INSIGHTS TO UNDERSTANDING MALARIA PARASITE BIOLOGY: CHARACTERIZATION OF THE *PLASMODIUM* PROTEIN, MAL13P1.319

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By

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INSIGHTS TO UNDERSTANDING MALARIA PARASITE BIOLOGY: CHARACTERIZATION OF THE *PLASMODIUM* PROTEIN, MAL13P1.319

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DEDICATIONS

I dedicate this dissertation to the best mentors of my life, who always believed in me and loved me unconditionally: Dr. Denzil Roberts, my family, Dr. Fatma Helmy, the late Dr. Jocelyn Spragg, and Dr. Teresa Singleton. I also dedicate this dissertation to the people who always strive to win the game that life deals you and believe that God is in everything we do.

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I can do all things through Christ which strengtheneth me. Phillipians 4:13

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ABSTRACT

Malaria is a mosquito-borne disease responsible for approximately 250 million human infections and about a million deaths annually, with most deaths occurring in African children. Primarily endemic in third world countries, efforts to control malaria are increasingly demanding because of drug-resistant parasites, insecticide-resistant mosquitoes and economic instability. Therefore, the research and development of additional malaria control methods are crucial. In humans, malaria is caused by five species of the protozoan parasite *Plasmodium*, with *P. falciparum* the most pathogenic and deadliest. During parasite development, there are many critical stages that are essential for host-parasite interactions such as the erythrocytic stages that contribute to causing disease in the host and the sporozoite stage that infects vertebrate and mosquito host tissue. Surface antigens and secreted proteins from these essential stages are likely to be involved in infectivity and invasion of host tissues and therefore can be effective targets for control by vaccines, drug therapy, or novel mosquito control methods. However, many of these proteins have yet to be characterized.

In an effort to discover molecules that aid in parasite invasion (specifically during the sporozoite stage), a *P. falciparum* sporozoite gene known as MAL13P1.319 was identified by a search of the annotated *Plasmodium* genome database (PlasmoDB) using specific criteria, i.e., presence of a signal peptide sequence, expression as a sporozoite protein as shown by mass spectrometry

and predicted to be a surface or secreted protein. The *P. falciparum* MAL13P1.319 (PfMAL13P1.319) protein demonstrates significant similarity with orthologs in other *Plasmodium* spp. and appears to be unique to Apicomplexans (i.e., a group of parasitic protozoa that use an apical complex to invade host cells). The PfMAL13P1.319 transcript expression profile demonstrated transcript expression during the erythrocytic stages, oocyst sporozoites, and salivary gland sporozoites via DNA microarray analyses and reverse-transcriptase PCR (RT-PCR). To assess if transcript presence correlated with protein expression, immunfluorescence assays and western blot analysis were performed and results demonstrated that the PfMAL13P1.319 protein was present during the erythrocytic stages; however there was minimal to no expression observed in the sporozoite stages. Additionally, PfMAL13P1.319-GFP trafficking studies confirmed PfMAL13P1.319 protein expression during erythrocytic stages with no expression during the mosquito stages (i.e., ookinetes/zygotes, hemolymph sporozoites, oocyst sporozoites and salivary gland sporozoites).

In determining the functional role of PfMAL13P1.319, multiple attempts at disrupting the gene failed to produce a clonal population, thereby suggesting that the PfMAL131.319 protein may have an important function for intraerythrocytic parasites. A comparative study of the *P. berghei* ortholog of MAL13P1.319 (PbMAL13P1.319) discovered a 2.0-kb gene that was predicted to encode a signal peptide. The PbMAL13P1.319 protein also was predicted to have two transmembrane domains and to be either a surface/secreted antigen that

localizes to the plasma membrane or endoplasmic reticulum. PbMAL13P1.319 transcript expression was detected during the erythrocytic stages but was not detected in oocyst sporozoites and salivary gland sporozoites of the mosquito stages, suggesting a different role of PbMAL13P1.319. Overall, this dissertation describes the characteristics of MAL13P1.319 in parasite biology. Chapter 1 provides an overview of the various parasite stages with an emphasis on the intraerythrocytic stages since PfMAL13P1.319 is expressed during this stage and is potentially important for parasite survival. Since PfMAL13P1.319 is predicted to have a signal peptide and be a surface or secreted antigen, Chapter 1 addresses the *Plasmodium* secretory system and gives insight to how recently studied *Plasmodium* proteins are processed and transported to specific organelles. In addition, current information about vaccines and drugs is discussed.

