al*., 2006). Current efforts are focused on pre-erythrocytic stage, blood stage, and transmission blocking vaccines (Girard *et al*., 2007). Additionally, there has been a renewed interest in using intact sporozoites as vaccines (Kanoi and Ewang, 2007).

10.1 Pre-erythrocytic stage vaccines

These vaccines target the sporozoites in an effort to prevent their invasion of liver hepatocytes and their subsequent development of liver stage parasites. Currently, the CSP, Liver stage antigen-1 and 3 (LSA-1 and LSA-3), and TRAP/SSP2 are the main focus for this group of vaccines. These vaccines are prepared in several ways, and include recombinant fusion protein vaccines, DNA vaccines, and live attenuated

recombinant vaccines. The ultimate goal is to provide either sterilizing humoral immunity or induce a cell-mediated humoral response that will inhibit sporozoite maturation inside the liver cells (Doolan and Hoffman, 2000; Walther, 2006).

10.1.1 CSP and other vaccines

The Walter Reed Army Institute (WRAIR) in collaboration with Glaxo Smith Kline (GSK) developed RTS, S, a vaccine that comprises the C-terminus (amino acids 207-295) of the CSP from *P. falciparum* fused to the hepatitis B surface antigen (Stoute *et al*., 1997; Kester *et al*., 2001). This molecule is expressed in *Saccharomyces cerevisiae* as a virus-like particle (VLPs) and in a Phase I trial it prevented further infection from *P. falciparum* in 85% of the experimental population (six out of seven individuals) (Stoute *et al*., 1998). Other clinical trials have demonstrated up to 70% protective efficacy against infection for the first two months with a rapid decline thereafter (Stoute *et al*., 1998). A more recent Phase I/IIb trial, conducted in Mozambique by the PATH Malaria Vaccine Initiative, showed that the RTS,S/AS02D vaccine is safe and reduced new infections in infants by 65% (Macete *et al*., 2007). Other molecules such as the LSA-1 and LSA-3 are currently in preclinical development (WHO, 2006).

10.1.2 DNA vaccines and live attenuated recombinant vaccines

This group of vaccines consists of plasmid DNA vaccines and live recombinant vaccines that use attenuated modified vaccinia Ankara (MVA) strain virus, adenovirus, Sindbis virus, fowlpoxvirus (FPV), yellow fever virus or a cold-adapted attenuated influenza virus as a vector for the CSP (Girard *et al*., 2007). Studies testing the plasmid DNA vaccines and MVA-based vaccines with the CSP did not show evidence of

protection against sporozoite challenge in humans (Wang *et al*., 1998; Le *et al*., 2000; Parker *et al*., 2001; Wang *et al*., 2001; Moore and Hill, 2004; Dunachie *et al*., 2006).

10.2 Asexual blood stage vaccines

Asexual blood stage vaccines target the erythrocytic stage parasites. Efforts to find an asexual blood stage vaccine have focused on MSP-1, 2, and 3, AMA-1, the glutamate-rich protein (GLURP), EBA-175, region II of the duffy binding protein, PfEMP1, and RAP-2 (Girard *et al*., 2007; portfolio of candidate malaria vaccines currently in development: http://www.who.int/vaccine_research/documents/malaria_table.pdf).

Several combinations have been tested in clinical trials in humans and have been shown to be safe and immunogenic (Okech *et al*., 2004; Pichyangkul *et al*., 2004; Saul *et al*., 2005; Hermsen *et al*., 2007; Stoute *et al*., 2007). A Phase I/IIb trial, with the combination MSP-1, MSP-2 and ring-stage infected-erythrocyte surface antigen (RESA) vaccine, showed that 5-9 year old children in Papua New Guinea had a reduction in parsite density by 62% after vaccination (Genton *et al*., 2007).

10.3 Transmission blocking vaccines

Transmission blocking vaccines target the sexual stages in an effort to prevent transmission of the malaria parasite by the mosquito; however, they are not intended to confer protection in the vaccinated individual. The main candidate antigens are Pfs25, Pfs28, and their homologs in *P. vivax* (Hisaeda *et al*., 2000; Arevalo *et al*., 2005; Arakawa *et al*., 2005) and currently the Malaria Vaccines Section at the National Institutes of Health (NIH) is developing these vaccines as yeast-secreted proteins (Girard *et al*., 2007). The gametocyte surface antigens Pfs48/45 and Pfs230 also are being

investigated as transmission blocking vaccines and are currently in vaccine trials (Bustamante *et al*., 2000; Milek *et al*., 2000; Gozar *et al*., 2001; Malkin *et al*., 2005 Collins *et al*., 2006). It is also possible that mosquito antigens may one day be vaccine candidates (Lal *et al*., 1994).

10.4 Sporozoites as vaccines

Several years ago, irradiated sporozoites were tested for their ability to confer protection against malaria. Studies by Nussenzweig *et al* (1967) found that irradiated *P. berghei* sporozoites provided protection against malaria infection in rodents. Since then, several studies have shown that irradiated *P. falciparum* and *P. vivax* sporozoites can confer protection in humans (Clyde *et al*., 1973a, 1973b, 1975, 1990; Rieckman *et al*., 1974, 1979, 1990; McCarthy and Clyde, 1977). However, advances in recombinant DNA technology shifted the focus to developing a protective recombinant subunit vaccine (Kanoi and Ewang, 2007). To date, there is still no viable recombinant vaccine against malaria, so there has been a renewed interest in using whole attenuated sporozoites as a vaccine. Radiation attenuated sporozoites (*RAS*) and genetically attenuated sporozoites (*GAS*) are currently being investigated as possible vaccine candidates (Kanoi and Ewang, 2007).

10.4.1 Radiation-attenuated sporozoites (*RAS*)

Irradiated sporozoites are generated by subjecting a *Plasmodium*-infected mosquito to radiation. The sporozoites then are injected into the vertebrate host via the bite of irradiated mosquitoes and they migrate to the liver in the same fashion as their non-irradiated counterparts. Once in the liver cells they can remain there for weeks, but are unable to undergo nuclear division (Hoffman *et al*., 2002). This series of events

triggers a cytotoxic CD8 T-cell response against the infected hepatocytes and an antibody response against the sporozoite surface proteins that is protective against future sporozoite challenge (Doolan *et al*., 2006; Hafalla *et al*., 2006).

Hoffman *et al.* (2002) found that *P. falciparum* RAS sporozoites protected human volunteers from developing a malaria infection but the protection was only for one year. The short-lived protection poses a huge obstacle because the production of sporozoites requires the use of a large amount of resources and the cost would not be practical for poor families in developing countries (Kanoi and Egwang, 2007). Another concern is that the sporozoites will be co-administered with unknown pathogens from the mosquito which could be life threatening to already immuno-compromised individuals, such as people with HIV (Hoffman *et al*., 2002). As a result of these concerns, recently the Sanaria vaccine company led by Dr. Stephen Hoffman, with funding by the Bill and Melinda Gates Foundation, is attempting to develop an irradiated sporozoite vaccine that includes isolating sporozoites from mosquito salivary glands (Coghlan, 2006). Trials for this irradiated sporozoite vaccine are due to begin in 2008 (Coghlan, 2006).

10.4.2 Genetically attenuated sporozoites (*GAS*)

GAS sporozoites also have an arrested development in the liver, but the lack of nuclear division is due to the deletion of a target gene that is essential for liver stage parasite development (Kanoi and Egwang, 2007). In a study by Mueller *et al.* (2005b), *P. berghei* sporozoites with a disrupted *UIS-3* (upregulated in infective sporozoites gene 3) gene, which is essential for early liver stage development, were unable to develop into liver stage schizonts. These mutant parasites were found to confer protection against malaria infection in Sprague-Dawley rats. In another study, *UIS-4* disrupted sporozoites

protected mice from subsequent infection with wild-type sporozoites (Mueller *et al*., 2005a). More recently, Tarun *et al.* (2007) demonstrated that GAS vaccines have different potencies. In those studies, BALB/c mice were immunized with 10,000 *P. yoelii Pyuis4*(-) or *Pyuis3*(-) sporozoites and then challenged with 10,000 wild-type sporozoites. Only one dose of *Pyuis4*(-) sporozoites was required for complete protection for 60 and 180 days, while at least 2 immunizations of *Pyuis3*(-) sporozoites were necessary for complete protection for 180 days. Jobe *et al.* (2007) showed that double knockout *Pbuis3*(-)/*Pbuis4*(-) parasites conferred complete protection in C57BL/6 mice for six months after the last immununizing boost.

Van Dijk *et al.* (2005) and Douradinha *et al.* (2007) found that sporozoites deficient in P36p, a *Plasmodium* surface protein, provide long-lasting protection against *P. berghei* infection in rodents because the mutant parasites fail to prevent host cell apoptosis. As a result, the apoptosis triggers the host immune response and leads to parasite clearance. Currently, *P. falciparum* GAS sporozoites have not been generated, but the future looks promising for this new technology (Kanoi and Egwang, 2007).

11. Dissertation research

There is still a great deal to be learned about the *Plasmodium* sporozoite and because this stage exhibits dual infectivity for mosquito salivary glands and vertebrate tissue, it is an excellent target for intervention efforts. Therefore, the goal of this dissertation research was to obtain more information about sporozoite biology and host-parasite interactions through the characterization of the sporozoite and erythrocytic stages (SES) protein.

The avian malaria parasite, *P. gallinaceum,* and the mosquito *Ae. aegypti* were used to conduct these studies. Unlike its human counterparts, in the chicken vertebrate host, *P. gallinaceum* exoerythrocytic schizogony takes place in the macrophages instead of the liver with the parasite eventually invading all tissues including the brain (Garnham, 1966). The *P. gallinaceum* system was used for several reasons including: 1) *P. gallinaceum* is phylogenetically closely related to *P. falciparum*, the most pathogenic form of human malaria (Waters *et al*., 1991; McCutchan *et al*., 1996), 2) molecules found to be secreted and/or expressed on the sporozoite surface and play a role in host tissue invasion in *P. gallinaceum* likely have a homolog with a similar function in *P. falciparum*, and 3) this system is tractable and molecules can be studied easily in both the vertebrate host and the vector.

The PgSES protein was discovered after screening a *P. gallinaceum* sporozoite cDNA library (see Chapter 2 for more details). It is expressed during the erythrocytic stages and on the surface of the salivary gland sporozoite (LaCrue *et al*., 2006). Recent antibody blocking studies suggest that it may play a role in sporozoite invasion of mosquito salivary glands (Chapter 3).

Studies by Pandey *et al.* (2006) investigating the role of the SES protein in the *P. falciparum* erythrocytic stages showed that the PfSES protein, referred to as falstatin in those studies, is a cysteine protease inhibitor which has a punctate staining pattern similar to PgSES in the erythrocytic stages. These same studies suggest that falstatin may be involved in merozoite invasion of erythrocytes (Pandey *et al*., 2006). The role of falstatin/SES during the mosquito stages remains to be determined and is, therefore, the focus of this Ph.D. dissertation project.

There is still a significant amount of information that is unknown about the function of *SES* and cysteine protease inhibitors in the malaria parasite, especially the mosquito stage parasites. Therefore, this project focused on the characterization of the SES protein during the invasive sporozoite stage, with additional information provided on the pre-sporozoite and erythrocytic stages. Ultimately, this research should provide a better understanding about the role of the SES protein in sporozoite development and host tissue infectivity.

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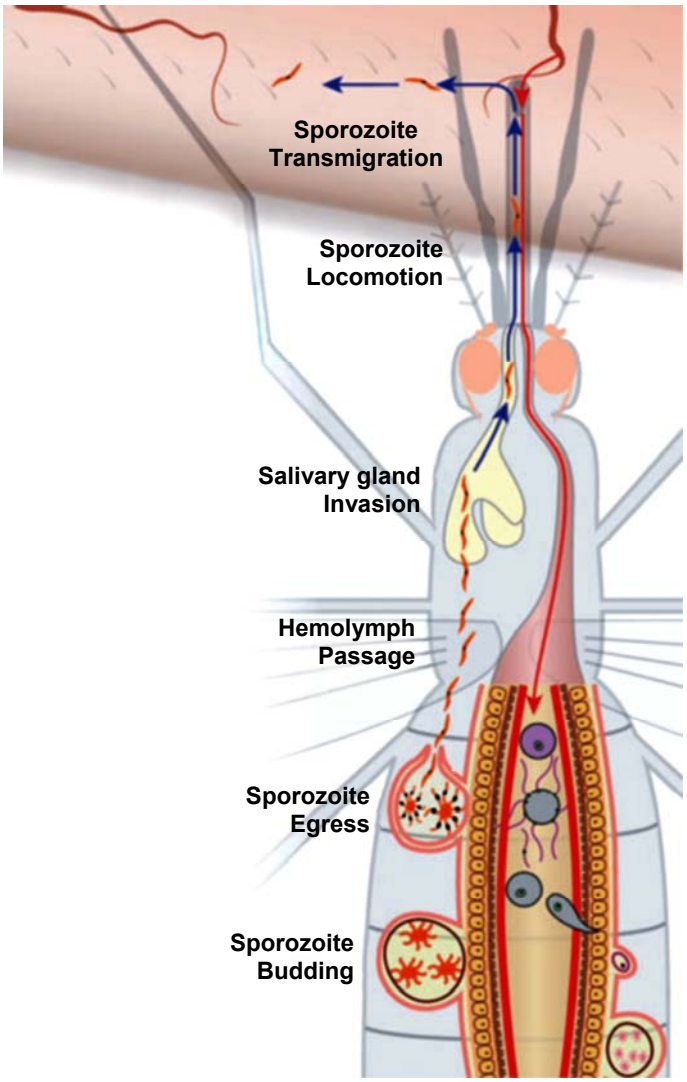

Figure 1.1 Development of *Plasmodium* within the mosquito. The female mosquito takes a blood meal and ingests infective gametocytes. Fertilization occurs in the midgut and the result is a diploid zygote that elongates into a motile ookinete. The ookinete migrates through the basal lamina of the mosquito midgut and develops into an oocyst. Within the oocyst, sporozoite buds form and elongate into mature sporozoites that are eventually released and make their way through the hemolymph to the salivary glands. There the sporozoite can be transmitted to the vertebrate host during the next blood meal. This figure was adapted from Matuschewski (2006) with permission from the publisher.

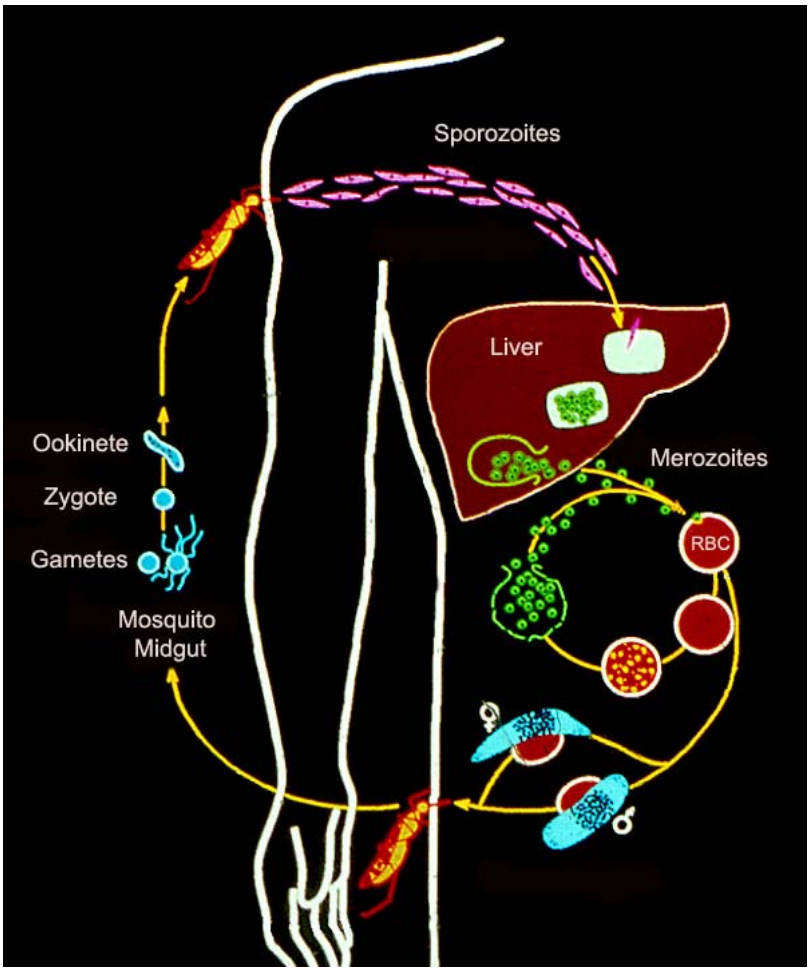

Figure 1.2. Life-cycle of the human malaria parasite. When the female mosquito takes a blood meal, she injects sporozoites into the bloodstream of the vertebrate host. The sporozoites invade the liver and develop into schizonts containing merozoites that are released and invade red blood cells. Within the red blood cells, more merozoites develop that when released infect more RBCs and either continue their asexual development or develop into male and female gametocytes that are infective to the mosquito.

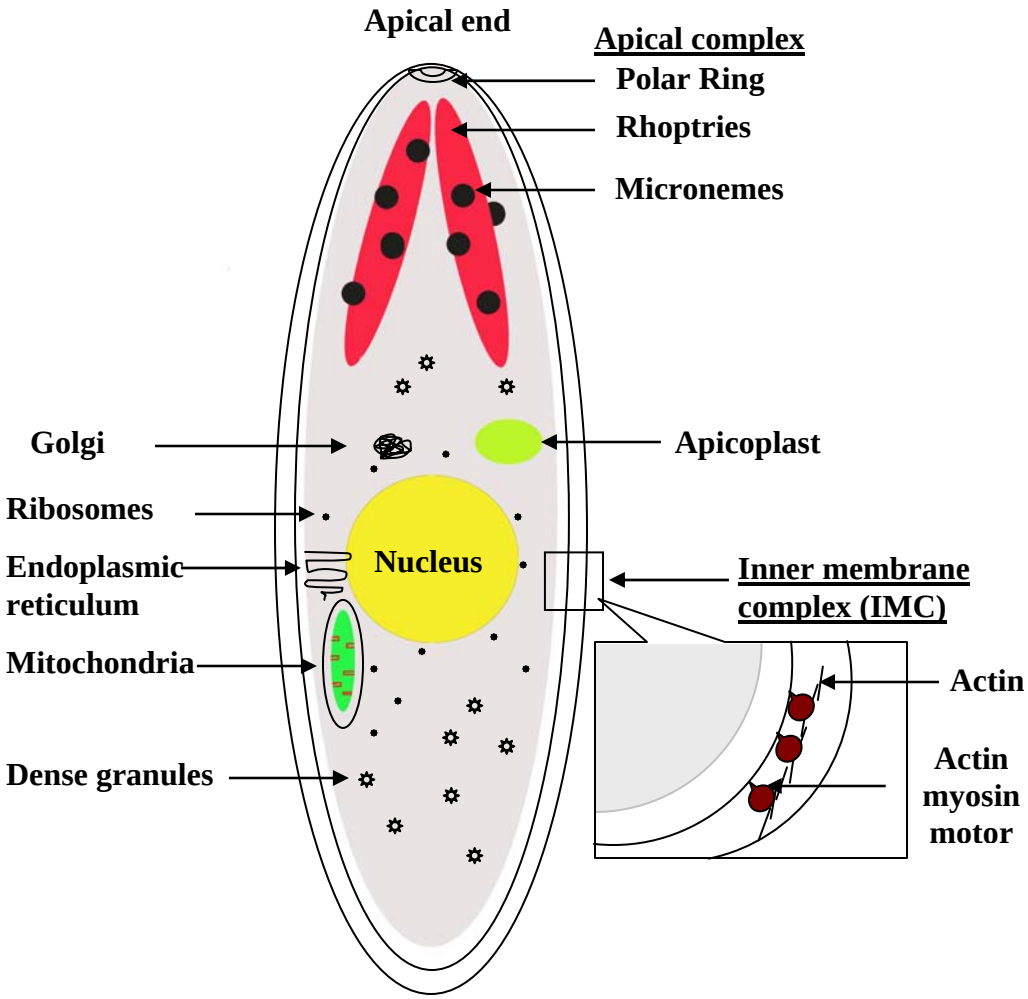

Figure 1.3. Anatomy of the *Plasmodium* sporozoite. The *Plasmodium* sporozoite is an apicomplexan parasite that contains an apical complex consisting of a polar ring, rhoptries, and micronemes. The sporozoite also contains an apicoplast that is derived from the chloroplast of plants, dense granules, a nucleus, golgi apparatus, ribosomes, endoplasmic reticulum, and mitochondria. The inner membrane complex (IMC) consists of three membranes. Sporozoite locomotion is mediated by the actin-myosin motor located between the two outermost membranes of the IMC. This figure was created by Alexis N. LaCrue and based upon figures from Kappe *et al*. (2004) and Frevert (2004).

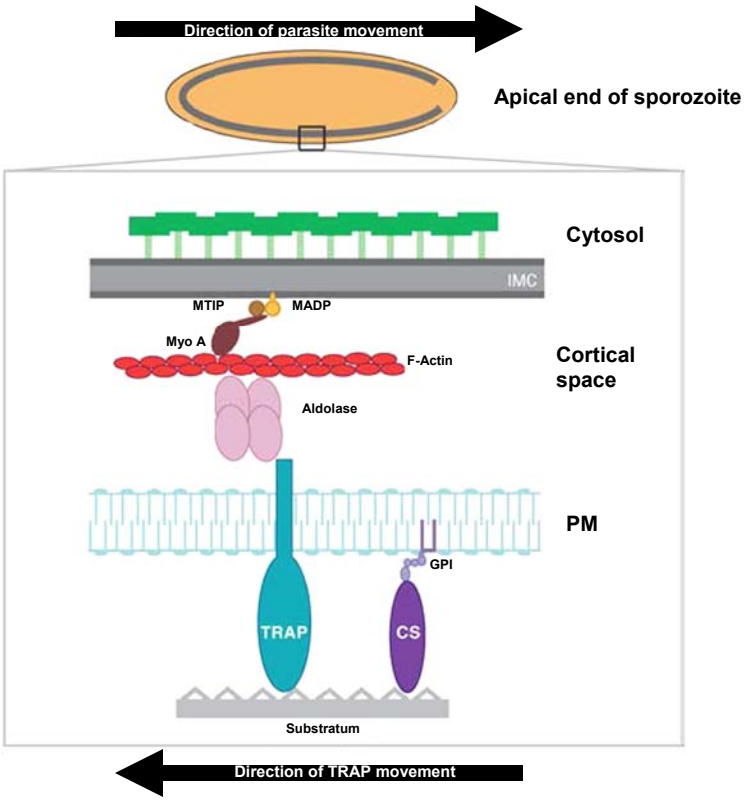

Figure 1.4. Model for sporozoite motility. The thrombospondin-related adhesive protein (TRAP) family of adhesins provide the link between the extra-cellular surface and the actin-myosin motor of the parasite. The myosin tail interacting protein (MTIP) is anchored to the outer membrane of the IMC via an interaction with the myosin-A (MyoA) docking protein (MADP). MTIP binds to MyoA that then binds to actin filaments (F-actin). The actin filaments are connected to the cytoplasmic domain of the TRAP via aldolase. Movement of myosin along actin filaments results in a forward motion of the parasite across the substrate or cell surface. The glycophosphatidylinositol (GPI) anchored circumsporozoite (CS) protein is released as the sporozoite glides. PM= Plasma membrane. This figure was adapted from Kappe *et al*. (2004) with permission from the publisher.

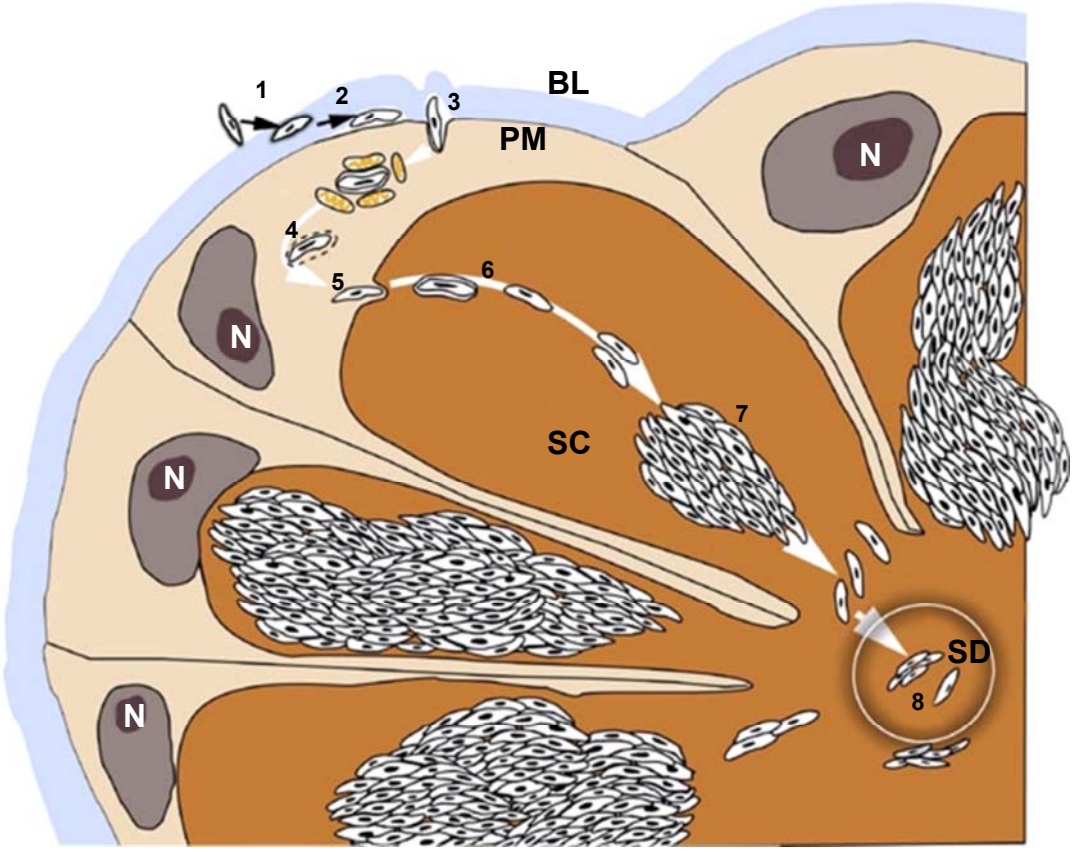

Figure 1.5. Sporozoite migration through the mosquito salivary glands. A model for *P. gallinaceum* sporozoite invasion of *Ae. aegypti* salivary glands based on ultrastructural studies by Pimenta *et al* (1994) is depicted. The eight steps for invasion are as follows: The sporozoite 1) attaches to the salivary gland basal lamina (BL), 2) migrates to the space between the basal lamina and basal plasma membrane (PM), loses its cell coat, 3) then pentrates the basal plasma membrane, 4) becomes surrounded by mitochondria, 5) enters the secretory cavity (SC), 6) becomes surrounded by a vacuole, 7) arranges into bundles, and 8) enters the secretory duct (SD). N= nucleus. Figure adapted from Sherman, 1998.

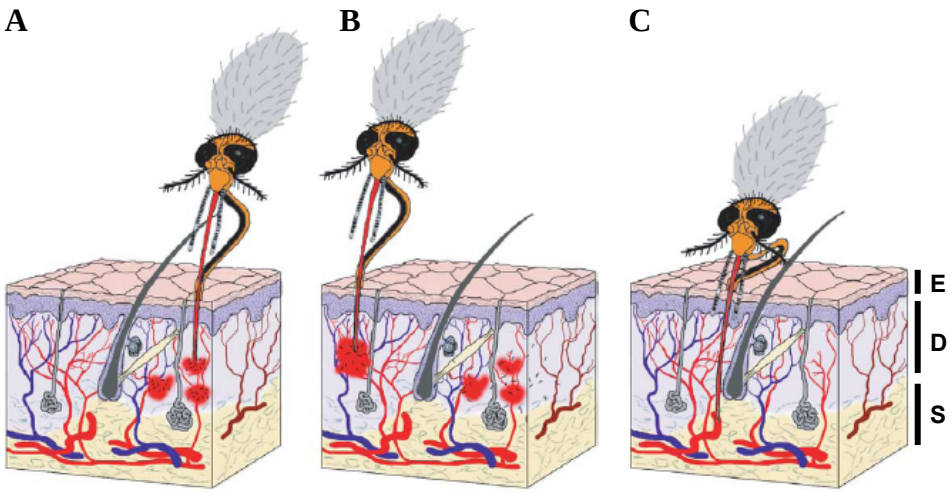

Figure 1.6. Mosquito probing behavior. A) The mosquito usually feeds from pools of blood generated by damaged capillaries. B) If the probing is unsuccessful, the mosquito will retract her proboscis and find a new site. C) The mosquito takes a blood meal by probing deeper into the subcutaneous layer where there are larger blood vessels. As the mosquito feeds, she releases saliva containing sporozoites that migrate through the circulatory system and make their way to the liver to establish an infection. Epidermis (E), dermis (D), and subcutaneous tissue (S). This figure was reproduced from Frevert, 2004 with permission from the publisher.

CHAPTER 2. A UBIQUITOUS *PLASMODIUM* PROTEIN DISPLAYS A UNIQUE SURFACE LABELING PATTERN IN SPOROZOITES

ABSTRACT

The *Plasmodium* sporozoite is infective for mosquito salivary glands and vertebrate host tissues. Although it is a key developmental stage of the malaria parasite, relatively few sporozoite surface or secreted proteins have been identified and characterized. Herein, we describe the molecular and cellular characterization of a novel surface molecule that is preferentially-expressed in salivary gland sporozoites as compared to oocyst and hemolymph sporozoites. This molecule, designated the sporozoite and erythrocytic stages (SES) protein (formerly known as Pg4), exhibits a spiral surface labeling pattern that spans over a known sporozoite surface antigen, the circumsporozoite protein, with only minor co-localization. SES consists of 551 amino acids encoding a putative 63.2 kDa protein that has been shown to be expressed not only on particular sporozoite stages, but also during the asexual and gametocyte stages. This novel protein also has three domains of unknown function that are conserved in at least eight *Plasmodium* spp. that represent human, avian, non-human primate, and rodent malarias.

Key words: Malaria; Sporozoite; Preferential expression; Surface protein

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This chapter was published in *Molecular and Biochemical Parasitology*. The full citation is as follows: LaCrue AN, Sivaguru M, Walter MF, Fidock DA, James AA, Beerntsen BT. A ubiquitous *Plasmodium* protein displays a unique surface labeling pattern in sporozoites. *Mol Biochem Parasitol.* 2006 Aug;148(2):199-209.

1. Introduction

Malaria, which is exhibiting a resurgence, is a devastating disease caused by *Plasmodium* spp. that results in significant morbidity and mortality for millions of people throughout the world [1]. During the life cycle of the malaria parasite, the sporozoite is a key developmental stage that is infective for both the mosquito salivary glands and vertebrate host tissues. Although host tissue invasion is likely mediated by molecules expressed on the surface or secreted, there are few well-characterized sporozoite molecules of this type. The circumsporozoite (CS) protein and thrombospondin-related adhesive protein (TRAP)/sporozoite surface protein (SSP) are surface molecules that have been well-characterized and shown to play vital roles in sporozoite biology [2–4]. The CS protein has been demonstrated to not only be critical for sporozoite budding during oocyst differentiation, but it also has been implicated in the invasion of mosquito salivary glands and vertebrate liver tissue [5–8]. Similarly, mutant sporozoites lacking the TRAP/SSP gene product have shown impaired gliding motility and poorly invaded host tissue [9]. In addition, MAEBL, an erythrocyte binding protein that originally was shown to be involved in merozoite invasion of erythrocytes, has recently been demonstrated to be abundantly expressed in the sporozoite stages and implicated in the invasion of mosquito salivary glands as well as vertebrate host tissue [10–13]. Other sporozoite surface molecules, such as the *Plasmodium falciparum* sporozoite antigen Pfs16 [14], sporozoite and liver stage antigen (SALSA) [15], and the sporozoite threonine-and asparagine-rich protein (STARP) [16] also have been identified and characterized to varying degrees.

The MB2 protein is a *P. falciparum* sporozoite surface molecule that was isolated from a sporozoite expression library following screening with immune serum from a protected human volunteer [17]. The PfMB2 protein is differentially-expressed with a distribution on the surface of the sporozoite, in the cytoplasm and parasitophorous vacuole (PV) of the liver stages, and in the cytoplasm, nucleus, and PV of the asexual blood stages. On the sporozoites, the *PfMB2* gene encodes a 120 kDa putative surface antigen with a transmembrane domain at the amino terminus and six copies of a nine-amino acid tandem repeat at the carboxyl terminus [17]. Although other *Plasmodium spp.* homologs recently have been identified [18], initial BLAST analyses of *PfMB2* indicated a significant lack of homology with identified genes in the databases. Therefore, efforts were made to isolate its *Plasmodium gallinaceum* homolog and test its ability for mediating invasion of mosquito or vertebrate tissues in the accessible *P. gallinaceum*–chicken–*Aedes aegypti* model system. During this screening, another novel sporozoite gene, designated SES for sporozoite and erythrocytic stages gene, was identified and it was determined to have no sequence identity with the *MB2* gene [17,18].

Herein, we describe the molecular and cellular characterization of *PgSES* that has protein homologs, containing three conserved regions of identity/similarity with unknown function, in at least eight *Plasmodium* species including the human species, *P. falciparum* and *P. vivax*. The PgSES protein is expressed in the erythrocytic stages and is preferentially-expressed on the surface of salivary gland sporozoites as compared with other sporozoite populations. A particularly interesting feature of this protein is that in the salivary gland sporozoites it exhibits a unique surface labeling pattern that spirals around the sporozoite.

2. Materials and methods

2.1 Mosquito maintenance and the parasite transmission cycle

Aedes aegypti (red strain) mosquitoes used for the transmission cycle were 3–10 days old. The mosquitoes were maintained as previously described [19]. *Plasmodium gallinaceum* strain 8A was maintained in White Leghorn chickens by exposure to *P. gallinaceum* sporozoite-infected mosquitoes [20]. All animal care and the experiments described herein were approved by and performed in accordance with guidelines of the National Institutes of Health and the University of Missouri-Columbia Animal Care and Use Committee.

2.2 Sporozoite cDNA library construction

Salivary gland sporozoites (25 million) were isolated from dissected mosquito salivary glands (700 pairs) using gentle homogenization with a pipette tip. Following total RNA extraction and subsequent Poly A^+ RNA (134 ng) isolation, cDNA was made and directionally cloned into the Lambda Zap Express phagemid vector and packaged following the supplier's protocol (Stratagene) [21].

2.3 Isolation of the novel sporozoite molecule PgSES

The sporozoite cDNA library was used to produce plaque filters that were screened following a described protocol [22]. The *PfMB2* cDNA probe (25 ng) was radio-labeled with ^{32}P-dATP using the Megaprime DNA labeling kit (Amersham Biosciences) and hybridized in aqueous conditions to the filters overnight at 42°C [23]. Following hybridization, the filters were washed using low stringency conditions at 42°C and exposed to X-ray film as previously described [23]. Those plaques that hybridized with the radio-labeled probe were isolated, phagemid DNA obtained, and the DNA

sequence determined [22]. A 1528 bp cDNA clone, designated *PgSES* (formerly known as Pg4), was isolated as a result of this heterologous screening. It contained a 5'-untranslated region and encoded an open reading frame (ORF) (1468 bp) that included an initiating methionine but no stop codon. Sequence analysis indicated that this cDNA clone was novel and that it had no sequence identity with the *PfMB*2 probe, suggesting that it was likely isolated due to the non-stringent hybridization conditions employed during the screening protocol. This partial *PgSE*S cDNA then was radio-labeled and used to isolate a full-length cDNA (1653 bp, GenBank accession number DQ068022) from the salivary gland sporozoite cDNA library using aqueous conditions and a hybridization temperature of 60°C [22].

2.4 DNA isolation and sequence determination of PgSES

DNA was isolated from overnight cultures using Wizard® mini-prep (Promega) and Qiagen midi-prep kits. Sequence of the isolated *PgSE*S cDNA clones was determined either by using commercially available ^{33}P sequencing kits (Amersham Biosciences) or by the University of Missouri sequencing facility. DNA and protein sequence alignments were done with Lasergene (DNASTAR Inc.) or Vector NTI (Explorer or Contig Express, Invitrogen). DNA and translated protein sequences were subjected to BLAST analysis [24] using the National Center for Biotechnology Information (NCBI) and PlasmoDB databases [25]. To find homologs to the PgSES protein, a PlasmoDB BLAST search was performed using the NCBI BLASTP program and "All *Plasmodium* ORFs" as the target database. The Apicomplexa database at NCBI also was searched. PSORT I, a protein localization program, was used to predict protein localization sites [26]. The ExPASy Proteomics tools also were used for additional protein analysis [27].

2.5 Sporozoite and sporozoite-infected tissue isolation

To assess *PgSES* transcript presence in pre-sporozoite and sporozoite stages, *P. gallinaceum*-infected *A. aegypti* abdomens and thoraces were collected on days 1–10 post-exposure (PE) and on days 12–14 PE. The infected tissues as well as control samples were isolated as previously described [19]. For immuno-localization and immuno-blot studies, *P. gallinaceum* mature oocyst sporozoites were isolated at 8–10 days PE, hemolymph sporozoites at 9–10 days PE, and salivary gland sporozoites at 12–14 days PE. The exact time for isolating sporozoites was determined by examining a sample of midguts, hemolymph, and salivary glands from the same infected cohort. Oocyst sporozoites and salivary gland sporozoites were isolated as previously described [19] and hemolymph sporozoites were obtained by perfusing the body cavity of infected mosquitoes with *Aedes* saline [28] and collecting the first drop of perfusate. Hemolymph sporozoites collected from a pool of mosquitoes (15–20) then were centrifuged at 10,000 × g for 10 min, the supernatant removed, and the sporozoite pellet washed twice with *Aedes* saline. Hemolymph sporozoites were collected from individual mosquitoes, via perfusion, directly onto a ringed slide and allowed to air dry prior to the immuno-fluorescence (IFA) experiments.

2.6 RNA isolation

Total RNA from mosquito tissues and blood samples were isolated using TRIzol reagent (Invitrogen) and TRI Reagent BD (Sigma) according to the manufacturer's instructions, respectively. To remove residual genomic DNA from all samples, the purified total RNA was treated with amplification grade DNase I using 1 U DNAase/µg RNA per the manufacturer's instructions (Promega).

2.7 Transcriptional expression analysis by reverse transcription PCR (RT-PCR)

To examine *PgSES* transcript presence in tissues infected with oocyst sporozoites and salivary gland sporozoites, RT-PCR was carried out using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) following a protocol previously described by LaCrue et al. [19]. PCR amplification was performed with the following gene-specific forward (F) and reverse (R) oligonucleotide primers: *PgSES* (F, 5-CTGGTTTATTGTCTTCTTTTCC-3 and R, 5-AGGTGGATTATTAGTTGATTATG-3). The primer sequences for the *P. gallinaceum* circumsporozoite gene (*CS*) [29], a control to show sporozoite presence, and *A. aegypti* muscle actin gene [30], a control to show RNA presence, were previously provided [19]. This experiment was performed in triplicate.

To assess *PgSES* transcript presence in pre-sporozoite stages through the sporozoite stages in a time-line experiment, total RNA (3 μg/sample) was used to make first-strand cDNA that then was used in an amplification reaction with the same gene-specific forward (F) and reverse (R) oligonucleotide primers as were used above for *PgSES* and the actin gene. Each reaction was prepared and performed as described previously [19]. This time-line experiment was done in duplicate.

To determine if *PgSES* transcripts were present in asexual blood stages and gametocytes, total RNA was isolated from *P. gallinaceum*-infected chicken blood (0.2 ml) on days 7–8 post-infection (PI) and from non-infected chicken blood (0.2 ml), and the RT-PCR reactions were performed as described previously [19]. The *PgSES* primers were the same as those used in the time-line RT-PCR. The primers for the *P. gallinaceum* asexually expressed small-subunit ribosomal RNA (*SSU rRNA*) [31], a control to show

the presence of RNA from erythrocytic stage parasites, and for the chicken carbonic anhydrase II (*CA II*) gene [32], a control to demonstrate the presence of RNA in all samples, were as previously described [19]. This experiment was performed in triplicate.