CHAPTER 1: INTRODUCTION

1. Background and significance

Malaria is a mosquito borne-disease caused by a protozoan parasite from the genus *Plasmodium*. There are more than 100 species of *Plasmodium*, which can infect many animals such as reptiles, birds, and various mammals; however, only five species of *Plasmodium* infect humans: *P. vivax, P. falciparum, P. malariae, P. knowlesi and P. ovalae*. *P. falciparum* and *P. vivax* are the most common human infections of the five, but *P. falciparum* is the most pathogenic and lethal (CDC, 2010). Human malaria can be transmitted when an infective female anopheline mosquito ingests a blood meal. The anopheline mosquito prevalence is dependent on the geography and climate of the region, thereby contributing to how malaria is distributed throughout various regions (CDC, 2010). According to the World Health Organization (WHO, 2011b), half of the world's population is at risk of contracting malaria where most of this population resides in endemic countries in Africa, South America and Southeast Asia. According to the World Malaria Report 2010, there were 225 million cases of malaria and an estimated 781,000 deaths in 2009, with most deaths occurring in African children under the age of five (contributing to 20% of childhood deaths in Africa) (WHO, 2011b).

Depending on the immune system of the individual and the type of *Plasmodium* infection, symptoms of human malaria can range from no signs or

mild cases of illness such as fever, headache, chills and vomiting within seven or more days after the mosquito transmits the parasite. However, if the disease is left untreated, severe symptoms (e.g. liver and kidney failure, seizures, convulsions, coma and even death) may develop. Individuals with malaria that live in endemic regions are likely to develop some immunity to the parasite; however studies have shown that continued exposure to *Plasmodium* antigens (i.e., high transmission/repeatedly getting infected) is required for the generation and persistence of memory and effector cells (Gupta et al., 1999; Langhorne et al., 2008). On the other hand, those persons who are not immune to the disease or have suppressed immune systems are at high risk of developing severe symptoms of the disease. Infected pregnant women are also at risk of having miscarriages and low-birth weight babies. According to WHO, approximately 200,000 infant deaths are caused by malaria infection during pregnancy (WHO, 2011b).

In addition to the health severity of malaria, economically, malaria can contribute to high health expenses for a country (e.g., almost half of impatient hospital admissions and more than 60% of outpatient clinic visits) and has decreased the gross domestic product (GDP) in many endemic countries (WHO, 2011a). In addition, endemic countries suffer from loss of investments such as tourism, markets and businesses, and farming since many people do not want to risk contracting malaria (WHO, 2011a). Personal/individual expenses for people living in endemic countries (e.g., purchasing insecticide treated mosquito nets

(ITNs), preventative medicines and treatments, hospital fees, and transportation to health facilities) also has created a health disparity among malaria-risk groups.

Novel therapeutic intervention methods and other ways to complement or replace existing malaria control methods (e.g., vaccines and innovative vector control strategies) are a necessity to combat malaria especially when drug resistant parasites and insecticide resistance are a continuing issue and there is no effective malaria vaccine available (Laufer et al., 2007; Noedl et al., 2009; Wondji et al., 2011; Yadouleton et al., 2009).

2. Life cycle

The life cycle of *Plasmodium* requires both a vertebrate and mosquito host for survival (Figure 1.1). Attracted to the heat, moisture, carbon dioxide, and other factors of the human host, a female anopheline mosquito lands on a human host and ingests a blood meal that contains female and male gametocytes. The change in pH, decreased temperature and exposure to xanthurenic acid within the mosquito midgut stimulates gametocytes to differentiate into sexual stages known as male and female gametes (Talman et al., 2004). The female and male gametes fuse together to form a zygote, which then elongates to form a motile ookinete. Ookinetes penetrate and invade midgut epithelial cells and remain in the basal lamina where they develop into an oocyst. Then oocysts grow and develop thousands of sporozoites which burst from the oocyst and travel through the hemocoel cavity to invade the salivary glands. From the salivary glands, sporozoites can be transmitted to the next vertebrate host. Studies performed in

the rodent malaria model system demonstrated that injected sporozoites may arrest in the skin for hours or invade a blood vessel and enter the bloodstream (Amino et al., 2006; Ejigiri and Sinnis, 2009; Gueirard et al., 2010; Sinnis and Coppi, 2007; Yamauchi et al., 2007). Once in the bloodstream, the sporozoites travel to and invade liver cells, differentiate and undergo asexual reproduction (i.e., also known as the exoerythrocytic stages) resulting in tens of thousands of merozoites. These merozoites invade red blood cells (RBCs) and undergo multiple rounds of asexual reproduction inside the RBC, i.e., known as the intraerythrocytic cycle. The asexual blood stage parasites are associated with disease symptoms and are directly related to severe clinical problems such as severe anemia, cerebral malaria and placental malaria (Chang and Stevenson, 2004; Haldar and Mohandas, 2009; Mens et al., 2010). While some merozoites that enter the RBC can asexually reproduce into schizonts, others can differentiate into female and male gametocytes which are the infective stage for the mosquito.

3. Vertebrate host parasite stages

3.1 Liver stages

An infected mosquito probes and penetrates the skin using its stylet mouthparts and injects saliva that contains sporozoites into the blood-feeding site. Sporozoites are released into the dermal layer where they reside for hours before invading blood and lymphatic vessels (Amino et al., 2006; Ejigiri and Sinnis, 2009; Gueirard et al., 2010; Sinnis and Coppi, 2007; Yamauchi et al.,

2007). About 70% of the *Plasmodium berghei* sporozoites that leave the injection site enter the blood stream and travel to the liver endothelium where they migrate through many liver cells before invading a final hepatocyte (Amino et al., 2006; Mota et al., 2001; Vaughan et al., 2008). When the parasite travels from the skin into the circulatory system and then to the liver, host cell traversal is mediated by the "sporozoite microneme protein essential for cell traversal-1" (SPECT-1) and -2 (SPECT-2) proteins. These proteins are important for sporozoites exiting from the dermal site and entering the circulatory system, *in vivo*, in the rodent malaria system (i.e., 90% of SPECT-1 and -2 mutant parasites were immotile at the site of injection) (Amino et al., 2008).

Once the sporozoite enters the circulatory system, the parasite must find the liver sinusoid, which is a vessel that is surrounded by Kupffer cells (i.e., liver macrophages) and endothelial cells, cross the liver sinusoid barriers and traverse through many hepatocyes before invading a final hepatocyte. The mechanism of how the parasite invades the hepatocytes is not yet known. However, parasite proteins such as the circumsporozoite (CS) protein have been shown to directly interact with the hepatocyte via highly sulfated heparan sulfate proteoglycans (HSPGs) in *P. falciparum* and in the rodent malaria model (Aly et al., 2009; Frevert et al., 1993; Pinzon-Ortiz et al., 2001; Vaughan et al., 2008). Highly sulfated HSPGs are abundant in the liver organ and remain an important feature to the liver sinusoid since it may signal to the sporozoite to invade the cell (Aly et al., 2009; Sinnis and Coppi, 2007).

After the sporozoite has invaded the hepatocyte, a protective parasitophorous vacuole membrane (PVM) develops and essential liver stage genes such as "upregulated in infectious sporozoites" (UIS) and genes involved in metabolism and nutrition are expressed. In rodent malaria model systems (i.e., *Plasmodium yoelli* and *P. berghei*), the UIS gene was important for liver stage development since UIS gene deletions showed growth arrest, thereby preventing blood stage parasite development (Mueller et al., 2005; Tarun et al., 2007). In addition, a *P. falciparum* liver fatty acid binding protein (L-FABP) was shown to affect liver stage growth and may directly or indirectly interact with UIS to assist in transporting lipids to the parasite (Sharma et al., 2008).

3.2 Intraerythrocytic stages (also known as erythrocytic or asexual blood stages)

3.2A Intraerythrocytic surface and secreted proteins involved in parasite entry/binding and invasion

During liver stage development, which may last from approximately seven to ten days in humans, the parasite progresses through asexual development and ultimately tens of thousands of merozoites are released into the blood stream (Smith and Craig, 2005). Once a merozoite invades a blood cell, the parasite develops into rings, then trophozoites, and schizonts, which ultimately releases merozoites. Invasion is initiated when a merozoite stage parasite binds to the red blood cell (RBC), reorients itself so that the apical end of the parasite is in contact with the RBC surface, and develops a tight junction for parasite

invasion (Baumeister et al., 2010; Iyer et al., 2007). During the invasion process, there are many parasite protein binding receptors and ligands that are localized and secreted at the apical end (i.e., specifically from the apical complex) and play a significant role in interaction (Figure 1.2).