2.8 Recombinant protein expression and purification

In order to produce polyclonal antibodies for immunoblotting studies and localization assays, recombinant PgSES fusion protein (rPgSES) with GST and HIS tags was generated using pAKSS, a dual-affinity expression vector [33]. A fragment, representing amino acids 31–550, was selected and primers were used to amplify this region. The primers used were forward, 5'-GGATCCATGGGTTTATTAAATCAGTCCACCTGG-3' and reverse, 5'-CATCCCCGGGTCAGATACTATTAAATGAACAATCC-3' and the amplification, cloning and subsequent protein expression were as described previously [19].

Because large amounts of purified rPgSES were difficult to obtain, it was necessary to isolate the protein from SDSPAGE gels. The lysate was applied to 10% SDS-PAGE gels and the appropriate bands representing the rPgSES protein were excised and lyophilized. The lyophilized rPgSES protein (~80 µg) was pulverized, resuspended in distilled water, and then mixed with an equal volume of Complete Freund's adjuvant before being injected subcutaneously into a rabbit. Following three additional booster injections of resuspended, lyophilized rPgSES (~70 µg/injection), anti-PgSES serum was obtained. Smaller amounts of affinity column-purified rPgSES fusion protein in Complete Freund's adjuvant were injected into mice (5 µg/injection) and following three booster injections, containing 5 µg of protein in Incomplete Freund's adjuvant, anti-PgSES mouse immune serum was obtained.

2.9 Immuno-blots

To determine if the native PgSES protein could be detected in isolated sporozoite tissues, a Western blot analysis was conducted. One million sporozoites were isolated from *P. gallinaceum*-infected salivary glands and midguts as described previously [19]. Salivary glands and midguts also were isolated from non-infected mosquitoes and mosquitoes exposed to a control blood meal. All samples, including the rPgSES fusion protein and GST fusion protein, were solubilized in sample buffer, subjected to acrylamide gel electrophoresis and then electroblotted to a nitrocellulose membrane (Optitran, Schleicher and Schuell). After an incubation in 1% milk-fat blocking solution, the membrane was incubated in the anti-PgSES mouse immune serum (1:350) that had been pre-absorbed by incubation with a filter containing GST-fusion protein and salivary gland and midgut proteins [19]. Following washes in TBST (TBS/0.05% Tween 20), the membrane was incubated in peroxidase-conjugated anti-mouse IgG (Fc fraction, 1:10,000) and visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Following visualization, the membrane was stripped following the manufacturer's instructions and incubated with the anti-CS MAb (1:500) to confirm that sporozoite proteins were present in both sporozoite populations [34]. A separate membrane containing the same protein samples was incubated with mouse non-immune serum.

2.10 Immuno-fluorescence assays and confocal microscopy

Immuno-localization studies were conducted to determine the expression pattern of the PgSES protein in sporozoites. For the IFAs, sporozoites were isolated from infected salivary glands and midguts or collected in a body cavity perfusate (pooled

hemolymph sporozoites) and washed twice in 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4). Approximately 10,000 sporozoites were spotted onto a ringed slide and allowed to air dry. For analysis of hemolymph sporozoites from individual mosquitoes, a perfusate (~15 μl) containing the sporozoites was collected directly onto a ringed slide. In all IFA experiments, the sporozoites were washed, blocked with a 1–2% non-fat milk solution in PBS pH 7.0/0.075% NP40, and incubated in the primary antibody (1:100 for the anti-PgSES mouse serum and non-immune mouse serum; 1:250 for the anti-CS MAb) [34]. After a washing step, the sporozoites were incubated in a Texas Red-conjugated secondary antibody (Fab fragment, 1:200 dilution) (MP Biomedicals), washed, and then briefly incubated in DAPI (1:1000). A drop of Fluoromount G (Electron Microscopy Sciences) was added to each ring and a coverslip applied. The sporozoites were examined using an Axioskop epifluorescence microscope (Carl Zeiss) with a Texas Red filter and images were merged and then digitally enhanced using PathVysion software (Applied Imaging). These IFA experiments were done in duplicate using independent sporozoite populations with at least 100 sporozoites examined in each group per experiment.

To confirm the sporozoite surface expression of the PgSES protein, confocal microscopy was performed. Sporozoites, isolated from salivary glands, were incubated in methanol-free 4% paraformaldehyde (Electron Microscopy Sciences) in 1× PBS/0.075% NP40 for 30 min, centrifuged at 3000 × g for 3 min, and washed twice via re-suspension in 1× PBS/0.075% NP40. Sporozoites then were incubated in 2% goat serum/2% bovine serum albumin, centrifuged, and incubated in primary antibody (1:50 dilution) (anti-PgSES rabbit, rabbit pre-immune serum, or anti-CS MAb) overnight at 4°C [34]. After

washing, the sporozoites were incubated with an Alexa-488-labeled goat anti-rabbit or Alexa-568 goat anti-mouse antibody (1:200) (Molecular Probes) and TO-PRO-3 (nuclear stain at 1:50 dilution) (Molecular Probes) for 4 h at room temperature (RT). The triple-labeled sporozoites then were washed twice, resuspended in Mowiol (EMD Biosciences Inc.), a water-based mounting media, and mounted on slides followed by a coverslip. At no point in the above labeling protocol were the sporozoites allowed to dry. The red, green, and blue fluorescence were visualized simultaneously using 488 nm light from an Argon laser and 568 nm light from a Krypton/Argon laser on a Radiance 2000 confocal system (Bio-Rad Cell Sciences) coupled to an Olympus IX70 inverted microscope under a 60× (1.2 NA) water immersion objective with digital zoom. A three-dimensional set of data was acquired in all three channels at 0.3 μm intervals comprising 14–16 images per channel. The individual raw data sets, consisting of all planes, were deconvolved under 20 constrained iterations using a 3D blind-algorithm [35] for confocal images using Autodeblurr software (Autoquant Inc.). The product of the deconvolution planes was uploaded into Imaris Imaging Suite software and analyzed after projecting all planes in three channels under standard and isosurface 3D volume rendering algorithms (Bitplane Inc.). This confocal microscopy experiment was repeated three times with an estimated 100 sporozoites examined in each group per experiment. Both the 3D images and the supplemental 3D movies were made from representative sporozoites from each experiment.

To determine if the PgSES protein was expressed during the erythrocytic stages, confocal microscopy was performed using blood smears that were prepared from *P. gallinaceum*-infected chickens. The blood smears were incubated in ice-cold methanol

for 10 min at −20°C and then blocked in 10% fetal calf serum diluted in PBS for 30 min at RT. The smears were rehydrated in PBS for 30 min and then incubated with the primary antibody (1:50 for the mouse anti-PgSES serum and mouse non-immune serum) for 2 h at RT. After the primary antibody incubation, the slides were washed twice with PBS and incubated with the secondary antibody for 1 h at RT (1:400 of Alexa-488 goat anti-mouse). The smears were washed, incubated with TOPRO-3 (1:50), rinsed with deionized water and then mounted with Prolong gold anti-fade reagent (Molecular Probes). The labeled samples were imaged as described above using a confocal laser scanning microscope. In addition, after obtaining the images of fluorescent labels, the blood cells were visualized using Nomarski optics (DIC) to locate the asexual stages and gametocytes in the perspective of blood cells. The DIC images were obtained using the 637 nm excitation line of red diode laser with a transmission detector attached to the confocal microscope with appropriate DIC optics and settings. The DIC images were further enhanced using Autodeblurr software as described above before reconstructing the overlay.

3. Results and Discussion

3.1 PgSES gene identification and sequence analysis

Following screenings of a salivary gland sporozoite cDNA library with a heterologous *PfMB2* probe, a *PgSES* cDNA sequence (1653 bp) encoding a putative 551 amino acid (aa) protein with an approximate molecular mass of 63 kDa was isolated. This PgSES sequence was found to have no identity or similarity with the *PfMB2* gene, suggesting that it was isolated as a result of the low stringency hybridization and wash conditions employed during the screening. SignalP analysis of the PgSES amino acid

sequence predicted a signal peptide (aa 1–21) and the Target P and PSORT I analysis programs predicted *PgSES* to encode a secretory protein that localizes to the plasma membrane [26,36,37] (Fig. 2.1). More specifically, PSORT I predicted the PgSES protein to have an uncleavable N-terminal signal sequence. The ScanProsite program detected multiple putative sites for *N*-myristolation, *N*-glycosylation, and phosphorylation (data not shown) [38] for the PgSES protein and all of its homologs (see below for more information). However, it seems that there is limited post-translational modifications of the PgSES protein, because its observed molecular weight (65 kDa), as determined by immuno-blot analysis, closely approximates particularly its predicted molecular weight with its non-cleavable signal peptide sequence (63.2 kDa).

The *PgSES* gene has orthologous sequences in human, *P. falciparum* and *P. vivax*, rodent, *P. berghei*, *P. chabaudi*, and *P. yoelii*, and non-human primate, *P. reichenowi* and *P. knowlesi* malaria parasites [25, PlasmoDB BLAST analysis, Pathogen Sequencing Unit, Wellcome Trust Sanger Institute] (Fig. 2.1). A particularly interesting feature of this group of proteins is that there are three conserved domains (CSDs) of unknown function found in the PgSES protein and its homologs. BLAST analysis of the domains, using the short, nearly exact match NCBI database, indicated that they have no significant similarity with other known proteins. In addition, Prosite and Profam results further confirm that these three conserved regions do not represent any known domains or motifs. Because of the highly conserved/similar nature, it seems likely that the CSDs are critical for the function of the protein in some manner, but sequence analysis alone is not sufficient at this time to provide any definitive answers.

In addition to *PgSES*, further sequence analysis using Target P predicted that *PfSES*, *PbSES*, *PcSES*, and *PySES* encode signal peptides, indicating that they also are likely to enter the secretory pathway and be either secreted or expressed on the sporozoite's surface. Because *PvSES*, *PkSES*, and *PrSES* are incomplete at the 5-end and do not encode any initiating methionines, it is unclear as to whether they contain signal peptides. Analysis of the orthologous protein sequences further demonstrated that PgSES is the only one to contain an amino acid repeat region consisting of ten tandem, six-amino acid imperfect repeats (aa 436–495). Repeat regions have been observed in such malarial antigens as CS, TRAP, and merozoite surface antigens (MSA) [39] as well as in three of the six MB2 proteins reported previously [18]. In malaria parasites, significant antibody responses are directed to repeat regions and it has been hypothesized that such regions in parasite surface proteins serve as decoys that divert immune responses away from other more important epitopes [39]. Although the specific purpose of the repeat region in the *P. gallinaceum* parasite is unknown, its presence supports other data that indicate that it is a surface molecule.

3.2 Transcriptional analysis of PgSES via RT-PCR

The *PgSES* gene transcript was present in both sporozoite-infected mosquito thoraces, containing mature salivary gland sporozoites, and in abdomens, containing mature oocyst sporozoites (Fig. 2.2A). There was no *PgSES* transcript expression noted in non-infected tissues. Additional transcriptional studies assessing *PgSES* transcript presence in pre-sporozoite stages showed that there were *PgSES* transcripts present in the *P. gallinaceum*-infected *A. aegypti* midguts throughout the ookinete, oocyst, and early oocyst sporozoite stages (Fig. 2.2B) [40]. It is possible however, that the transcripts

observed at days 1–2 PE were from the erythrocytic stage parasites ingested during the blood meal and did not represent new gene expression. On day 12 PE, when sporozoites were present in the salivary glands, a large amount of the *PgSES* transcript was observed. Transcriptional studies assessing *P. gallinaceum*-infected chicken blood showed that *PgSES* transcripts also were present during the erythrocytic stages (Fig. 2.2C).

3.3 Immuno-localization of PgSES in the erythrocytic stages

Subsequent confocal microscopy and deconvolution analysis using the anti-PgSES antibodies confirmed the RT-PCR findings and showed that the PgSES protein is expressed during the ring, schizont, and gametocyte stages (Fig. 2.3). The PgSES protein appears to have some co-localization with the nuclear stain in both the early ring and schizont stages. In gametocytes, PgSES appears to localize to three distinct regions. However, it also was noted that PgSES protein expression was not universal in these stages. There were parasite-infected red blood cells that did not show PgSES expression, indicating that PgSES may be more highly expressed at different times of development in the erythrocytic stage parasites.

3.4 Immuno-blot and immuno-localization studies with sporozoites

Native PgSES protein was present in salivary gland sporozoites and not observed in oocyst sporozoites as determined by immuno-blot studies (Fig. 2.4A). Immuno-blot analysis showed that when equal numbers of sporozoites were assayed, the anti-PgSES antibodies recognized an ~65 kDa protein in the salivary gland sporozoite sample and did not recognize any protein in the oocyst sporozoite sample. As expected, the antibodies recognized the recombinant PgSES (rPgSES) positive control protein and did not recognize any proteins in the negative control samples, consisting of control blood meal

tissues and naive mosquito tissues. When the blot was stripped and re-probed with an anti-CS MAb there was parasite protein present in both the salivary gland sporozoites and oocyst sporozoite samples (Fig. 2.4B) [34]. Non-immune mouse serum did not recognize any proteins in the samples (data not shown).

Immuno-fluorescence assays revealed that anti-PgSES antibodies preferentially recognize salivary gland sporozoites as compared with hemolymph and oocyst sporozoites (Fig. 2.5A), consistent with the interpretation that PgSES is preferentially expressed in salivary gland sporozoites. Every salivary gland sporozoite observed (>100/experiment) exhibited intense labeling with the anti-PgSES antibodies while the oocyst sporozoites (>100/experiment) labeled faintly, at best, with the same antibodies. Hemolymph sporozoites, collected in a pooled sample from 15 to 20 mosquitoes, exhibited a mixed labeling pattern with the PgSES antibodies. Over 37% (117/316) of the pooled hemolymph sporozoites showed no labeling with the anti-PgSES antibodies and an additional 10% (33/316) were labeled only weakly. The remainder of the hemolymph sporozoites (166/316; 52.5%) exhibited a moderate to strong labeling pattern with the anti-PgSES antibodies. The CS MAb control strongly labeled every hemolymph sporozoite examined (183/183).

Thus, the PgSES protein appears to be expressed at a very low level in oocyst sporozoites as shown by IFA and immunoblot analyses, where the latter demonstrated that there was no detectable PgSES protein in the oocyst sporozoite sample even though comparable numbers of salivary gland sporozoites produced an observable protein product. In the hemolymph sporozoites, PgSES protein expression was varied, as compared with salivary gland sporozoites where all such sporozoites showed a uniform,

intense PgSES expression pattern via IFA. In initial studies, hemolymph sporozoites were pooled from a large sample of infected mosquitoes and were not collected from individual mosquitoes prior to examination, thereby making it difficult to assess if the varied expression pattern was the result of delayed or abnormal sporozoite development in a few mosquitoes as compared with the other infected mosquitoes. Alternatively, the varied PgSES expression in the pooled hemolymph sporozoites could have been the result of an asynchronous developmental process ongoing in all of the infected mosquitoes with newly released sporozoites showing no expression and then, as they mature and circulate in the hemolymph, exhibiting increasing amounts of the PgSES protein prior to entry into the salivary glands [41]. Subsequent studies using hemolymph sporozoites isolated from individual mosquitoes (n = 15 mosquitoes from two independent experiments) demonstrated that in 14 of the mosquitoes, PgSES protein expression was varied in a given mosquito with different levels of PgSES observed on the sporozoites (data not shown). In only one mosquito were hemolymph sporozoites displaying no PgSES expression at the time that the sporozoites were isolated. Also, it is noted that those sporozoites that express the PgSES protein in the hemolymph may be incapable of salivary gland invasion or there may be a population of hemolymph sporozoites that never express the PgSES protein and whether these sporozoites can invade the salivary glands is unknown.

The IFA data also suggested that the PgSES protein was expressed on the salivary gland sporozoite surface. Therefore, to confirm the surface localization, additional immunofluorescence and immuno-electron microscopy techniques were performed. Initial efforts using immuno-gold labeling coupled with transmission electron microscopy

resulted in weak labeling of the sporozoite surface (data not shown), potentially due to either the destruction of antigenic determinants on the sporozoite as a result of the fixation protocol or almost complete removal of PgSES during critical point drying and carbon coating. Thus, we focused on indirect immuno-fluorescence labeling coupled with confocal microscopy, because this technique involves relatively mild fixation and sample preparation procedures with excellent tissue preservation.

The PgSES protein was found to be expressed on the surface of salivary gland sporozoites with a unique surface distribution as confirmed by confocal microscopy, followed by deconvolution analysis and 3D reconstruction (Fig. 2.5B). As expected, the CS MAb positive control showed surface labeling of the sporozoite. TO-PRO-3, a nuclear stain, was used to show the presence of sporozoites. The rabbit pre-immune serum was negative for sporozoite labeling (data not shown). Interestingly, the PgSES protein has a spiral-like pattern and it appears to wrap or span around the CS protein, which uniformly covers the surface of the sporozoite, yet it does not co-localize extensively with the CS protein as indicated by the absence of a yellow color.

The surface location of the PgSES protein could enable it to play a role in adhesion, invasion or motility as has been shown or suggested for other sporozoite surface proteins [8,9,12,42]. Because of its localization to the outside surface of the plasma membrane, it is possible that the PgSES protein might be a component of the extracellular matrix, like laminin or fibronectin, which are proteins involved in cell to cell adhesion. However, this awaits further experimentation. In addition to adhesion, its surface location also could enable the PgSES protein to play a role in salivary gland or vertebrate host cell invasion as has been shown for other *Plasmodium* sporozoite surface

molecules like CS, TRAP/SSP, and MAEBL [8,9,12]. Furthermore, *Plasmodium* sporozoites have demonstrated a well-characterized gliding motility as well as a recently described corkscrew-like forward motility, and it remains to be determined as to whether the PgSES protein, with its unique spiral surface pattern, influences sporozoite motility [42,43].

Collectively, the protein and transcriptional data for *PgSES* are consistent with PlasmoDB expression data for *PfSES* which showed, via transcriptional analysis and mass spectrometry, that RNA levels were elevated in salivary gland sporozoites and asexual blood stages [44] and that the PfSES protein was expressed in the merozoite and salivary gland sporozoite stages [45]. Currently, there is neither oocyst nor hemolymph sporozoite transcriptional data available for *PfSES*, and there also is no mass spectrometry data available for these parasite stages for the PfSES protein [45].

In summary, the molecular and cellular characterization of *PgSES* has resulted in the discovery of a novel gene with conserved domains that is found in at least eight *Plasmodium* spp. and that is expressed in multiple parasite stages including asexuals, gametocytes and particular sporozoite populations. In the latter, it is preferentially expressed in some hemolymph sporozoites and on all salivary gland sporozoites with little to no expression observed in oocyst sporozoites. PgSES exhibits a unique spiral labeling pattern on the surface of the sporozoite and its surface location could enable it to play a role in the invasion of mosquito salivary glands or vertebrate host tissue. Therefore, in vivo blocking studies with antibodies and PgSES recombinant protein are planned and functional assays using gene disruption technology with *P. falciparum* and/or *P. berghei* will be performed to determine the role of this novel protein in

Plasmodium and, more specifically, sporozoite biology. Ultimately, this novel protein could be another target towards which efforts to control malaria and its transmission could be directed.

Acknowledgments

The partial *P. reichenowi* sequence data were produced by the Pathogen Sequencing Unit at the Wellcome Trust Sanger Institute and can be obtained from http://www.sanger.ac.uk/ Projects/P reichenowi/. This study was supported by the National Institutes of Health (NIH) grants AI 01657, AI 53156, and F32 AI 09731 to B.T.B and a Minority Biomedical Research Support (MBRTI)/NIH grant (R25 GM56901) and Missouri Alliance for Graduate Education and the Professoriate (MAGEP)/NSF Fellowship to A.N.L.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2006.03.016.