3.2A.1 Apical complex proteins

The apical complex of the parasite consists of organelles known as the rhoptries, micronemes and dense granules, which are known to secrete proteins involved in merozoite invasion. For example, *P. falciparum* rhoptry protein 3 (RHOPH3) is expressed during the schizont and merozoite stage and manipulates the RBC membrane for parasite invagination and parasitophorous vacuolar membrane (PVM) formation (Perkins and Ziefer, 1994). Another rhoptry protein known as apical membrane antigen 1 (AMA1) is also a late-erythrocytic stage protein essential for merozoite invasion (specifically PVM formation). Triglia et al. (2000) attempted to delete the *P. falciparum* AMA1 gene, however, they were not able to isolate AMA1-deleted parasites suggesting that AMA1 was essential for *P. falciparum* survival during the intraerythrocytic stages (Triglia et al., 2000). According to Srinivasan et al. (2011), PfAMA1 binds to another rhoptry protein (RON2) and collectively promotes invasion. More importantly, when using anti-RON2 antibodies to block RON2 from binding with AMA1, the authors discovered that junction formation was inhibited, however, it did not affect the rhoptry bulb from releasing its contents into the cell and forming

vacuoles. Therefore, this study showed how two different molecules that interact can affect two different processes of merozoite invasion (Srinivasan et al., 2011).

Many erythrocyte-binding like (EBL) proteins are micronemal proteins involved in merozoite and erythrocyte interaction. For example, a 175-kDa erythrocyte-binding antigen (EBA-175) is a well-characterized protein in *P. falciparum* that localizes in micronemes (Sim et al., 1992) and displays a sialic-acid dependent binding to Glyphorin A on red blood cells (Duraisingh et al., 2003). Other EBLs such as EBA-140/BAEBL and EBA-181 also contribute to merozoite binding and form a tight junction with the red blood cell (Maier et al., 2003). Unlike the other organelles that secrete proteins before invasion, dense granule proteins are likely to be secreted once the parasite invades the erythrocyte and may play a role in development. A *P. falciparum* ring infected erythrocyte surface antigen (RESA or Pf155) is released from the dense granules, localizes to the PV and erythrocyte membrane where it interacts and stabilizes RBC membrane skeleton (Foley et al., 1991; Pei et al., 2007).

3.2A.2 Surface proteins and proteases

When the merozoite binds to the RBC surface, there are many protein-protein interactions that take place to efficiently allow the parasite to invade the cell. One of the most abundant and well-studied proteins of merozoite stage parasites is the merozoite surface protein (MSP). There are currently ten *P. falciparum* MSPs (i.e., MSP1-MSP10) that are usually synthesized during the late erythrocytic stages and can either be membrane-bound/integral proteins (i.e.,

specifically GPI-anchored) or peripheral proteins. The MSP1 precursor protein is cleaved and processed into many fragments thereby giving rise to MSP subunits that may localize and interact differently (Maier et al., 2009). For example, the $MSP1_{19}$ subunit was shown to initially be GPI-anchored at the surface but when the parasite becomes internalized, $MSP1_{19}$ is transported to the food vacuole where it may serve a possible function (Dluzewski et al., 2008). In addition to MSP1 being proteolytically cleaved into different fragments, MSP 8 and 10 proteins are also precursors (Black et al., 2003).

Although there are many proteases that have yet to be identified or fully characterized, it is known that proteases are important for merozoite invasion into the host cell. During invasion, proteases are specifically involved in altering parasite proteins and the RBC cell surface and cytoskeleton (Maier et al., 2009). Studies performed by Dluzewski et al. (1986) displayed a significant reduction in *P. falciparum* merozoite invasion when serine and cysteine protease inhibitors (leupeptin and chymostatin) were introduced, thereby suggesting that proteases were important for parasite attachment and invasion (Dluzewski et al., 1986). Although the targets for this study were not identified, there have been identified proteases such as the *P. falciparum* falcipains and PfSUB1 (i.e., a subtilisin-like protease) that have specific roles in hemoglobin degradation (Sijwali et al., 2006) and proteolytic cleavage of itself (Sajid et al., 2000), respectively.

3.2B Clinical manifestations of intraerythrocytic stages

A key feature of red blood cells (RBCs) is their dependence on the host immune system for protection against foreign invaders (Hanssen et al., 2010) When parasitized RBCs proceed to the spleen, the spleen has macrophages that are able to detect deformed RBCs which are destroyed and removed from the circulatory system. On the other hand, the parasite has developed mechanisms to evade splenic clearance. *Plasmodium* proteins (e.g., the exportome which are proteins exported to the RBC cytoplasm and membrane) can alter the host RBC so that it may have adhesive properties and bind to vascular endothelium, thereby evading the spleen and causing severe symptoms within the host (Deitsch and Wellems, 1996; Hanssen et al., 2010; Maier et al., 2009). The intraerythrocytic stages are known to manifest in many clinical symptoms for the infected individual. The average incubation period of *P. falciparum* is approximately 9-14 days where individuals may be asymptomatic while others may develop symptoms of malaise, fatigue, fever, mild jaundice and anemia. These symptoms are primarily due to infected blood cell lysis and the host immune detection of the parasites. When the parasites adhere and sequester in the vessels, there is a decrease in blood and oxygen flow to the brain, thereby resulting in convulsions and/or coma (i.e., cerebral malaria) (Chang and Stevenson, 2004). Although the mechanism of sequestration is poorly understood, studies have shown that a parasite surface protein on infected RBCs known as *Plasmodium falciparum* erythrocyte membrane protein (PfEMP1)