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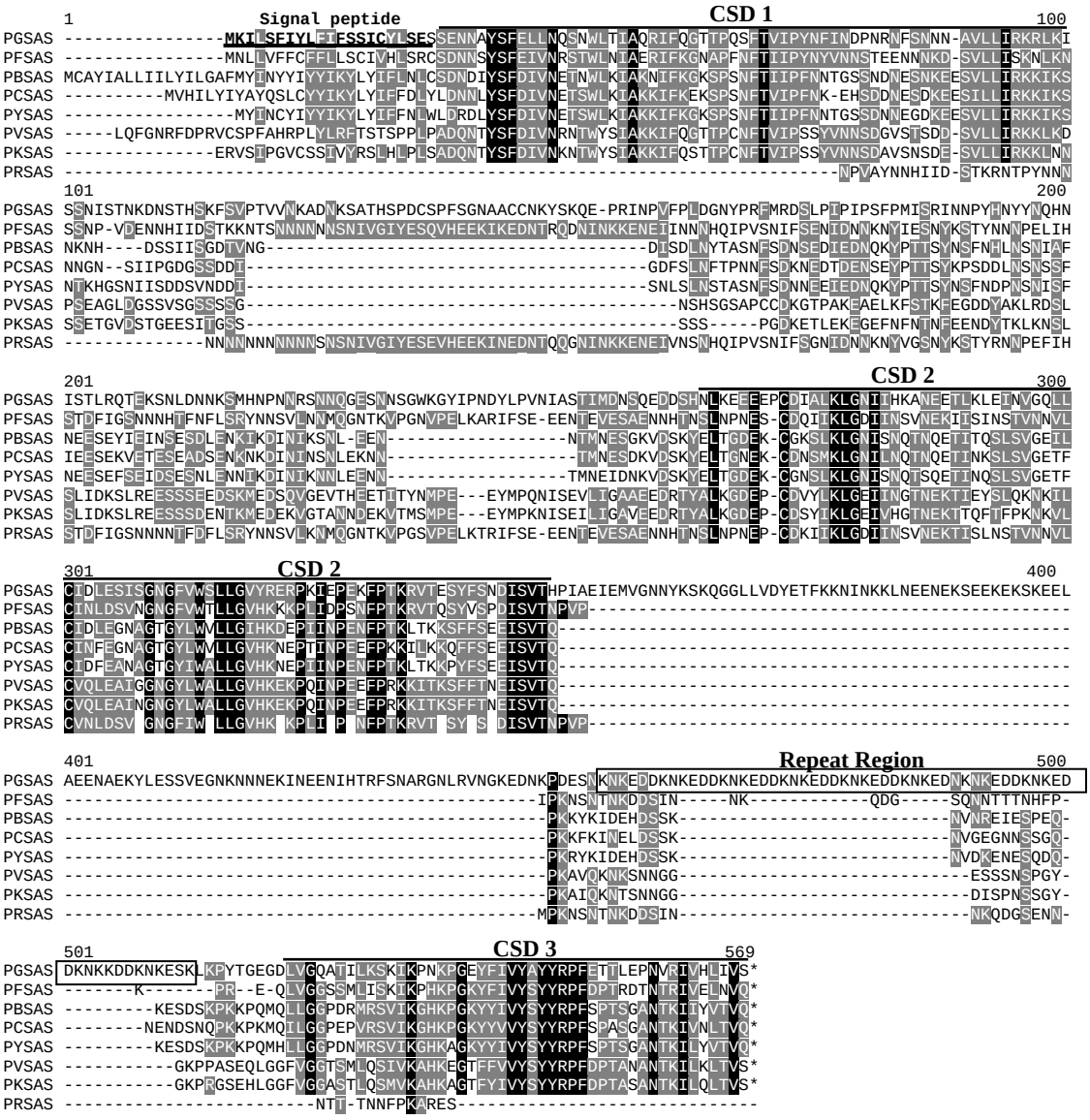

Fig. 2.1. Alignment of the novel PgSES amino acid sequence and its homologs identifies three conserved domains of unknown function. *P. falciparum* (PfSES orf = PFI0580c in PlasmoDB), *P. viva*x (PvSES orf = Pv 3868-2-131333-132457 in PlasmoDB), *P. knowlesi* (PkSES orf = Pk 209h02p1c-3-1143-2222 in PlasmoDB), *P. berghei* (PbSES orf = Pb 5602-4-8083-6944 in PlasmoDB), *P. yoelii* (PySES orf = chrPyl 00985-3-13503-14624 in PlasmoDB), *P. chabaudi* (PcSES orf = Pc 48462-1292-2428 in PlasmoDB), and *P. reichenowi* all have distinct regions of identity with the PgSES protein as determined

by aligning the translated proteins with the Vector NTI Explorer program. The signal peptide sequence in PgSES, as determined by SignalP analysis, is underlined. The PgSES repeat region is boxed. Amino acids highlighted in black are conserved in all proteins and those highlighted in gray are conserved in a majority of the proteins. The three conserved unknown domains (CSD) have a line above them.

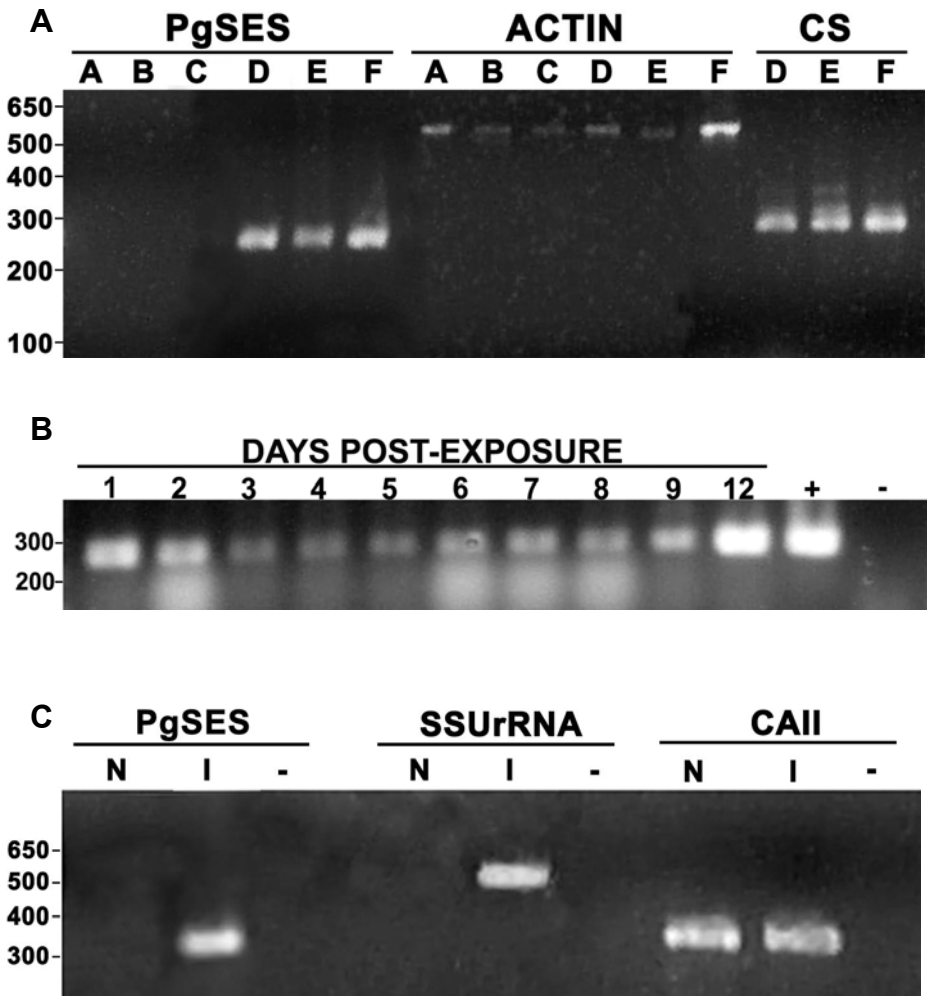

Fig. 2.2. Reverse-transcription PCR (RT-PCR) analysis of *PgSES*. (A) *PgSE*S transcripts are present in both oocyst sporozoites and salivary gland sporozoites as determined by RT-PCR analysis. The amplifications were performed with gene-specific *PgSES*, *A. aegypti* muscle actin, and *P. gallinaceum* circumsporozoite (*CS*) primers that are listed above each set of templates. A lanes represent non-infected whole *A. aegypti* mosquitoes; B lanes represent thoraces isolated from *A. aegypti* exposed to a non-infected blood meal; C lanes represent abdomens isolated from *A. aegypti* exposed to a non-infected blood meal; D lanes represent thoraces isolated from *A. aegypti* infected with salivary gland

sporozoites; E lanes represent abdomens isolated from *A. aegypti* infected with mature oocyst sporozoites; F lanes represent positive control reactions for each gene. DNA size markers in base pairs (bp) are shown to the left of all figures. (B) PgSES transcripts are present in pre-sporozoite and sporozoite stages of *P. gallinaceum*-infected *A. aegypti* (days 1–9 PE) with the greatest abundance observed when sporozoites are present in the salivary glands (day 12 PE). *PgSE*S cDNA was used for the positive control reaction (+) and PCR reagent mix without DNA was used for the negative control reaction (−). (C) Analysis of *P. gallinaceum*-infected chicken blood indicates that *PgSE*S transcripts are present in the erythrocytic stages. The amplifications were performed with first-strand cDNA representing non-infected chicken blood (N) and infected chicken blood (I). Primers specific for *PgSES*, *SSUrRNA*, and *C*A II are listed above each set of templates. Negative control reactions without template (−) also were performed.

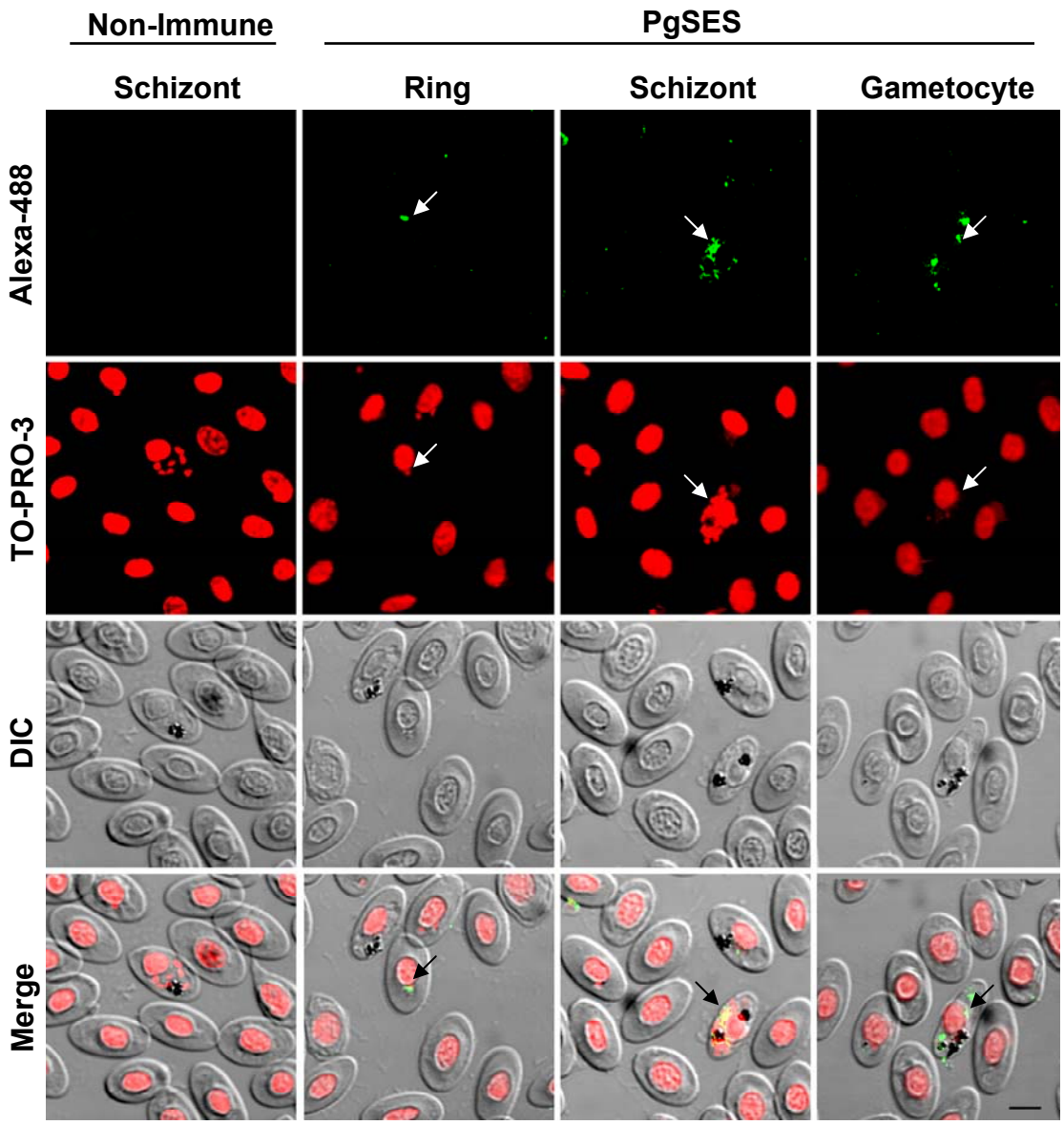

Fig. 2.3. The PgSES protein is expressed in erythrocytic stage parasites as demonstrated by immuno-localization studies using confocal microscopy. The PgSES protein is expressed during some ring, schizont, and gametocyte stages within infected blood cells. *P. gallinaceum*-infected red blood cells were labeled with an anti-PgSES mouse antibody (primary), Alexa-488 antibody (secondary) and TO-PRO-3 (a nuclear stain). Confocal raw data were first 3D reconstructed and then merged with the Nomarski/DIC images to

show localization of the PgSES protein during the different parasite stages in the blood cells. The presence of PgSES in the erythrocytic stages was confirmed as it co-localized with the nuclear stain in both ring and schizont stages and it appeared to label three distinct regions of a gametocyte. The mouse non-immune serum showed no cross-reactivity with the parasites. The scale bar represents 5 μm.

Fig. 2.4. The PgSES protein is expressed in salivary gland sporozoites, but not in oocyst sporozoites. (A) Immuno-blot analysis was performed using one million *P. gallinaceum* salivary gland sporozoites and oocyst sporozoites isolated from infected midguts. Anti-PgSES antibodies (primary antibody) recognized an ~65 kDa protein in the salivary gland sporozoites (arrow) but not in the oocyst sporozoites. S designates sporozoites, B denotes tissues isolated from mosquitoes exposed to a non-infected blood meal, and N designates tissues isolated from naive mosquitoes. The (−) control lane contained GST fusion protein and the (+) control lane contained recombinant PgSES/GST fusion protein (*M*r = 89 kDa). (B) After stripping of the blot, an anti-CS MAb recognized the CS protein in the sporozoite samples. The anti-CS MAb detected proteins of ~64 and 76 kDa in the salivary gland (SG) sporozoites and an ~76 kDa protein in the oocyst sporozoites isolated from midguts (MG), indicating that sporozoite proteins were present in the samples. S designates sporozoites and N designates tissues isolated from naive mosquitoes. Molecular weight markers in kilodaltons (kDa) are shown to the right of the figures.

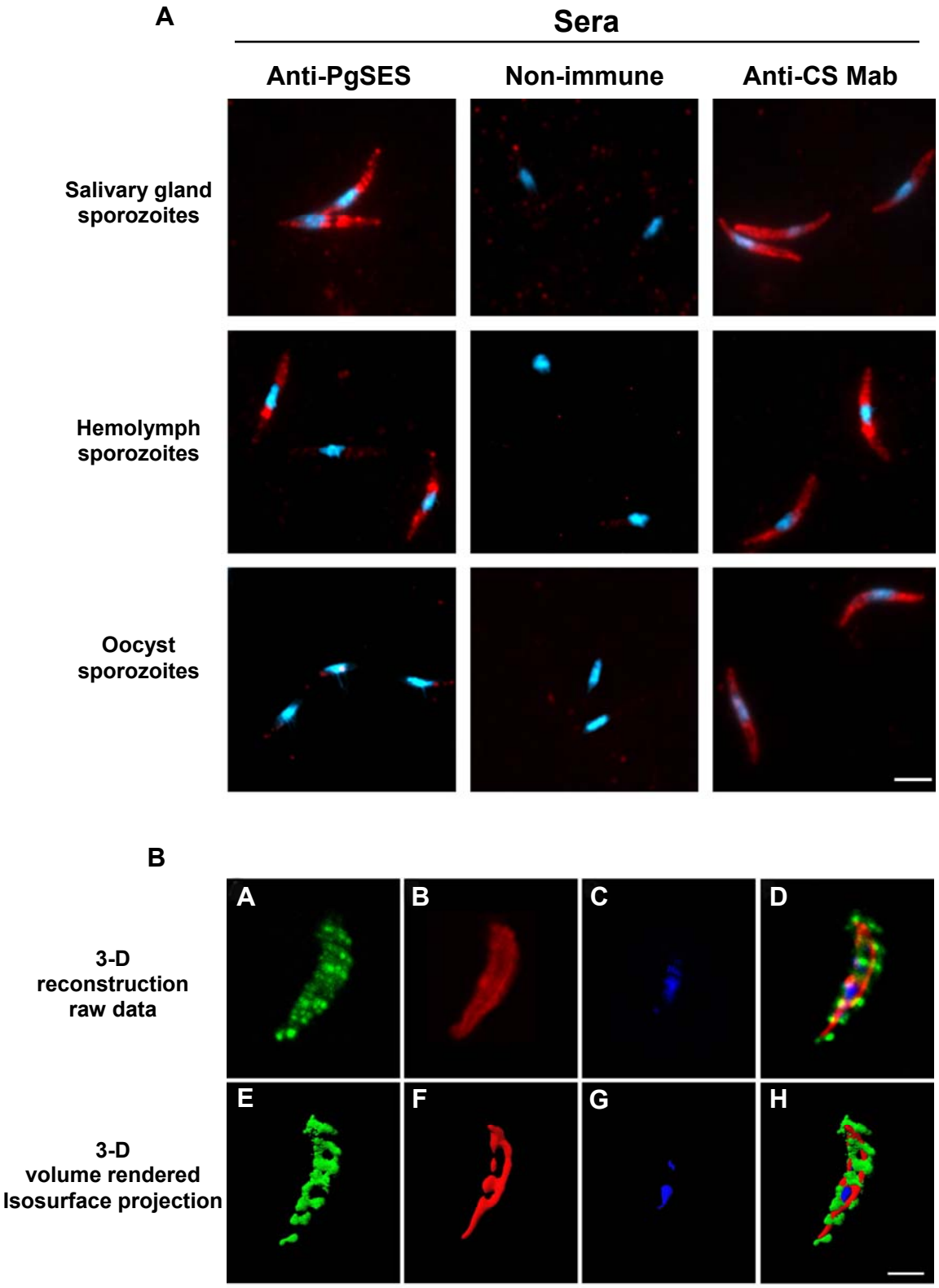
A
Sera
Anti-PgSES
Non-immune
Anti-CS Mab
Salivary gland sporozoites
Hemolymph sporozoites
Oocyst sporozoites
B
3-D reconstruction raw data
3-D volume rendered Isosurface projection
A
B
C
D
E
F
G
H

Fig. 2.5. Immuno-localization of the novel PgSES protein in different sporozoite populations. (A) The PgSES protein is preferentially-expressed in salivary gland sporozoites as compared with oocyst and hemolymph sporozoites. Immuno-fluorescence assays show that anti-PgSES antibodies preferentially label salivary gland sporozoites as compared with oocyst and hemolymph sporozoites. The anti-PgSES mouse serum (PgSES), anti-circumsporozoite (CS) mouse MAb (positive control), and non-immune mouse serum (non-immune) were used as primary antibodies. The scale bar represents 5 μm. (B) The PgSES protein localizes to the surface of salivary gland sporozoites with a unique spiral labeling pattern as determined by confocal microscopy. Sporozoites were triple-labeled with anti-PgSES rabbit polyclonal antibodies, anti-CS mouse MAb, and TO-PRO-3 (a nuclear stain). Confocal raw data were first 3D reconstructed (panels A–D) and then deconvolved and isosurface volume rendered (panels E–H). The surface labeling of the novel *P. gallinaceum* sporozoite protein, PgSES, is shown in green (panels A and E) and the circumsporozoite protein, CS, is shown in red (panels B and F). TO-PRO-3, shown in blue, was used to stain the sporozoite nucleus (panels C and G). PgSES appears to spiral outside around the CS protein, thus confirming its surface expression (panels D and H). The scale bar in H represents 3 μm.

CHAPTER 3. PGSES APPEARS TO PLAY A ROLE IN SPOROZOITE INVASION OF MOSQUITO SALIVARY GLANDS

ABSTRACT

Previously, we described PgSES, a ubiquitously expressed *Plasmodium gallinaceum* sporozoite surface protein. PgSES contains homologs in various *Plasmodium* species including the most pathogenic form of human malaria, *P. falciparum.* In *P. falciparum*, SES, also referred to as Falstatin, acts as a cysteine protease inhibitor and anti-falstatin antibodies have been show to block new ring formation in erythrocytes. To determine if PgSES plays a role in host tissue invasion, IgG- and Fab-purified anti-PgSES antibodies were injected into *P. gallinaceum*-infected mosquitoes. Infected mosquitoes were injected with antibodies on Day 8 post-exposure to an infected blood meal and salivary glands were dissected 48 hours post-injection to determine sporozoite loads. Salivary gland infections were reduced by 49-87% in anti-PgSES antibody injected mosquitoes, suggesting a possible role for PgSES in sporozoite invasion of mosquito salivary glands.

Key words: Malaria; Sporozoite; Antibody blocking; SES

1. Introduction

Malaria sporozoite invasion of the mosquito salivary glands is thought to be mediated by specific receptor-ligand interactions (Rosenberg, 1985; Touray *et al*., 1992; Barreau *et al*., 1995; Brennan *et al*., 2000; Myung *et al*., 2004). According to Pimenta *et al*. (1994), the sporozoite must bind to the salivary glands before invasion occurs and Korochkina *et al*. (2006) showed that antibodies against an *Ae. aegypti* salivary gland specific protein (aaSGS1) block *P. gallinaceum* sporozoite invasion of the mosquito salivary glands by ~60%. Collectively, these data suggest that mosquito salivary glands have receptors that interact with sporozoite proteins, thereby facilitating salivary gland invasion.

Molecules expressed on the surface, or secreted to the sporozoite surface have been shown to play a role in host tissue invasion. Studies analyzing the major sporozoite surface protein, the circumsporozoite protein (CS), have shown that the N2H6D5 anti-CS monoclonal and single-chain antibodies block *P. gallinaceum* sporozoite invasion of *Ae. aegypti* salivary glands by 97-99.0% (Warburg *et al*., 1992; de Lara Capurro *et al*., 2000). Ramirez *et al*. (1995) showed that the N5G3H6 monoclonal anti-CS antibody blocks *P. gallinaceum* sporozoite invasion of *Gallus gallus* host tissue by 100%. Other studies investigating sporozoite and salivary gland interactions have shown that in *P. falciparum* conserved region I of the CS protein preferentially binds to *An. stephensi* salivary glands (Sidjanski *et al*., 1997). More recently, in *P. gallinaceum* the SES protein has been shown to be expressed on hemolymph sporozoites and on the surface of salivary gland sporozoites (LaCrue *et al*., 2006). Also, studies have shown that the PgSES homolog in *P. falciparum*, SES/Falstatin, is a cysteine protease inhibitor and that antibodies against

Falstatin block new ring formation in erythrocytes by ~80% (Pandey *et al*., 2006). Based on these and the previously described studies, we set out to determine if PgSES plays a role in sporozoite invasion of the mosquito salivary glands.

2. Materials and Methods

2.1 Mosquito maintenance and the parasite transmission cycle

Aedes aegypti (Red strain) mosquitoes used for the transmission cycle were 3-10 days old. The mosquitoes were maintained as previously described (LaCrue *et al*., 2005). *Plasmodium gallinaceum* strain 8A (Hewitt, 1949) was maintained in White Leghorn chickens by exposure to *P. gallinaceum* sporozoite-infected mosquitoes. All animal care and the experiments described herein were approved by and performed in accordance with guidelines of the National Institutes of Health and the University of Missouri-Columbia Animal Care and Use Committee.

2.2 Antibodies and antibody production

Antibodies against PgSES were generated as previously described (LaCrue *et al*., 2006). The anti-PgSES rabbit polyclonal antibodies and pre-immune serum were IgG-purified on a protein A/G column (Pierce, Rockford Illinois) per the manufacturer's instructions. Fab digestion of the IgG-purified antibodies was performed using a Fab preparation kit (Pierce) per the manufacturer's instructions. The N5G3H6 and N2H6D5 monoclonal antibodies, directed against the *P. gallinaceum* CS protein, were a gift from Dr. A. Krettli (Krettli *et al*., 1988).

2.3 Sporozoite invasion assay

A *P. gallinaceum*-infected White Leghorn chicken was used to infect 4-5 day old female *Ae. aegypti* mosquitoes. Briefly, one day prior to sporozoite release from oocysts

(*i.e.,* day 8 post-exposure), 0.5 µl (0.2 µg / µl) of the purified IgG or Fab fraction of the rabbit polyclonal anti-PgSES antibody was injected intra-thoracically into a cold-anesthetized mosquito using a finely pulled capillary needle attached to a micromanipulator (Hillyer *et al*., 2003). The monoclonal anti-CS antibodies, either N5G3H6 or N2H6D5, were used as positive injection controls (Krettli *et al*., 1988; Warburg *et al*., 1992). When the IgG blocking studies were conducted, the N5G3H6 anti-CS mAb antibody, which has been shown to prevent *Plasmodium gallinaceum*-infection in chickens (Ramirez *et al*., 1995) was the only anti-CS antibody available in the lab; however, its blocking capability in the mosquito had not previously been tested. The N2H6D5 anti-CS mAb, shown to be successful in blocking sporozoite invasion of the mosquito salivary glands in other studies, became available for use as a positive control just prior to the Fab injection studies (Warburg *et al*., 1992; de Lara Capurro *et al*., 2000). Mosquitoes injected with pre-immune IgG were used as negative injection controls.

Forty-eight hours post-injection (PI), mosquito salivary glands were dissected and sporozoite invasion was assessed. Briefly, 5 pairs of salivary glands were dissected in a drop of *Aedes* saline on a slide, combined into a 1.5 ml microcentrifuge tube containing 15 µl of *Aedes* saline, homogenized with a siliconized pipette tip to release sporozoites, and sporozoites counted via a hemacytometer using phase-contrast microscopy. Six to ten groups of five were counted for each treatment in the IgG experiment and seven groups of five mosquitoes were counted for each treatment in the Fab group. The IgG and Fab experiments were done in triplicate. The injected mosquito mortality rate for all experiments was ~10-15%. Data were analyzed by one-way analysis of variance

(ANOVA) to test for variation between groups and by the Tukey test to identify pairwise differences between groups. Differences were considered significant at a P-value <0.05.

3. Results and Discussion

3.1 Sporozoite invasion assay

PgSES is expressed in some hemolymph sporozoites and on the surface of salivary gland sporozoites. The expression of SES on hemolymph sporozoites suggested a possible role in salivary gland invasion; therefore, we investigated the role of PgSES in sporozoite invasion by injecting *P. gallinaceum*-infected female mosquitoes with IgG-purified anti-PgSES rabbit polyclonal antibodies before oocyst rupture. Salivary gland sporozoites then were counted 48 hours PI to assess antibody blocking efficiency. As expected, the preimmune serum for both the IgG and Fab experiments did not block sporozoite invasion of the mosquito salivary glands and therefore, the level of sporozoite invasion in the preimmune control was defined as 100% invasion. The IgG-purified anti-PgSES polyclonal antibodies blocked sporozoite invasion by 49-61% in comparison to the preimmune control (Table 3.1), with experiments 1 and 3 being statistically significant (Figure 3.1A) [ANOVA for between-group variations $F = 14.3$, $P < 0.001$; Tukey test for pairwise multiple comparison, anti-PgSES IgG versus preimmune control, $q = 6.02$, $P = 0.001$]. The studies were later repeated using a Fab-purified fraction of the anti-PgSES antibodies to confirm that sporozoite blocking was not due to steric hindrance caused by the large IgG molecule. Data from those experiments showed that the Fab-purified anti-PgSES antibodies blocked sporozoite invasion by 52-87% in comparison to the preimmune control (Table 3.2), with experiments 2 and 3 being statistically significant (Figure 3.1B) [ANOVA for between-group variations $P < 0.001$; Tukey test

for pairwise multiple comparison, anti-PgSES Fab versus preimmune control, $q = 3.57$, $P = 0.001$]. There was no significant difference in blocking capability between the IgG and Fab treatment groups ($P = 0.102$). These data suggest that the PgSES protein may play a role in sporozoite invasion of the mosquito salivary glands.

Interestingly, the *P. falciparum* homolog, PfSES/Falstatin, acts as a cysteine protease inhibitor of parasite and host proteases during the erythrocytic stages (Pandey *et al*., 2006). In those studies, anti-PfSES antibodies inhibited new ring formation by ~80%, suggesting a possible role for SES/Falstatin in merozoite invasion (Figures 3.2 A and B; Pandey *et al*., 2006). The authors suggested that Falstatin, as a cysteine protease inhibitor, most likely targets parasite cysteine proteases such as the well characterized falcipains that, if left unchecked, could disrupt the limited proteolysis by serine proteases that is required for erythrocytic invasion (Pandey *et al*., 2006). Additionally, Falstatin also inhibits host cysteine proteases, such as cathepsin L and calpain-1, most likely so that merozoites can invade the erythrocytes and continue their development. The role of the SES protein in mosquito stage parasites is not clear. However, based on antibody blocking results from both the PfSES/Falstatin and PgSES studies, it is possible that within the mosquito SES also may work to protect the sporozoite from parasite and mosquito proteases so that sporozoites can invade the salivary glands (Figures 3.3 A and B).

Sporozoite invasion of mosquito salivary glands appears to be an active process that involves very intricate steps. When released from the oocyst, sporozoites must make their way through the hemocoel, invade the salivary glands, and avoid being targeted by the mosquito immune response (*i.e.*, phagocytosis by hemocytes, melanization, initiation

of apoptosis by proteases) (Beerntsen *et al*., 2000; Hurd and Carter, 2004; Hillyer *et al*., 2007). The exact mechanism of sporozoite clearance is unclear and phagocytosis of sporozoites does not appear to be an efficient process (Hillyer *et al*., 2007). Consequently, it is speculated that instead of clearing the sporozoite burden from the mosquito, phagocytosis by hemocytes may play a role in processing the sporozoite to initiate immune signaling, thereby leading to a proteolytic cascade and ultimately the destruction of the parasite (Beerntsen *et al*., 2000; Hillyer *et al*., 2007).

3.2 Possible role of SES in the mosquito

Only 20% of *P. gallinaceum* sporozoites released from the oocyst actually invade *Ae. aegypti* salivary glands and after 8 hours no additional sporozoites invade, but instead become degraded by the mosquito immune response (Hillyer *et al*., 2007). LaCrue *et al*. (2006) showed that ~ 60% of *P. gallinaceum* hemolymph sporozoites express the SES protein, so it is possible that the sporozoites expressing the SES protein have the best chance of invading the salivary glands because they are protected from degradation. For example, Hillyer *et al*. (2007) proposes that the mosquito immune system may attack critical sporozoite motility machinery to incapacitate the parasites so that they are opsonized and degraded in a fashion similar to what has been observed for the ookinete in the mosquito midgut (Blandin *et al*., 2004). The role of SES as a cysteine protease inhibitor in the mosquito may be to regulate mosquito proteases in an effort to prevent the disruption of important motility and host binding molecules, such as TRAP and CS, respectively (Warburg *et al*., 1992; Sultan *et al*., 1997; Ramirez *et al*., 1995). In doing so, the sporozoite is able to migrate through the hemocoel, invade the salivary glands, and then the vertebrate host tissue within a narrow window of time.

Recent studies have shown that in *Plasmodium berghei*, a cysteine protease of parasite origin plays a role in sporozoite egress from oocysts of *Anopheles stephensi* (*i.e.,* Egress cysteine protease-1) and the cleavage of CS so that sporozoites can invade the liver (Aly and Matuschewski, 2005; Coppi *et al*., 2005). Based on this information, it is possible that a protease of parasite origin (*e.g.,* serine protease), that is not inhibited by SES, also plays a role in parasite invasion of mosquito salivary glands. The role of SES may be to inhibit parasite cysteine proteases that might otherwise interfere with the sporozoites ability to invade the salivary glands and, once in the glands, SES expression continues to prevent parasite and mosquito proteases from destroying the sporozoites.

Another possible role for SES is based on studies investigating the role of apoptosis in *Plasmodium* and mosquitoes. Apoptosis (programmed cell death) is a rapid first response to invading pathogens in vertebrates that involves many proteases including cysteine proteases such as caspases, metacaspases, cathepsins and calpains. The potential role of apoptosis in insect immunity is unknown; however, current literature indicates that molecules such as the metacaspases and caspases are present in both *Plasmodium* and mosquitoes, respectively (Hurd *et al*., 2006). In *Plasmodium* it has been shown that *P. berghei* metacaspase-1 (PbMc1) is involved in apoptosis and is expressed in all mosquito stage parasites, but not in the asexual stages (Le Chat *et al*., 2007). Recently, a novel initiator caspase, *Aedes* Dredd, has been characterized that may play a role in *Ae. aegypti* apoptosis of *Plasmodium*-infected cells by activating downstream effector caspases that cleave specific proteins and ultimately lead to cell and parasite death (Cooper *et al*., 2007). It is possible, that like Falstatin in the erythrocytic stages, SES binds to and blocks the activity of parasite and host proteases so that either the sporozoite

does not inititate its own cell death or become victim to apoptosis while traversing through the hemolymph and subsequently invading the salivary glands.

In this study, the antibody blocking results suggest that in *P. gallinaceum* the SES protein plays a role in sporozoite invasion of the mosquito salivary glands in a manner similar to what has been proposed for the role of SES/Falstatin in the invasion of red blood cells by *P. falciparum* merozoites (Pandey *et al*., 2006). These and other studies indicate that parasite invasion of host tissues appears to be a well-timed event that involves proteases and inhibitors, as well as the recognition and evasion of several parasite and host molecules.

Future studies include assessing the role of SES in apoptosis by incubating live hemolymph and salivary gland sporozoites with markers of apoptosis such as acridine orange and/ or annexin, followed by incubation with anti-SES antibodies and a fluorescently-labeled secondary antibody to determine if the SES protein is present on sporozoites that are undergoing apoptosis. In the hemolymph, the possible outcomes include: 1) none of the sporozoites will label with the apoptosis marker, suggesting that expression of the SES protein is developmentally-regulated, 2) some sporozoites will express SES and not label with the apoptosis marker and non-SES expressing parasites will label with the apoptosis marker. This result would suggest that only parasites expressing the SES protein are viable and have the best chance of invading the salivary glands, 3) some sporozoites will express SES and label with the apoptosis marker, while the non-SES expressing parasites will not label with the apoptosis maker. This result would suggest that sporozoites not expressing the SES molecule are the viable parasites that make it into the salivary glands, and 4) all of the parasites label with the apoptosis

marker, suggesting that the method of harvesting and handling the sporozoites triggered apoptosis.

Additionally, the role of SES in vertebrate tissue invasion will be determined by injecting chickens with sporozoites incubated in anti-SES antibodies and assessing parasitemias in the chickens. If the chickens fail to develop a parasitemia, then it would suggest that SES plays a role in sporozoite invasion of host tissues. It is also possible that there could be a significant delay in parasitemia suggesting that SES may play a role in host tissue invasion; however, redundant invasion mechanisms (*e.g.* other proteases or parasite invasion molecules not hindered by the absence of the SES inhibitor) eventually resulted in parasite invasion of host tissues.

Note/Acknowledgment

The research reported in this chapter is the foundation for a paper to be submitted at a later date. The authors for this paper will be: LaCrue AN, Lowery RJ, Roberts RN, and Beerntsen BT.

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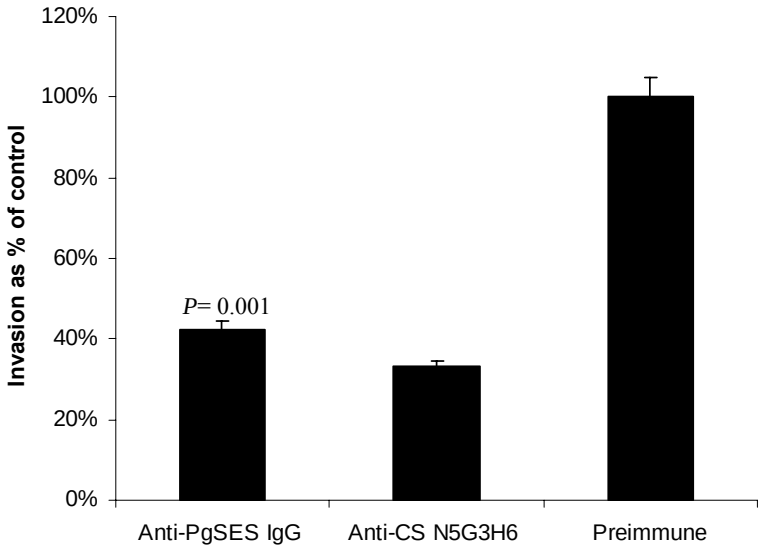
Experiment	Group	Prevalence of midgut infection (%)	Range of oocysts in midgut	Mean intensity of oocysts in midgut	Average number of sporozoites per salivary gland pair
1	**Non-injected**	100 (29/29)	1-77	21	646
8 sets of 5	**Pre-immune**				590
	Anti-PgSES				232
	% reduction				**61%** *
	Anti-CS mAb				343
	% reduction				42%
2	**Non-injected**	90 (36/40)	1-73	16	1318
6 sets of 5	**Pre-immune**				1313
	Anti-PgSES				672
	% reduction				**49%**
	Anti-CS mAb				312
	% reduction				76%
3	**Non-injected**	87.5 (42/48)	1-87	25	955
10 sets of 5	**Pre-immune**				985
	Anti-PgSES				389
	% reduction				**61%** *
	Anti-CS mAb				294
	% reduction				70%

Table 3.1. Anti-PgSES IgG antibodies partially block sporozoite invasion of mosquito salivary glands. IgG-purified anti-PgSES antibodies were injected intrathoracically into *P. gallinaceum*-infected mosquitoes on Day 8 post-exposure (PE) to an infected blood meal. Two days post-injection, salivary glands were dissected in groups of five, homogenized, and sporozoite numbers were assessed using a hemacytometer. Analysis of variance (ANOVA) and a Tukey Test were used to determine statistical significance, $P < 0.05$. Experiments with statistically significant blocking of sporozoite invasion by anti-PgSES antibodies as compared with the pre-immune serum are indicated by an asterisk. The anti-CS monoclonal antibody (N5G3H6) was used as a positive injection control. The preimmune control was not statistically different from the non-injected positive control.

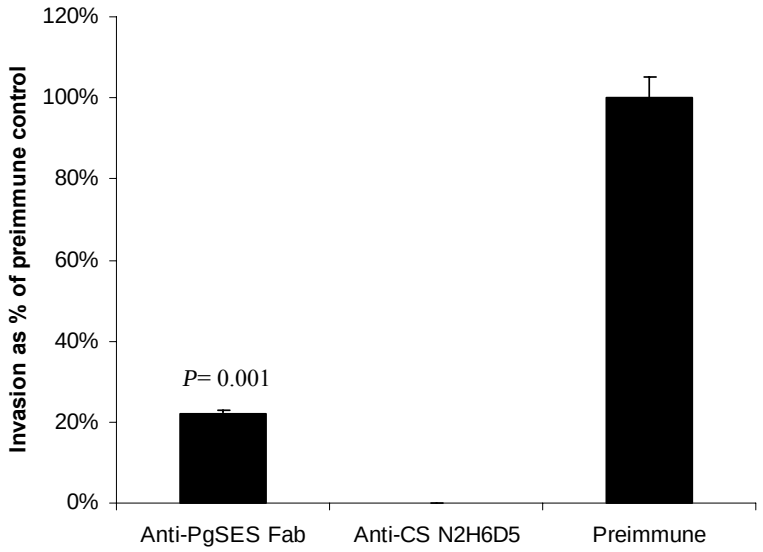
Experiment	Group	Prevalence of midgut infection (%)	Range of oocysts in midgut	Mean intensity of oocysts in midgut	Average number of sporozoites per salivary gland pair
1	**Non- injected**	100 (40/40)	1-95	32	45129
7 sets of 5	**Pre-immune**				43393
	Anti-PgSES Fab				20874
	% reduction				**52%**
	Anti-CS mAb				60
	% reduction				99.9%
2	**Non- injected**	93 (26/28)	1-104	21	19686
7 sets of 5	**Pre-immune**				16529
	Anti-PgSES Fab				2191
	% reduction				**87% ***
	Anti-CS mAb				5
	% reduction				99.97%
3	**Non- injected**	100 (20/20)	2-98	30	9026
7 sets of 5	**Pre-immune**				11571
	Anti-PgSES Fab				3973
	% reduction				**66% ***
	Anti-CS mAb				45
	% reduction				99.6%

Table 3.2. Fab-purified anti-PgSES antibodies partially block sporozoite invasion of mosquito salivary glands. Fab-purified anti-PgSES antibodies were injected intrathoracically into *P. gallinaceum*-infected mosquitoes on Day 8 post-exposure (PE) to an infected blood meal. Two days post-injection, salivary glands were dissected in groups of five, homogenized, and sporozoite numbers were assessed using a hemacytometer. Analysis of variance (ANOVA) and a Tukey Test were used to determine statistical significance, $P < 0.05$. Experiments with statistically significant blocking of sporozoite invasion by anti-SES Fab antibodies as compared with the preimmune serum are indicated by an asterisk. The anti-CS monoclonal antibody (N2H6D5) was used as a positive injection control. The preimmune control was not statistically different from the positive control.