interacts with various host endothelial molecules such as the intracellular adhesion molecule (ICAM), CD36, heparan sulfate (HS), chondroitin sulfate A (CSA) and thrombospondin in order to promote parasite virulence and severe clinical symptoms (Flick and Chen, 2004; Kraemer and Smith, 2006; Miller et al., 2002). Various host receptors of PfEMP1 are associated with specific disease pathologies; hence, PfEMP1 is a major player in most severe clinical pathologies of the disease (Ochola et al., 2011; Pasternak and Dzikowski, 2009). In addition to the role of PfEMP1 in cytoadherence, PfEMP1 is a major antigen exposed on the RBC surface that is involved in antigenic variation in order to evade the host immune system (Pasternak and Dzikowski, 2009). Although PfEMP1 is an important target for the host immune system to prevent severe disease, the antigenic variation of PfEMP1 allows the parasites to evade host antibodies.

3.3 Gametocytogenesis

While there are merozoites that can invade new RBCs, there are also merozoites that may differentiate into male and female gametocytes which are the infective stage for the mosquito (i.e., gametocytogenesis). Although it is not known what exactly triggers gametocytogenesis, many studies have shown that environmental stresses such as parasite number and host environment can influence gametocyte formation, thereby suggesting a signaling mechanism between the parasite and environment (Dixon et al., 2008; Kuehn and Pradel, 2010). Bruce et al. (1990) showed that merozoites from a single schizont are committed to produce asexual or gametocyte stage parasites, however the

parasite switch from asexual to sexual stage remains unclear (Bruce et al., 1990). While researchers have identified a small subset of genes that are stage-specific, a majority of the genes expressed between early sexual- and asexual-stage parasites are similar, suggesting other mechanisms of gene regulation that may contribute to stage-switching (Eksi et al., 2005; Silvestrini et al., 2005). The small subset of upregulated genes also had possible roles in host cell remodeling, metabolism and cell signaling to promote stage-switching (Eksi et al., 2005). There are five gametocyte stages that are characterized by shape/morphology, maturity and gender. Once a mosquito ingests gametocytes, parasites are exposed to many environmental changes (e.g. temperature and presence of xanthurenic acid) that signal the parasite to leave the protective RBC shield and transform into male and female gametes (Billker et al., 1997; Talman et al., 2004). Male gametes activate and differentiate into eight flagellated microgametes (i.e., exflagellation) that fertilize with the female gamete to form a zygote.

4. Mosquito host parasite stages

4.1 Zygote/ookinete development, ookinete invasion and oocyst development

Approximately an hour after ingesting an infected blood meal, female and male gametes fertilize to develop a spherical shaped zygote which elongates to become a crescent-shaped ookinete. The zygote stage is a diploid parasite stage that proceeds through meiosis. The cellular structure of the zygote changes to

form a different cytoskeleton and apical complex end which is important for ookinete invasion (Sinden et al., 1985). The ookinete has gliding motility that allows it to travel to the midgut wall.

There are many well studied factors that contribute to ookinete invasion and development such as the *P. berghei* calcium-dependent protein kinase (CDPK3) which may play a role in ookinete motility and ookinete invasion of the *Anopheles stephensi* midgut (Ishino et al., 2006; Siden-Kiamos et al., 2006). *P. berghei* circumsporozoite and thrombospondin-related anonymous protein-related protein (CTRP) is a micronemal protein that also is important for ookinete motility and mosquito midgut invasion (Dessens et al., 1999; Yuda et al., 1999). Other micronemal proteins such as secreted ookinete adhesive protein (SOAP) and membrane attack ookinete protein (MAOP) are important for ookinete invasion/development and ookinete entry into *A. stephensi* midgut epithelial cells in the *P. berghei* rodent malaria system, respectively (Dessens et al., 2003; Kadota et al., 2004). *P. berghei* ookinete surface proteins P25/P28 bind to anopheline mosquito laminin and are essential for ookinete development and entry into midgut cells (Siden-Kiamos et al., 2000; Tomas et al., 2001). These factors and many other receptor-ligand interactions allow the ookinetes to first penetrate the peritrophic matrix (i.e., the thick extracellular sheath that surrounds the blood meal), traverse the midgut epithelium and arrest between the basal lamina and midgut epithelium where it develops into an oocyst. It is important for the ookinete to be associated with the basal lamina since studies have shown

that components (i.e., macromolecules and short peptides) in the basal lamina trigger ookinete transformation and oocyst development (Arrighi and Hurd, 2002; Paulsson, 1992).