A



B



Figures 3.1 A and B. IgG- and Fab-purified anti-PgSES antibodies partially block sporozoite invasion of mosquito salivary glands. *Plasmodium gallinaceum*-infected *Aedes aegypti* mosquitoes were injected with the IgG or Fab fraction directed against

PgSES, preimmune IgG (negative control), or monoclonal anti-CS (N2H6D5 or N5G3H6) antibodies (positive control). Salivary glands were dissected and sporozoites counted 48 hours post-injection. When the statistically significant experiments were combined, the anti-PgSES IgG and Fab antibodies blocked sporozoite invasion by 61% and 78%, respectively. Analysis of variance (ANOVA) and a Tukey Test were used to determine statistical significance, $P < 0.05$. The pairwise significant difference between the anti-PgSES antibodies and the preimmune control is represented by the *P*-values. There was no significant difference between the PgSES IgG and Fab fractions, indicating that sporozoite blocking was due to the properties of the anti-PgSES antibodies and not steric hindrance. The preimmune IgG was not significantly different from the non-injected positive control (data not shown). The anti-CS monoclonal antibodies N5G3H6 and N2H6D5 blocked sporozoite invasion by 67% and 99%, respectively.

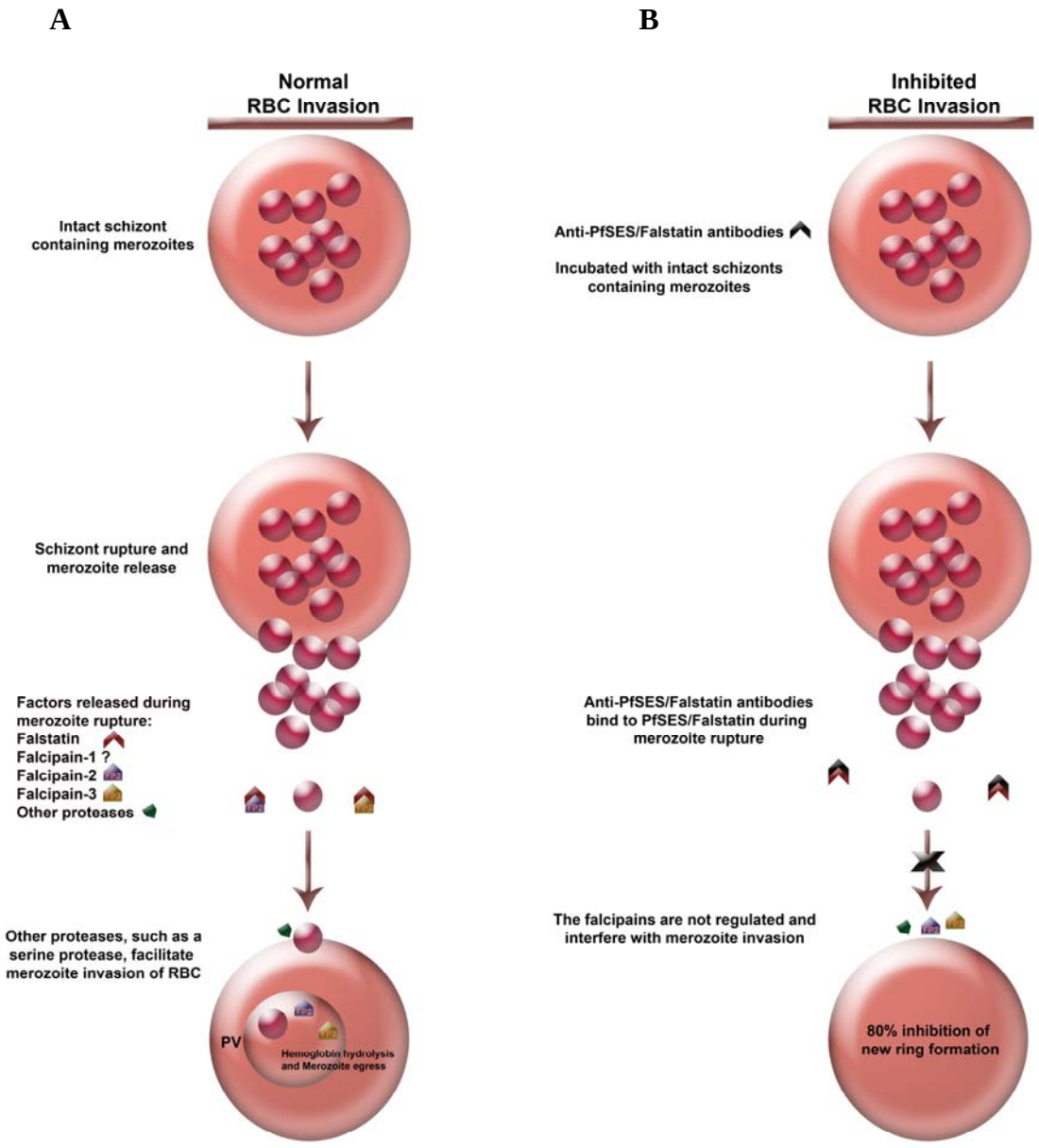
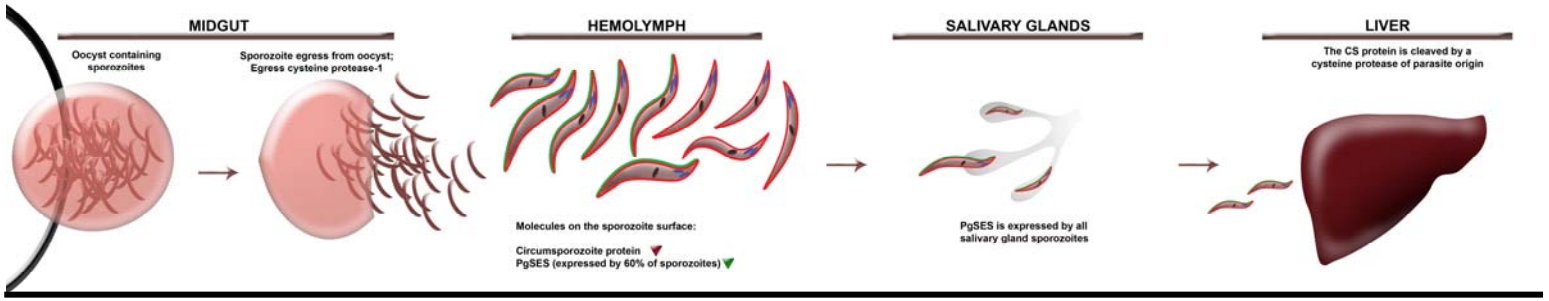


Figure 3.2 The proposed action of PfSES/Falstatin in the erythrocytic stages. A) In *Plasmodium falciparum*, under normal circumstances, the merozoite containing schizonts rupture and merozoite invade new red blood cells. Studies by Pandey *et al*. (2006), suggest that PfSES/Falstatin regulates cysteine proteases of parasite and host origin (*e.g.* falcipains, calpain-1, etc.) so that other proteases, such as serine proteases, can facilitate

merozoite invasion of erythroyctes. PV= parasitophorous vacuole B) Anti-Falstatin antibodies inhibited new ring formation by ~80%. It is speculated that in the absence of PfSES/Falstatin, that cysteine proteases, such as the falcipains, act non-specifically and interfere with merozoite invasion of the red blood cell. Figure created by Alexis LaCrue.

A

PgSES and sporozoite invasion



B

Anti-PgSES Inhibited sporozoite invasion

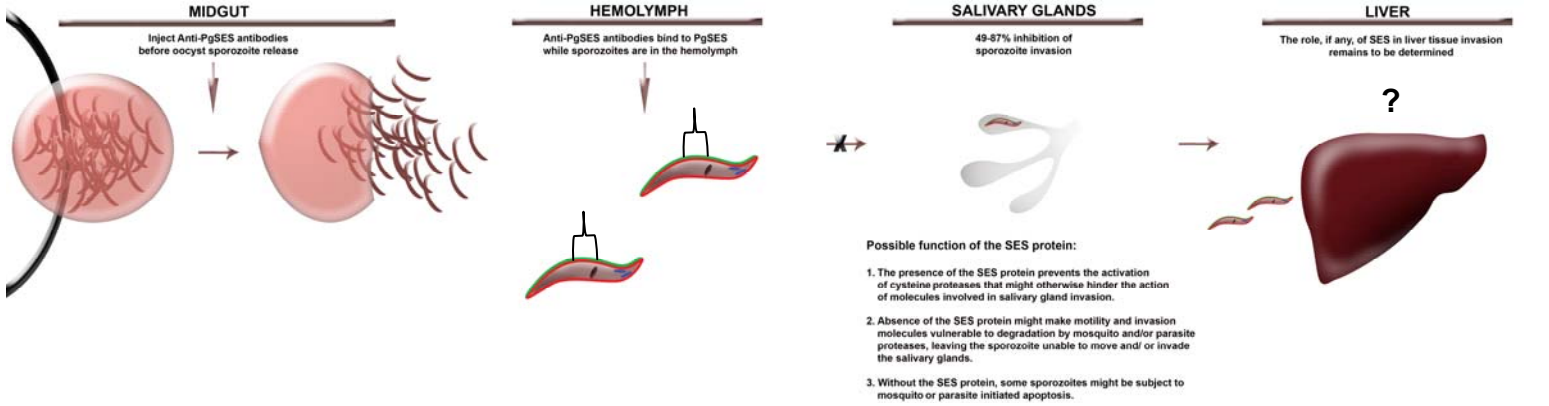


Figure 3.3 The PgSES protein is expressed by hemolymph and salivary gland sporozoites and antibody blocking studies suggest that the SES protein plays a role in sporozoite invasion of the salivary glands. A) LaCrue *et al*. (2006) showed that 60% of *Plasmodium gallinaceum* hemolymph sporozoites express a cysteine protease inhibitor, the SES protein. Studies have shown that in *P. gallinaceum* a cysteine protease of parasite origin, egress cysteine protease-1, plays a role in sporozoite egress from the oocyst (Aly and Matuschewski, 2005). Additionally, a cysteine protease of parasite origin cleaves the CS protein so that sporozoites can invade the liver (Coppi *et al*., 2005). B) Anti-PgSES antibodies inhibited sporozoite invasion of the mosquito salivary glands by 49-87%. The possible functions of the SES protein based on the antibody blocking results are listed in the figure. Figure created by Alexis LaCrue.

CHAPTER 4. THE INITIAL CHARACTERIZATION OF *PFSES*

ABSTRACT

Recently, the Beerntsen lab has characterized the PgSES protein, a ubiquitously expressed sporozoite surface molecule (LaCrue *et al*., 2006). Subsequent experiments suggest that the PgSES protein may play a role in sporozoite invasion of salivary glands (LaCrue *et al*., unpublished data). Studies by Pandey *et al* (2006) investigating the role of the SES protein in the *Plasmodium falciparum* erythrocytic stages showed that the PfSES protein, referred to as falstatin in these studies, is a cysteine protease inhibitor during the erythrocytic stages. These same studies suggest that falstatin may be involved in merozoite invasion of erythrocytes (Pandey *et al*., 2006). In this chapter, we show that not only is PfSES expressed during the asexual stages, but it is also expressed during the salivary gland sporozoite and gametocyte stages. Gene-disruption studies show that the PfSES disruption construct has integrated into the *P. falciparum* genome and subsequent studies will confirm this integration event. After obtaining a clonal line, the mutant parasites will be used in functional studies.

Key words: Malaria; Sporozoite; *Plasmodium falciparum*, transfections

1. Introduction

Protease inhibitors are molecules that regulate the function of peptidases and in protozoa they have been localized to different regions of an organism, including the cell surface, vesicles, and lysosomes (Monteiro *et al*., 2001, Besteiro *et al*., 2004; Riekenberg *et al*., 2005). Cysteine proteases (CPs) have been described in *Plasmodium* and studies have suggested that these proteases may play a role in parasite erythrocyte invasion, hemoglobin hydrolysis, and erythrocyte rupture (Hadley *et al*., 1983; Debrabant and Delplace, 1989; Francis *et al*., 1997, Rosenthal, 2004). The falcipains (FP), a family of papain-family (clan CA) enzymes, are well characterized *Plasmodium* cysteine proteases of which there are four (FP1, FP2, FP2´ and FP3) (Rosenthal, 2004). Gene disruption studies showed that FP1 and FP2´ are not essential to asexual parasite development, FP2 plays a role in hemoglobin hydrolysis (*i.e.* a process by which the parasite obtains essential amino acids for growth), and FP3 is essential for erythrocytic parasite development (Eksi *et al*., 2004; Sijwali *et al*., 2004a; Sijwali *et al*., 2004b; Sijwali *et al*., 2006).

In addition to the falcipains, several other cysteine proteases have been characterized in protozoan parasites, but until recently, the presence of cystatin-like inhibitors had not been demonstrated in lower eukaryotes (Monteiro *et al*., 2001). Monteiro *et al*. (2001) described the first protozoan cysteine protease inhibitor (CPI), known as Chagasin. Chagasin is a *Trypanosoma cruzi* CPI that tightly binds to and inhibits cruzipain, a cysteine protease of trypanosomes (Tomas and Kelly, 1996). Chagasin is expressed in the flagellar pocket and cytoplasmic pockets of trypomastigotes and on the cell surface of amastigotes. Besteiro *et al*. (2004) discovered a *Leishmania*

mexicana inhibitor of cysteine protease (ICP), which is a natural inhibitor of cathepsin-L like cysteine peptidases. ICP appears to play a role in modulating parasite CPs and perhaps in protecting the parasite from host CPs. ICP has a punctate staining pattern and localizes to the vesicles of early-stationary-phase promastigotes with some nuclear and kinetoplast staining. Studies also show that ICP is part of the secretory pathway and destined for the cell surface or lysosomes (Besteiro *et al*., 2004).

Pandey *et al*. (2006) used the Chagasin sequence to search for a homolog in *P. falciparum* and found that Chagasin has weak homology with domain three of a protein that has been referred to either as PfSES or falstatin (LaCrue *et al*., 2006; Pandey *et al*., 2006). Falstatin is 414 amino acids in length and encodes an approximately 47 kilodalton protein with a cleavable signal peptide. PlasmoDB mass spectrometry analysis indicates that falstatin is expressed during the sporozoite and merozoite stages (Florens *et al*., 2002). Kinetic studies investigating the role of the falstatin protein in the erythrocytic stages of *P. falciparum* showed that falstatin inhibits the activity of various cysteine proteases including the previously described falcipains, as well as the human protease calpain-1 (Pandey *et al*., 2006). Also, when anti-falstatin antibodies were incubated with *P. falciparum*-infected erythrocytes, an 80% blocking of new ring formation was observed. These studies suggest that falstatin may play a role in modulating the activity of erythrocytic-stage parasite proteases or host proteases by preventing premature cysteine protease activation, thereby facilitating erythrocytic invasion by free merozoites (Pandey *et al*., 2006).

Recent studies investigating the SES protein emphasize that there is still a significant amount of information that is unknown about the function of this protein and

cysteine protease inhibitors in the malaria parasite, especially the mosquito stage parasites. Therefore, this chapter will focus primarily on the characterization of the SES protein in the sporozoite stage, which is critical for invasion of mosquito salivary glands and vertebrate host tissue. The expression of SES during the erythrocytic stages is also investigated. Ultimately, this research should provide a better understanding about the role of the SES protein in sporozoite development and host tissue infectivity.

2. Materials and Methods

2.1 Mosquito maintenance and the parasite transmission cycle

The *Anopheles stephensi*, used to maintain the transmission cycle, were reared according to the Malaria Research and Reference Reagent Resource Center (MR4) protocols. For *P. falciparum* infections, 4- to 5-day-old mosquitoes were allowed to feed on a blood meal containing cultured *P. falciparum* (NF54 strain) in human blood supplemented with 10% human serum. The infected and control blood meals were fed to the mosquitoes through a 37°C water-jacketed membrane feeder with pig intestine used as a membrane. Mosquitoes were allowed to feed for approximately 20 min to ensure that the majority of insects were engorged. The use of human blood components for these procedures is in compliance with all federal guidelines and institutional policies. All animal care and the experiments described herein were approved by and performed in accordance with guidelines of the National Institutes of Health and the University of Missouri-Columbia Animal Care and Use Committee.

2.2 Parasite strain and culture

The *P. falciparum strain* NF54 was a gift from Shirley Luckhart (University of California at Davis, CA). *P. falciparum* NF54 parasites were cultured in type O^+ human

red blood cells and maintained at 6% hematocrit in complete RPMI 1640 medium supplemented with 25 mM HEPES, 0.5% Albumax (Invitrogen, Carlsbad, California) and 0.005% hypoxanthine (Sigma, St. Louis Missouri). Following a medium change, flasks were filled with a gas mixture (1% O_2, 5% CO_2, and 94% N_2) and maintained at 37°C. For mosquito infections, gametocytes were cultured in 10% human serum in complete culture medium

2.3 DNA isolation and sequence analysis

For all experiments, DNA was isolated from overnight cultures using Qiagen® mini-, midi-, or maxi-prep kits (Promega, Madison Wisconsin). Genomic DNA was isolated using the Qiagen DNeasy Tissue Kit. Sequence of the isolated clones was determined by using the University of Missouri sequencing facility. DNA and protein sequence alignments were done with Vector NTI (Explorer or Contig Express, Invitrogen). Microarray expression data was obtained from the *Plasmodium* genome database (PlasmoDB) (Kissinger *et al*., 2002).

2.4 Transcriptional analysis by reverse transcription PCR (RT-PCR)

To determine if *PfSES* transcripts were present in mosquito stage parasites (*i.e.*, sporozoites) and erythrocytic stage parasites, total RNA was isolated from *P. falciparum*-infected mosquito tissues day 10 post-exposure (PE) to an infected bloodmeal when sporozoites are present in the mosquito and from *P. falciparum*-infected red blood cells, respectively. The RNA was DNase-treated and the RT-PCR reactions were performed as described previously (LaCrue *et al*., 2005). The PfSES primers used for the asexual blood stage RT-PCR were forward, 5'-CGCGGATCCTATCGTGCATAGTCCATCTTTC-3' and reverse, 5'-AGTGCGGCCGCTTATTTTCTTCTGTAGAATTATTTAC-3'. Primers

used for the mosquito stage parasite RT-PCR were Forward 5'-GGATCCTAGATAATAACAGCTACTCATTTG-3' and Reverse 5'-GCGGCCGCTTATTTGATCACATGATTCATTGG-3'; *Bam*HI and *Not*I restriction sites are underlined. *P. falciparum* genomic DNA was used as a positive control to show the difference in size between the intron-containing *PfSES* genomic DNA and the cDNA transcript.

2.5 Recombinant protein expression, purification, and antibody production

To produce polyclonal antibodies for Western blot analysis and immuno-localization studies, recombinant PfSES fusion protein (rPfSES) was generated using the thioredoxin, HIS-tag containing pET32a expression vector (Novagen, San Diego California). A fragment, representing amino acids 23-414, was amplified using the following primers: forward 5'-GATCGGATCCGATAATAACAGCTACTCATTTG-3' and reverse, 5'-GATCCTCGAGTTATTGCACGTTTAACTCTAC-3'. *Bam*HI and *Xho*I restriction sites are underlined. *P. falciparum* (3D7A) genomic DNA (40 ng) was used as a template in a 50 µl PCR amplification as previously described (LaCrue *et al*., 2005) with the difference being that Vent polymerase (1U) (New England Biolabs, Ipswich Massachusetts and 0.2 mM $MgSO_4$ were used. The amplification consisted of 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 62°C for 1 minute 30 seconds with a final extension temperature of 62°C for 10 minutes. The amplification product was digested with *Bam*HI and *Xho*I following the manufacturer's protocol (New England Biolabs) and ligated into the double-digested pET32a expression vector followed by transformation into Rosetta-gami DE3 pLysS competent cells (Novagen). The PfSES fragment then was expressed as a 6xHis fusion protein. Purification of the rPfSES-fusion

protein was done via a His-Bind Kit protocol (Novagen) according to procedures in LaCrue *et al.* (2005) with the exception that the rPfSES protein was eluted with 100 mM EDTA. The purified rPfSES protein was used to produce rabbit polyclonal antibodies using previously described methods (LaCrue *et al*., 2005).

2.6 Immunoblotting

To determine if the native PfSES protein could be detected during the erythrocytic stages, a Western blot analysis was performed on sorbitol-synchronized parasites. Synchronization of the asexual stages was performed by three rounds of sorbitol treatment as described previously (Miao *et al*., 2006). Parasites were collected 2, 10, 18, 26, and 32 h post-sorbitol treatment to represent rings, early and late trophozoites, and early and late schizonts. Day 14 gametocytes also were collected. Infected erythrocytes were treated with 0.05% saponin to lyse the red blood cell membrane and the released parasites were pelleted at 1600 x g for 5 min and washed twice with 1x PBS. The lysed parasites were resuspended in reducing SDS sample buffer and separated by 10% SDS-PAGE with each lane containing equal numbers of parasites. The proteins were transferred to a membrane for 1 h and then the membrane was incubated in 5% non-fat dry milk for 1 h at room temperature. The membrane then was washed three times in TBS and incubated overnight with anti-PfSES antibodies (1:500) preabsorbed against non-infected red blood cells and naïve mosquito tissues according to previously described protocols (LaCrue *et al*., 2005). Following the overnight incubation, the membrane was washed, incubated with a secondary antibody, washed, and detected using previously described methods (LaCrue *et al*., 2005). After the membrane was exposed to film, it was stripped with NaOH (0.1 M) for 5 min, washed with TBS, blocked for 1 h, and reprobed

with anti-MSP1-19 (1:3000) to confirm the presence of parasite proteins (Blackman *et al*., 1991).

2.7 Confocal microscopy

To confirm the immunoblot analysis and determine where the PfSES protein was expressed in the sporozoite and erythrocytic stages, immuno-localization studies were performed according to previously described methods (LaCrue *et al*., 2006) with the only difference being that the fixed erythrocytic stage parasites were incubated with the primary antibody overnight and the secondary antibody for 4 h. The following antibodies were used at a 1:200 dilution: 1) preabsorbed rabbit anti-PfSES serum, 2) positive control antibodies, mouse anti-MSP-1 for the asexual stages and anti-Pfs230 for the gametocyte stages (Williamson *et al*., 1993), and 3) goat anti-rabbit Alexa-488 and goat anti-mouse Alexa-546 secondary antibodies. The nuclear stain, TOPRO-3, was used at a 1:50 dilution.

2.8 Construction of transfection plasmids

To construct the PfSES/falstatin gene disruption plasmid, a 702 bp DNA fragment of the *PfSES* gene was PCR-amplified from NF54 genomic DNA using FastStart high fidelity Taq DNA polymerase (2.5U) (Roche, Indianapolis Indiana), 3mM $MgCl_2$, and PCR parameters described in the recombinant protein section. The following primers were used: Forward 5'-GGATCCTAGATAATAACAGCTACTCATTTG-3' and Reverse 5'-GCGGCCGCTTATTTGATCACATGATTCATTGG-3'; *Bam*HI and *Not*I restriction sites are underlined. The PCR product was digested with *Bam*HI and *Not*I and cloned into the *Bam*HI and *Not*I digested pcamBSD to obtain pcamBSD-ΔPfSES. The pcamBSD plasmid expresses blasticidin S deaminase (*bsd*) (Mamoun *et al*., 1999) under

the control of a 0.6 kb *P. falciparum* calmodulin promoter (Crabb and Cowman, 1996). The transfection plasmid pcamBSD was a gift from David Fidock (Albert Einstein College of Medicine, NY).

2.9 Transfections

Prior to transfection, parasite cultures were synchronized with 5% D-sorbitol for 10 minutes followed by two washes with RPMI 1640 at 1600 x g for 5 min. Blasticidin S hydrochloride (2.5 μg/ml) (Invitrogen) was used to select for growth of transfected parasites carrying the resistance gene. D-sorbitol synchronized ring stage NF54 parasites were electroporated (BTX 600; 0.2 cm cuvette, 0.31 kV, 950 μF, maximum resistance) with 100 μg of pcamBSD-ΔPfSES plasmid DNA. Two days following electroporation, media containing blasticidin S hydrochloride (2.5 μg /ml) was added to the culture to select for transfected parasites. To enrich for recombinants and eliminate episomal plasmids, parasites were subjected to three rounds of growth (3 weeks each round) with and without blasticidin S hydrochloride (2.5 to 5.0 μg/ml).

3. Results and Discussion

3.1 Transcriptional analysis by reverse transcription PCR (RT-PCR)

The *PfSES* transcripts are present in both the *P. falciparum*-infected mosquitoes on Day 10 PE to an infected bloodmeal when oocyst, hemolymph, and salivary gland sporozoites are likely present (Figure 4.1A). Transcriptional studies assessing *P. falciparum*-infected red blood cells show that the *PfSES* transcripts also were present during the erythrocytic stages (Figures 4.1 B). Future studies include assessing the *PfSES* transcript profile using parasites isolated from the individual parasite stages in the mosquito or RBC.

3.2 Immuno-blot and immuno-localization studies with erythrocytic stage parasites

The native PfSES protein (~47 kDa) was present in the sorbitol-synchronized parasites during all of the collected time points representing ring, trophozoite, schizont, and gametocyte stage parasites (Figure 4.2 A). These data were similar to those reported in Pandey *et al*. (2006) with the exception that proteins were seen during the trophozoite stage (time-points 10-18). As expected, the antibodies did not recognize any proteins in the negative control sample, consisting of non-infected red blood cells. When the blot was stripped and re-probed with anti-MSP1-19, there was parasite protein present in all of the samples (Figure 4.2 B).

Subsequent confocal microscopy of parasites, prepared from the previously described time-points, confirmed the RT-PCR and Western blot findings, showing that the PfSES protein is expressed during the ring, schizont, and gametocyte stages (Fig. 4.3). The PfSES protein appears to localize to the parasite periphery during the ring and schizont stages. It also was noted that there was very little PfSES protein expression during the trophozoite stage (data not shown) which is in contrast to the time-line western. It is possible that even though the cultures were synchronized that there were small amounts of other parasite stages, such as the ring stage, whose proteins were detected by the anti-PfSES antibodies. These data are consistent with findings for falstatin expression during the asexual stages (Pandey *et al*., 2006), as well as the PlasmoDB mass spectrometry data which indicates expression of PfSES during the merozoite stage (Florens *et al*., 2002). In contrast to the falstatin studies, this study investigated the expression of PfSES in the gametocyte stages and found that *PfSES* appears on the surface and in the cytoplasm of stage III and V gametocytes. There also

appears to be some co-localization between PfSES and the hemozoin (malaria pigment). Combined, the asexual and gametocyte stage protein expression profile for PfSES is almost identical to its *P. gallinaceum* homolog, PgSES (LaCrue *et al*., 2006).

3.3 Immuno-localization studies with sporozoites

Immuno-fluorescence studies using fixed and air-dried sporozoites revealed that the PfSES protein is expressed during the salivary gland sporozoite stage and it appears to have some co-localization with the CS protein, as indicated by a yellow color (Figure 4.4). The partial co-localization of SES with CS suggests that it may be labeling the surface of the sporozoite. The sporozoite labeling pattern is reminiscent of that observed for PgSES and it suggests that PfSES may be labeling the surface of the sporozoite (Figure 4.5); however, replicates of this study must be conducted to confirm these findings (LaCrue *et al*., 2006). These results confirm the mass spectromentry analysis showing expression for PfSES in salivary glands sporozoites (Florens *et al*., 2002). Currently, there is no mass spectrometry data available for the oocyst and hemolymph sporozoite stages.

3.4 Transfections

To disrupt the *PfSES* gene, *P. falciparum*-infected red blood cells were transfected with the pcamBSD-ΔPfSES plasmid that is designed to integrate into the SES chromosomal locus via single crossover homologous recombination (Figure 4.6A). Transfected parasites were selected with blasticidin S hydrochloride until integration was detected, and then recombinants were enriched for through growth with and without blasticidin. Using primers specific for recombinant parasites, PCR analysis shows that the pcamBSD-ΔPfSES plasmid has integrated into the chromosomal locus (Figure 4.6B).

The *PfSES* mutant parasites are on their third round of drug selection and are continuing to grow and be detected via PCR analysis. The next step is to perform a Southern blot analysis to confirm the integration event, obtain pure recombinant parasites via dilution cloning and verify the presence of a single clone via Southern blot and RT-PCR analysis. Immuno-blotting will further confirm the absence of the PfSES protein in disrupted parasites. If the clone continues to propogate, then it can be concluded that *PfSES* is not lethal to asexual stage parasites, although it may be important for a particular process. For example, parasites with a disrupted falcipain-2 gene produce parasites that continue to develop into mature stage parasites that appeared to be normal and multiply like wild-type parasites; however, the trophozoites ability to perform hemoglobin hydrolysis was impaired (Sijwali *et al*., 2006). In those studies, it also was noted that the FP2-knockout parasites appear to be more sensitive to protease inhibitors.

In summary, *PfSES*/falstatin is a cysteine protease inhibitor that is expressed during multiple parasite stages. During the salivary gland sporozoite stage, PfSES exhibits a labeling pattern similar to its *P. gallinaceum* homolog, PgSES, and future studies include determining the expression profile of PfSES during the oocyst and hemolymph sporozoite stages. For the disruption studies, once a *PfSES* mutant clone is obtained and is shown not to be lethal to asexual stage parasites, the next step will be to produce gametocytes for infecting mosquitoes and determining the role of *PfSES* during the mosquito stages.

Note/Acknowledgment

The research reported in this chapter is the foundation for a paper to be submitted at a later date. The authors for this paper will be: LaCrue AN, Kariuki M, Ou R, and Beerntsen B.

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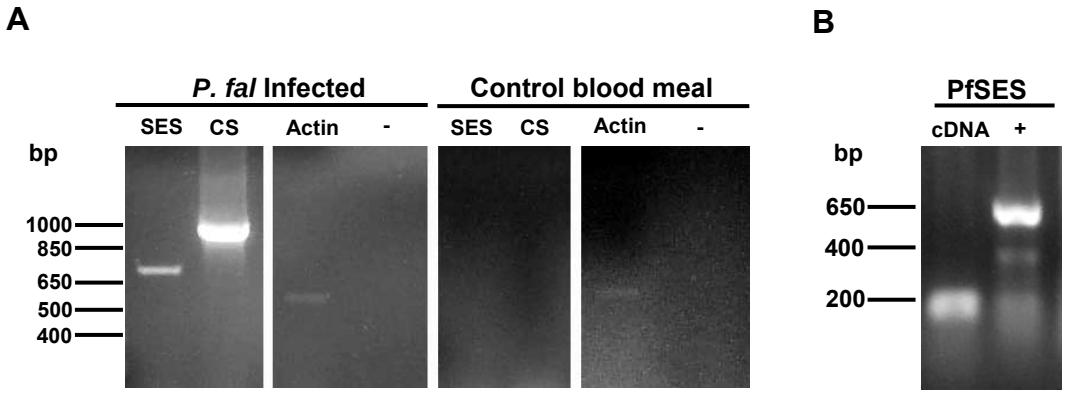

Figure 4.1. Reverse-transcription PCR (RT-PCR) analysis of *PfSES*. A) *PfSES* transcripts are present in *P. falciparum*-infected (*P.fal*) *An. stephensi* day 10 post-exposure (PE), when sporozoites are present, as determined by RT-PCR analysis. The gene-specific primers used for the PCR analysis are listed above each lane. As expected, a no RT control did not amplify genomic DNA (-). As expected, cDNA from mosquitoes exposed to a control blood meal did not amplify a product with either *PfSES* or *CS* gene-specific primers. Primers specific for the circumsporozoite (CS) gene were used as a positive control to show the presence of parasite RNA in the infected samples. Mosquito muscle actin (Actin) was used to show the presence of RNA in the *P. falciparum*-infected and control blood meal samples. B) *PfSES* transcripts are present during the erythrocytic stages as shown by RT-PCR analysis of RNA isolated from *P. falciparum*-infected red blood cells. The amplifications were performed with *PfSES* gene-specific primers. *P. falciparum* genomic DNA was used as a positive control (+). A no RT control was performed (data not shown).

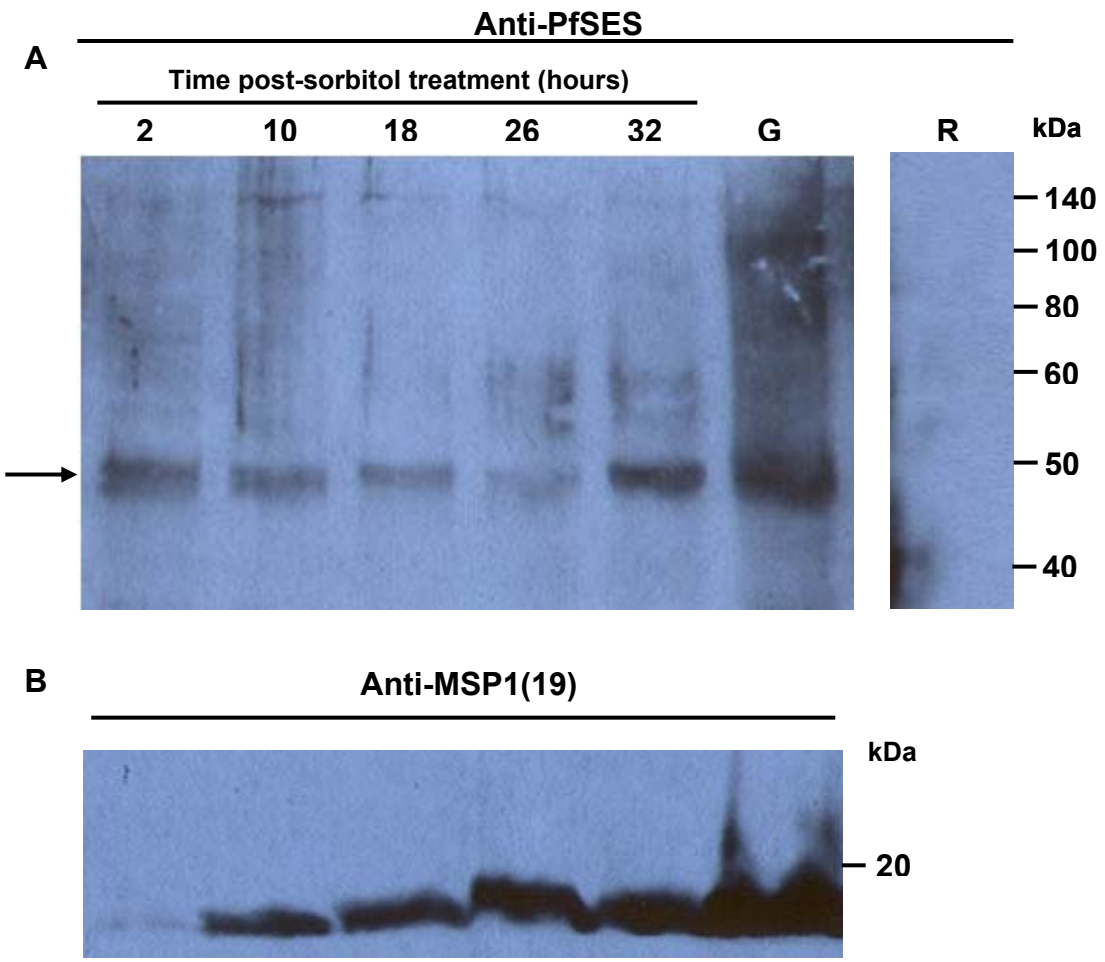

Figure 4.2. The native PfSES protein is present in the erythrocytic stages as shown by Western blot analysis. Anti-PfSES antibodies recognize the ~47 kDa SES protein (arrow) in sorbitol-synchronized *P. falciparum* parasites collected at time periods representing primarily the parasite ring (2), early and late trophozoite (10-18), early and late schizont (26-32) and also day 14 gametocyte (G) stages. Parasites were resuspended in reducing sample buffer and analyzed on a 10% SDS-polyacrylamide gel. Non-infected RBCs were included as a negative control (R). B) The membrane was stripped and reprobed with anti-MSP1-19 antibodies to confirm the presence of parasite proteins in the samples. Molecular weight markers in kilodaltons (kDa) are shown to the right of the

figure. As expected, the pre-immune serum did not recognize proteins in any of the samples (data not shown).

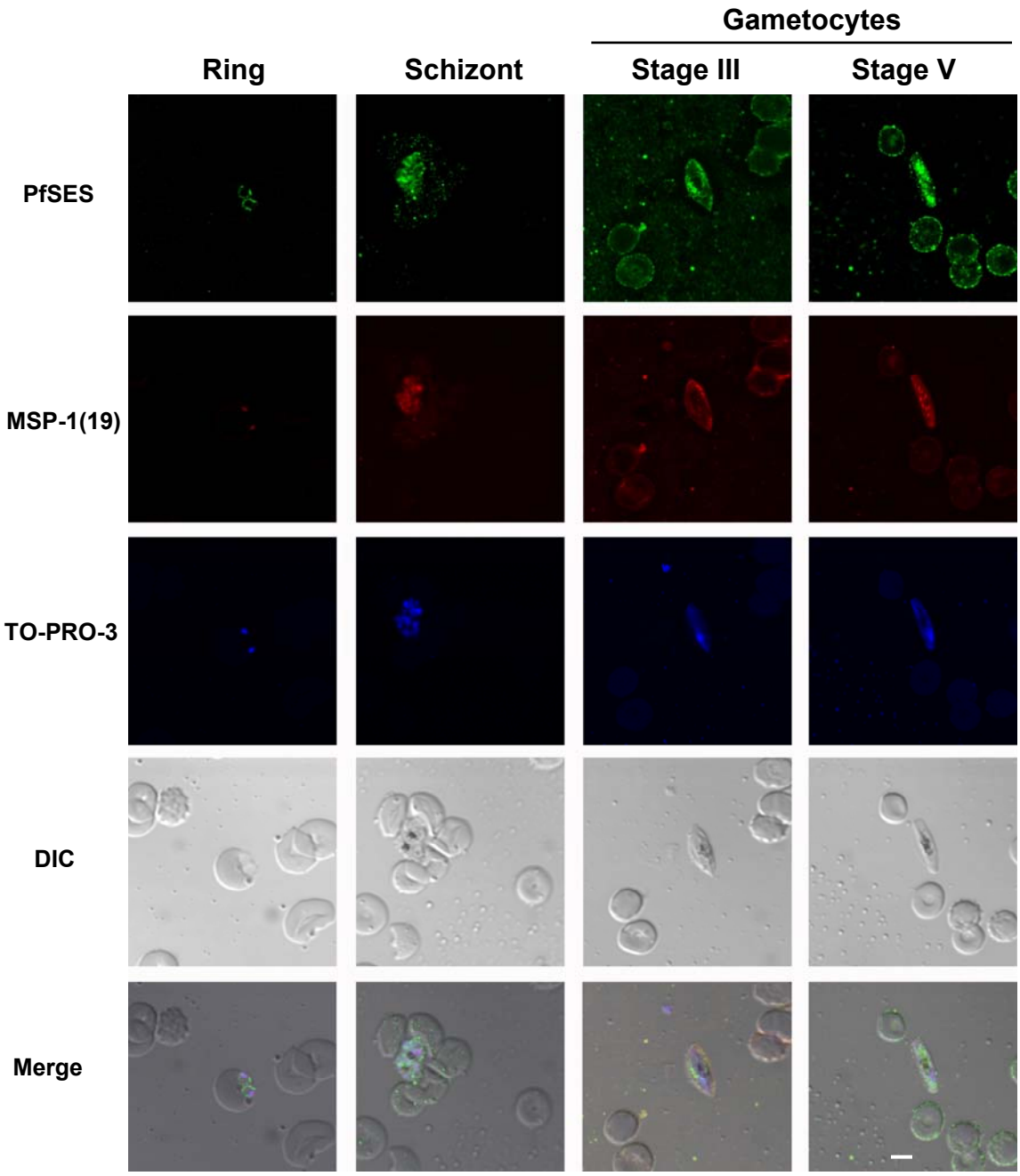

Figure 4.3. The PfSES protein is expressed in the erythrocytic stages as shown by confocal microscopy. The PfSES protein is expressed during the ring, schizont, and gametocyte stages. *Plasmodium falciparum*-infected red blood cells (RBC) were triple-

labeled with rabbit anti-PfSES (green) and mouse anti-MSP1-19 antibodies (red), followed by the nuclear stain TO-PRO-3 (blue). PfSES localizes to the periphery of the ring and schizont stage. During the gametocyte stage, PfSES appears to co-localize with the gametocyte specific anti-Pfs230 antibody (yellow color) and the hemozoin in Stage V gametocytes. The confocal image was merged with the Normarski/DIC image to show localization of the PfSES protein during the different parasite stages in the RBC. Scale bar = 5μm.