As the oocyst remains between the basal lamina and midgut epithelium layer, it will grow to approximately 40-60 μm and release thousands of sporozoites into the mosquito hemocoel. Within a developing oocyst, the cytoplasm begins to expand while the plasma membrane folds inwards and forms clefts that divide within the oocyst (i.e., sporoblasts) (Vlachou et al., 2006). Cytokinesis occurs within the sporoblasts and divides the budding haploid sporozoites (Vlachou et al., 2006).

4.2 Sporozoites

The resulting sporozoites generated within the oocyst are released into the hemocoel of the parasite where they then travel to the salivary glands to be transmitted to the vertebrate host. Well-characterized sporozoite surface antigens and apical complex proteins that are involved in sporozoite infectivity and invasion of host tissues are the circumsporozoite protein (CS) and thrombospondin-related adhesive protein/sporozoite surface protein 2 (TRAP/SSP2). *P. falciparum* CS is the most abundant protein expressed on the surface of the sporozoite and is required for sporozoite development and invasion of the mosquito salivary gland and vertebrate host tissue (Menard and Janse, 1997; Myung et al., 2004; Rathore et al., 2002; Sidjanski et al., 1997; Thathy et al., 2002; Yoshida et al., 1980). Studies have shown that *P. yoelii*

recombinant CS protein and a peptide from the N-terminal portion of CS can inhibit *P. yoelii* sporozoite binding to the salivary glands (Myung et al., 2004; Sidjanski et al., 1997). When analyzing *P. berghei* CS deletion mutants, sporozoites did not form in the oocysts and oocyst differentiation was affected (Menard and Janse, 1997; Thathy et al., 2002). Another well-characterized sporozoite surface protein is TRAP, with studies showing that TRAP is essential for sporozoite gliding motility and mosquito salivary gland and hepatocyte invasion (Muller et al., 1993; Robson et al., 1988; Rogers et al., 1992; Sultan et al., 1997). Specifically, Sultan et al. (1997) showed a substantial reduction of *P. berghei* TRAP mutant sporozoites invading the salivary glands and the liver of rodents in comparison to wild-type parasites. In addition, TRAP mutant sporozoites were non-motile and had no gliding movement in comparison to wild-type sporozoites. Other sporozoite surface proteins that have been characterized and have a role in sporozoite invasion are the apical membrane 3 antigen/erythrocyte binding-like protein (MAEBL) (Kariu et al., 2002), the sporozoite threonine-and asparagine-rich protein (STARP) (Fidock et al., 1994; Pasquetto et al., 1997), and the sporozoite and liver stage antigen (SALSA) (Bottius et al., 1996; Puentes et al., 2004). Targeted disruption of *P. berghei* MAEBL has demonstrated that this gene is critical for sporozoite attachment and invasion into the anopheline mosquito salivary glands but is not essential for *P. berghei* sporozoite motility (Kariu et al., 2002). The *P. falciparum* STARP protein is expressed on the sporozoite surface, liver stage and ring stage parasites and

was demonstrated to be important for sporozoite invasion into hepatocytes, since anti-STARP antibodies inhibited approximately 90% of sporozoites invading the liver cells (Fidock et al., 1994; Pasquetto et al., 1997). Like STARP, the SALSA protein is expressed on the *P. falciparum* sporozoite surface and liver parasite stages (Bottius et al., 1996). Antibodies against SALSA peptides/epitopes also inhibited sporozoite invasion of hepatic cells (Puentes et al., 2004). The sporozoite and erythrocytic stage (SES) protein, which was previously characterized by Dr. Alexis LaCrue in the Beerntsen lab, was discovered to be distributed on the surface of *P. gallinaceum* sporozoites. Antibody blocking studies using anti-PgSES antibodies demonstrated a significant reduction of sporozoite numbers in the mosquito salivary glands, which suggests that PgSES was needed for sporozoite invasion of the salivary glands (LaCrue et al., unpublished). Overall, sporozoites utilize various antigens for salivary gland invasion, sporozoite development and motility and recognition/binding to the mosquito salivary glands.

5. Molecular biology of the *Plasmodium* secretory system

Like many eukaryotic organisms, *Plasmodium* has a secretory system that allows proteins to be processed and manipulated through the endoplasmic reticulum and Golgi apparatus. However, unlike other organisms, *Plasmodium* has other specialized organelles that allow specific stages of the parasite to survive in both the vertebrate and invertebrate host and to help invade host tissues. Examples of these specialized organelles are the apicoplast,

micronemes, rhoptries and dense granules that are used to produce proteins important for metabolism and parasite invasion (Figure 1.3). Although trafficking to these specialized organelles remains poorly characterized, some studies support membrane-bound vesicles being transported from the ER/Golgi apparatus to the cytoplasm where the vesicles fuse to the organelles (Klemba et al., 2004). For example, Klemba et al. (2004) showed that the green fluorescent protein (GFP) tagged plasmepsin II (PMII) protein is trafficked through the secretory system to cytosolic vacuoles and ultimately to the food vacuole where it is proteolytically processed to mature PM II. *Toxoplasma gondii* (i.e., an apicomplexan sister of *Plasmodium*) studies have shown that proteins exported to rhoptries have a N-terminal signal peptide that allows them to proceed through the secretory system, however secondary signals such as special motifs direct them specifically to the rhoptry (Di Cristina et al., 2000; Hoppe and Joiner, 2000; Reiss et al., 2001). Although these signals have not been discovered in the *Plasmodium* system, there is speculation that apical complex proteins may have special motifs or are paired with "escorter" proteins that have a targeting motif (Przyborski and Lanzer, 2005). The latter hypothesis was based on a study by Baldi et al. (2000) who showed that the C-terminal end of *P. falciparum* rhoptry associated protein-1 (RAP1) was necessary for targeting RAP-2 to the rhoptries (Baldi et al., 2000). However, some suggest that there are no necessary signals for export and that proteins are dependent on a "time-point of expression" (Kocken et al., 1998; Przyborski and Lanzer, 2005).

6. Current research of vaccines

6.1 Vaccines

Many efforts are being made to develop a vaccine and to identify new malaria drug targets due to the increase of drug-resistant parasites, insecticide-resistance, and financial burdens contributed by numerous malaria cases. There are many reasons why a vaccine has not been developed, such as 1) parasite antigenic variation (i.e., where the exposed parasite molecule may have different variants of an antigen thereby creating difficultly to effectively recognize the foreign antigen) (Su et al., 1995) 2) a complex life cycle with stage specific proteins that may be expressed for a short time, thereby preventing antibody detection (Crompton et al., 2010) and 3) the complexity of the host immune system and how it interacts with the human malaria parasites (Epstein, 2002; Langhorne et al., 2008). Currently, there are different types of approaches for vaccine development that focus on different parts of the life cycle such as pre-erythrocytic stage vaccines, asexual stage vaccines and transmission-blocking vaccines.

Pre-erythrocyctic stage vaccines primarily focus on preventing sporozoites from entering the liver and inhibiting liver stage parasite development. Therefore these vaccines must provide complete protection against sporozoite invasion or liver stage development, because development of liver stage parasites and subsequent release of even one merozoite can result in a blood stage infection. One of the well known pre-erythrocytic stage vaccines is the GlaxoSmithKline

and Walter Reed Army Institute of Research (WRAIR) RTS,S vaccine which utilizes the central repeat and the thrombospondin domain of the *P. falciparum* circumsporozoite (PfCS) protein (i.e., the abundant sporozoite surface protein) fused to a hepatitis B surface antigen to induce a high immunogenic response (Nardin and Nussenzweig, 1993; Stoute et al., 1997), Currently in phase III trials, the RTS,S vaccine is immunogenic as patients given the vaccine demonstrate anti-CS antibodies for up to 45 months (Aide et al., 2011; Barbosa et al., 2009; Casares et al., 2010; Gordon et al., 1995). In addition, a study performed on Mozambican children showed a 35.3% risk reduction in clinical malaria and a 48.6% reduction in severe malaria when given RTS,S and follow-up studies showed the vaccine to be safe with no severe adverse effects (Alonso et al., 2005; Alonso et al., 2004; Sacarlal et al., 2008). In Kenya and Tanzania, two studies of infants and young children (17 months – 5years old) inoculated with the RTS,S vaccine showed that the risk of clinical episodes decreased by 53% with no severe adverse effects (Abdulla et al., 2008; Bejon et al., 2008). Overall, RTS,S appears to be a promising vaccine; however the efficacy against infection and clinical disease may be a potential weakness since the 2004 Mozambique study displayed efficacy for up to 45 months but antibodies decreased over time, which does not support the longevity of the immune response.