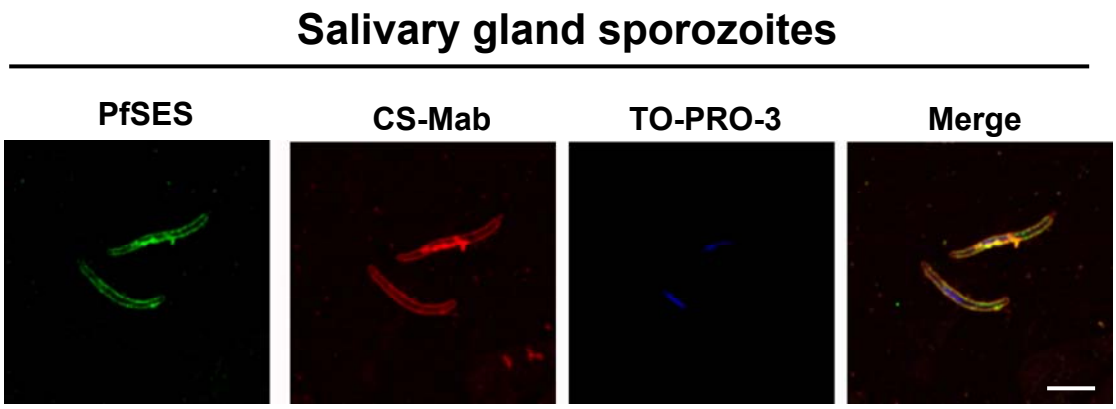

Figure 4.4. The PfSES protein is expressed during the salivary gland sporozoite stage as shown by confocal microscopy of fixed parasites. *P. falciparum* salivary gland sporozoites were triple-labeled with rabbit anti-PfSES and mouse anti-circumsporozoite (CS) protein antibodies, and the nuclear stain TO-PRO-3 (blue). Goat anti-rabbit-Alexa-488 (green) and goat anti-mouse Alexa-546 (red) were used as the secondary antibodies. The rabbit pre-immune serum showed no cross-reactivity with parasites. Co-localization between the PfSES and CS proteins is represented by a yellow color, suggesting surface localization of PfSES. Scale bar = 5 μm.

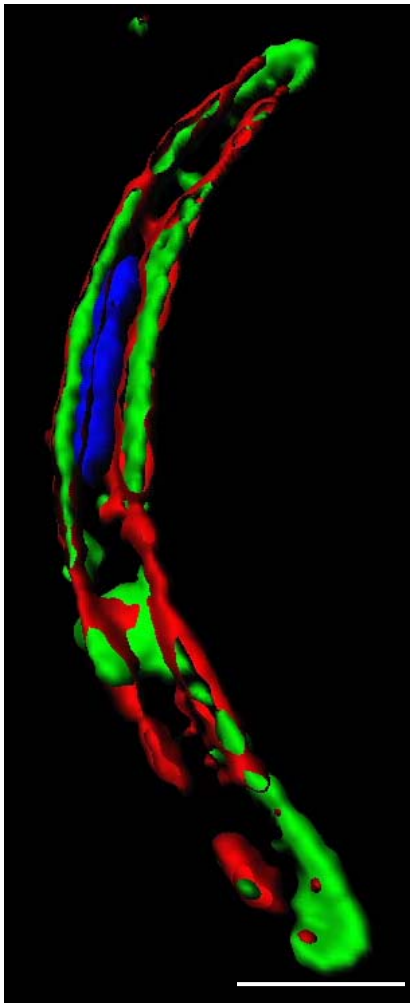

Figure 4.5. PfSES appears to localize to the surface of the salivary gland sporozoite as shown by confocal microscopy of non-air dried parasites. *P. falciparum* salivary gland sporozoites were triple-labeled with rabbit anti-PfSES and mouse anti-circumsporozoite (CS) protein antibodies and the nuclear stain TO-PRO-3 (blue). Confocal raw data were first 3D reconstructed and then deconvolved and isosurface volume rendered. Labeling of PfSES (green) appears to be in a similar location as the CS protein (red), suggesting that PfSES is expressed on the surface of the sporozoite. Scale bar = 2 μM.

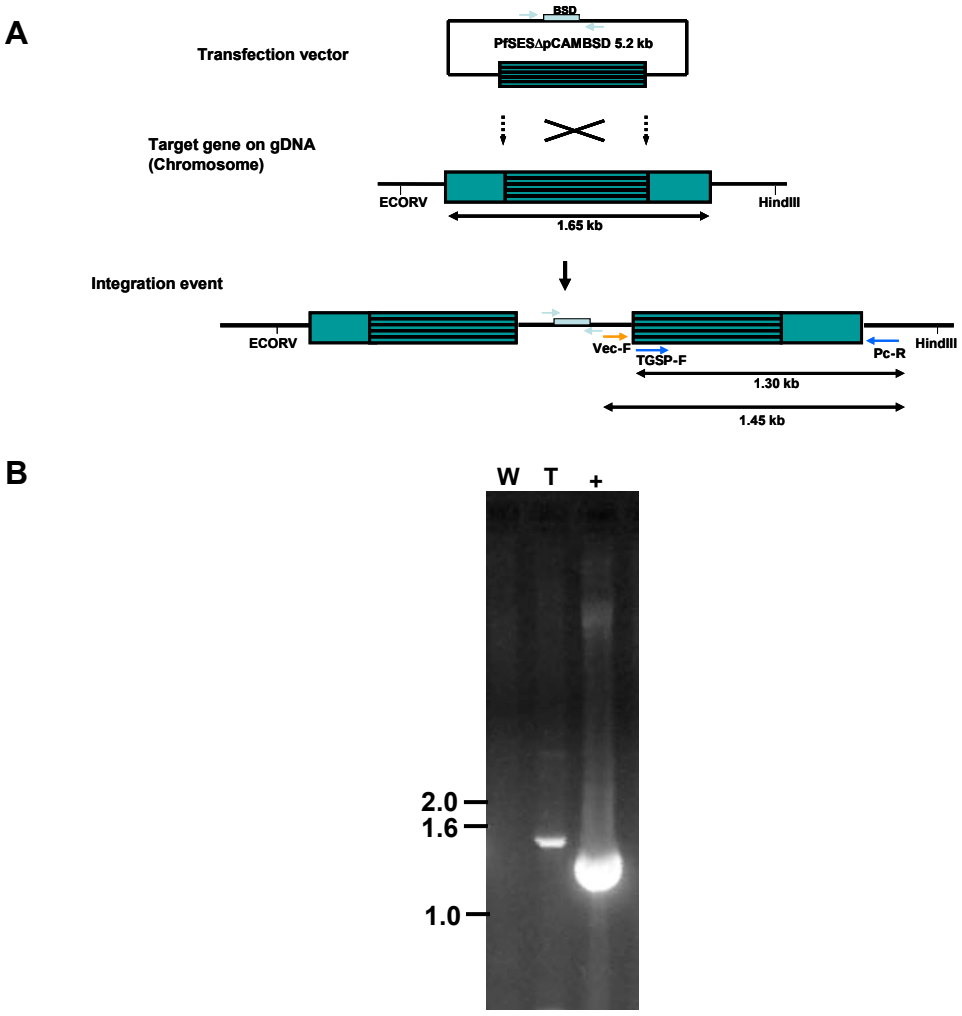

Figure 4.6. Disruption of the *PfSES/Falstatin* gene (PFI0580c). A) The pcamBSDΔ*PfSES* plasmid is shown with the blasticidin S-deaminase (*bsd*) drug selectable marker and the 702 bp PfSES fragment (striped box). The *bsd* cassette is inserted into the *PfSES* gene by a single cross over homologous recombination event, resulting in a disruption of the chromosomal *PfSES* gene. Relevant restriction enzyme sites are shown. B) The *PfSES* gene has been disrupted by the integration of the pcamBSDΔPfSES plasmid into the *PfSES* chromosomal locus (T) as shown by PCR analysis. As expected, the wild-type genomic DNA did not yield a product with integration specific primers (W). The positive control using wild-type genomic DNA and

gene-specific primers produced a product (+). PCR products were amplified using primers specific for wild-type: transfection gene-specific forward (TGSP-F) and chromosomal reverse (PcR) and integration: vector forward (VF) and chromosomal reverse primer (Pc-R).

CHAPTER 5. DISCUSSION

This dissertation describes the initial characterization of the SES protein, including expression during parasite development in the mosquito and its apparent role in salivary gland invasion. In *P. gallinaceum*, SES is expressed by some hemolymph sporozoites and on the surface of salivary gland sporozoites on which it has a unique spiral labeling pattern that surrounds the well-characterized sporozoite surface molecule, the CS protein (Chapter 2). Sporozoite surface molecules, such as the CS protein, have been shown to play a role in parasite development and host tissue invasion via gene-disruption and antibody blocking studies (Warburg *et al*., 1992, Ramirez *et al*., 1995; Menard *et al*., 1997). Gene-disruption studies are not possible in *P. gallinaceum;* therefore, antibody blocking studies to ascertain the function of PgSES were performed. Those studies suggested that SES may play a role in host tissue invasion (Chapter 3).

Interestingly, the *P. falciparum* homolog, PfSES/Falstatin, is a cysteine protease inhibitor during the erythrocytic stages (Pandey *et al*., 2006). In those studies, anti-PfSES antibodies inhibited new ring formation by ~80%, suggesting a possible role for SES/Falstatin in merozoite invasion (Pandey *et al*., 2006). As mentioned in Chapters 3 and 4, Falstatin most likely regulates the well-characterized *Plasmodium* cysteine proteases, the falcipains, so that the limited proteolysis by serine proteases that is required for parasite invasion of erythrocytes can occur. Additionally, Falstatin also inhibits host cysteine proteases, such as cathepsin L and calpain-1, most likely so that merozoites can invade the erythrocytes and continue their development. Based on antibody blocking results from both the PfSES/Falstatin and PgSES studies, it is possible that within the mosquito, SES may work to protect the parasite from the untimely action

of parasite and host proteases so that sporozoites can safely and successfully invade the salivary glands. To determine the exact role of SES in mosquito stage parasite development and host tissue invasion, gene-disruption studies, which are possible in *P. falciparum*, are currently underway (Chapter 4).

Malaria ookinete, sporozoite, and merozoite invasion of host tissue appears to be a well-choreographed process that involves several parasite molecules (Table 5.1) and their recognition of specific host molecules. Parasite proteases, such as the falcipains, appear to play key roles in parasite development and, as mentioned in Chapter 3, cysteine proteases of parasite origin play a role in sporozoite egress from the oocyst (*i.e.,* Egress cysteine protease-1) and the cleavage of CS so that sporozoites can invade the liver (Aly and Matuschewski, 2005; Coppi *et al*., 2005).

Additionally, studies by Hillyer *et al*. (2007) showed that a small percentage of *P. gallinaceum* sporozoites (~20%) released from the oocyst actually invade the salivary glands within a limited period of time (8 hours) after which no additional sporozoites invade, but instead become targeted by the host immune response (*i.e.,* phagocytosis, melanization, and apoptosis). In Chapter 2, it was shown that ~ 60% of *P. gallinaceum* hemolymph sporozoites express the SES protein; therefore, a possible role of SES as a cysteine protease inhibitor in the mosquito may be to regulate mosquito or parasite proteases in an effort to prevent the disruption of important motility and host binding molecules, such as TRAP and CS, respectively (Warburg *et al*., 1992; Ramirez *et al*., 1995; Sultan *et al*., 1997; Hillyer *et al*., 2007). By preventing the disruption of important molecules by the mosquito immune response, the sporozoite would be able to migrate

through the hemocoel, invade the salivary glands and then the vertebrate host tissue within a narrow window of time (Figure 3.3A, Chapter 3).

Future studies include using an *in vivo* model system to confirm that SES plays a role in *Plasmodium* infection in the vertebrate host. For this study, chickens would be injected with *P. gallinaceum* sporozoites incubated with anti-PgSES antibodies. If the anti-PgSES injected chickens fail to develop an infection or have a significantly lower infection as compared to the preimmune-injected chickens, it can be assumed that the SES protein plays a role in parasite invasion of vertebrate host tissues. Additional studies include obtaining a PfSES-disrupted clone via limiting dilution, propagating the mutant, using it to infect mosquitoes, and then determining the role of SES in mosquito stage parasite development and host tissue invasion. If gene-disrupted parasites fail to develop during the gametocyte stage, within the mosquito at the gamete or ookinete stages or are arrested during the oocyst sporozoite stage, then it can be assumed that the SES protein is critical for parasite development within the mosquito. If oocyst sporozoites develop, are released, and subsequently fail to invade the glands, then a role for SES in sporozoite invasion of mosquito salivary glands will be confirmed. It is also possible that the dirsrupted parasites will develop normally and complete their life-cycle within the mosquito. If this happens, then the ability of the mutant salivary gland sporozoites to invade HepG2 cells *in vitro* will be assessed.

Kinetic studies have shown that PfSES interacts readily with native falcipain-2 and -3 and weakly with falcipain-1 (Pandey *et al*., 2006). According to PlasmoDB, all three proteases have transcripts present during the *P. falciparum* salivary gland sporozoite stage, but only falcipain-1 has been shown to have protein present via mass

spectrometry analysis (Rosenthal, 2004). It is possible that the falcipain 2 and 3 proteins are expressed by salivary gland sporozoites, but the levels are too low to be detected via mass spectrometry. Western blot analysis could be used to determine if the falcipains are expressed during the sporozoite stage, and confocal microscopy could then confirm the Western analysis findings as well as determine where in or on the sporozoite the falcipains might be expressed.

The *P. gallinaceum* SES protein appears to play a role in sporozoite invasion of *Ae. aegypti* salivary glands; therefore protein-protein interaction studies could be performed to determine the interacting partners and further define the mechanism by which SES facilitates sporozoite invasion. The protein-protein interaction studies could be accomplished via a FAR Western or immuno-precipitation assay using antibodies against known molecules such as the CS protein, falcipains, and apoptosis molecules (*e.g.*, caspases, calpains, and metacaspases) first and then progressing to finding unknown parasite or mosquito binding partners. Using x-ray crystallography to obtain the structure of SES would help determine how SES interacts with other proteins and it could be beneficial for designing a drug to target the SES protein.

The SES/Falstatin protein is a *Plasmodium* cysteine protease inhibitor of parasite origin that appears to play a role in erythrocyte invasion by merozoites and salivary gland invasion by sporozoites in *P. falciparum* and *P. gallinaceum*, respectively. If through gene-disruption studies, which are currently in progress, SES is found to be critical for both salivary gland and/or vertebrate host tissue invasion, it would be an excellent target for drugs designed to target the SES molecule in the erythrocytic stages or vaccines designed to block its action in the erythrocytic and mosquito stage parasites.

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Ookinete molecules	Location	Mode of action	Antibody blocking	Knock-out defects	Vaccine candidate/ type
CTRP (Ref: 7,14,19,32)	Micronemes; apical region	Ookinete transformation to oocyst	Reduced oocyst formation 78-88%	No oocyst formation	Yes; transmission blocking
P25/P28 (Ref: 34,35)	Surface	Ookinete transformation to oocyst	n.d.	Reduced oocyst formation	Yes; transmission blocking
SOAP (Ref: 8)	Micronemes	Ookinete transformation to oocyst	n.d.	Impaired ability to invade mosquito midgut	n.d.
MAOP (Ref: 13)	Micronemes	Ookinete invasion of midgut	n.d.	Impaired ability to invade mosquito midgut	n.d.

Sporozoite molecules	Location	Mode of action	Antibody blocking	Knock-out defects	Vaccine candidate/ type
CS (Ref: 26,36,37)	Inner oocyst membrane; surface of sporozoite	Sporozoite invasion of mosquito salivary glands	Prevent sporozoite invasion of salivary glands by 99.0%	Sporozoites fail to develop within oocysts	Yes; block sporozoite invasion of liver
ECP1 (Ref: 1)	Upregulated in oocyst sporozoites	Egress of sporozoites from the oocyst	n.d.	Sporozoites fail to egress from oocysts	n.d.
MAEBL (Ref: 15)	Micronemes of oocyst sporozoites; some in salivary gland sporozoites	Sporozoite attachment to salivary glands	Block sporozoite invasion of HepG2 cell and primary hepatocytes	Impaired sporozoite invasion of salivary glands	n.d.
Pfs16 (Ref: 17,22)	Sexual stage and sporozoite	Sporozoite invasion of liver	Block sporozoite invasion of HepG2 cell and primary hepatocytes	Reduction in gametocyte production	Yes; transmission blocking
PfCCP1 (Ref: 25)	Plasma membrane of mature gametocytes	Sporozoite release from oocyst?	n.d.	Block in sporozoite transition to salivary glands	n.d.
PxSR (Ref: 5)	Sporozoites	Oocyst sporozoite development	n.d.	Sporozoites fail to develop within oocysts	n.d.
TRAP/SSP2 (Ref: 27,28,31)	Micronemes and secretory vesicles of apical complex	Sporozoite motility and invasion	n.d.	Impaired gliding motility; poor invasion of host tissues	Yes; block sporozoite invasion of liver

Liver stage molecules	Location	Mode of action	Antibody blocking	Knock-out defects	Vaccine candidate/ type
LSA-1 (Ref: 3)	Parasitophorous vacuole (PV) of intrahepatic trophozoites and schizonts	Impair host immune response upon hepatocyte rupture	Prevent liver-stage development	n.d.	Yes
LSA-3 (Ref: 24)	Sporozoite suface; PV of intrahepatic trophozoites and schizonts	Impair host immune response upon hepatocyte rupture	Prevent liver-stage development	n.d.	Yes
SALSA (Ref: 2)	Sporozoite membrane surface; granular material of parasitophorous vacuole (PV) membrane of intrahepatic sporozoites	Sporozoite invasion of hepatocytes	Prevent sporozoite invasion of HepG2 cells	n.d.	n.d.
STARP (Ref: 9)	Sporozoite surface; intrahepatic sporozoites ; early ring stages	Sporozoite invasion of hepatocytes	Prevent sporozoite invasion of human hepatocytes by 48-90%	n.d.	n.d.

Merozoite molecules	Location	Mode of action	Antibody blocking	Knock-out defects	Vaccine candidate/ type
AMA-1 (Ref: 21)	Apical complex of merozoites	Merozoite invasion of erythrocytes	Inhibit merozoite invasion of erythrocytes	n.d.	Yes
EMP-1 (Ref: 4,10)	Surface of infected erythrocytes 18 h after merozoite invasion	Bind to host endothelial receptors; plays a role in placental adhesion	Prevent sequestration of *P. falciparum*-infected erythrocytes	n.d.	Yes
GLURP-1 (Ref: 33)	PV of intrahepatic sporozoites; merozoite surface; mature schizonts	Parasite development	Promote monocyte-dependent inhibition of *P. falciparum* growth	n.d.	Yes
MSP-1 (Ref: 16,30)	Membrane of early rings; surface of merozoites	Merozoite invasion of erythrocytes	Inhibit merozoite invasion of erythrocytes	n.d.	Yes

Table 5.1 *Plasmodium* parasite molecules referred to throughout the text and their involvement in different aspects of host tissue invasion. No current data = n.d. and References = Ref.

VITAE

Alexis Nichole LaCrue was born in Temple Hills, Maryland and grew up traveling a lot because her mom was in the military. Alexis graduated from East High School in Cheyenne, Wyoming and then went on to pursue an undergraduate degree at New Mexico State University in Las Cruces, New Mexico. She earned Bachelor's degrees in Animal Science and Biology with a minor in human biology and graduated with honors in May of 2000. In August of 2000, she began her pursuit of a graduate degree as the first graduate student for Brenda T. Beerntsen, who at the time was a newly hired faculty member. The first year of graduate school involved helping to set-up the new lab and starting on a Master's project. She received her Master's degree in Veterinary Biomedical Science in May of 2003 and enjoyed the research so much that she decided to stay in the Beerntsen lab, work on a different project and pursue a Ph.D. in the Area Program in Pathobiology. In December of 2007, Alexis received her Doctor of Philosophy degree.