Other types of pre-erythrocytic vaccines are using irradiated or genetically attenuated sporozoites. In the 1970s, researchers discovered that immunizing human volunteers with irradiated mosquitoes infected with *P. falciparum* or *P.*

vivax could protect volunteers from future malaria infections (Clyde et al., 1973; McCarthy and Clyde, 1977; Rieckmann et al., 1979). However, the production of numerous sporozoites and exposure to hundreds of irradiated-infected mosquitoes was impractical. Hoffman et al. (2010) challenged this issue by purifying and cryopreserving large amounts of *P. falciparum* irradiated sporozoites that were made by exposing *P. falciparum*-infected mosquitoes to gamma radiation (Hoffman et al., 2002). Irradiated sporozoites are live attenuated sporozoites that can enter hepatocytes and partially develop when injected into the host (Hoffman et al., 2002). In efforts to create a live attenuated non-replicating sporozoite vaccine, Hoffman and other researchers at Sanaria Inc (a vaccine production company that aims to commercialize irradiated sporozoite vaccines against *P. falciparum*) collaborated with other institutions to test the first generation vaccine called *P. falciparum* sporozoite vaccine (PfSPZ) in human volunteers. The first clinical trials of PfSPZ demonstrated protection in volunteers however immunogenicity and protective efficacy were suboptimal, which could be due to poor efficacy of the vaccine or inefficient administration (Epstein et al., 2011; Hoffman et al., 2010). PfSPZ is currently in phase I/IIa clinical trials (Crompton et al., 2010).

Another type of pre-erythrocytic vaccine that utilizes genome information and genetic engineering of the parasite is genetically attenuated sporozoites. Genetically attenuated sporozoites are established by deleting an essential liver stage gene that allows the production of genetically engineered sporozoites but

with no development in the liver. In *P. falciparum*, the p52/36 genes were simultaneously deleted and did not affect sporozoite production and infectivity to the mosquito salivary glands (VanBuskirk et al., 2009). More importantly, p52/p36 deletion mutants demonstrated growth defects of the liver stage parasites using an *in vitro* liver stage growth assay and a chimeric mouse model carrying human hepatocytes (VanBuskirk et al., 2009). *P. falciparum* p52/p36 is currently in phase I/IIa clinical trials using human volunteers infected with genetically attenuated sporozoites via mosquito bite (Vaughan et al., 2010).

Asexual blood stage vaccines are targeted at the erythrocytic/blood stages of the parasite, specifically during merozoite invasion. Some of the current asexual vaccine candidates, which were also discussed earlier in the Intraerythrocytic Stages of Section 3 are AMA-1, EBA-175 and MSPs (El Sahly et al., 2010; Sheehy et al., 2011; Spring et al., 2009). Some of these asexual blood stage vaccine candidates (i.e., either alone or combined with other vaccine candidates) have established a safe and immunogenic response in volunteers (e.g., chimeric AMA1+$MSP1_{19}$ (Malkin et al., 2008) and recombinant EBA-175 (El Sahly et al., 2010), while others such as recombinant AMA-1 have not shown significant efficacy against *P. falciparum* infection (Sagara et al., 2009). Many challenges that researchers face when developing blood stage malaria vaccines are highly polymorphic proteins (e.g. AMA-1) and identifying new candidates that have redundant functions (Crompton et al., 2010; Hadley et al., 1987; Wellems et al., 2009).

Transmission-blocking vaccines are targeted primarily towards the early stages of the parasite in the mosquito (i.e., gametes, zygotes and ookinetes). Although these vaccines are not meant to relieve the burden of clinical disease manifestations, they are focused on preventing the transmission of malaria parasites, thereby reducing overall malaria cases. One transmission-blocking vaccine candidate is the ookinete surface protein, Pfs25. Since antibodies against Pfs25 are ingested by the mosquito during a blood meal, anti-Pfs25 antibodies can block parasite development in the mosquito and prevent transmission to other people. Kubler-Kielb et al. (2007) investigated the immunogenicity of Pfs25 plus adjuvant in mice in order to increase antibody levels for transmission blocking in the mosquito. They discovered that Pfs25 bound to itself or another protein could elicit high levels of transmission-blocking antibodies for the mosquito (Kubler-Kielb et al., 2007). In addition, Kubler-Kielb et al. (2010) combined two vaccine candidate molecules, Pfs25 and CSP, to generate antibodies against both mosquito and pre-erythrocyctic stages of the parasite. The results indicated that using a combined vaccine approach would induce high levels of antibodies that would provide immunity to sporozoite infections of the liver and prevent transmission by mosquitoes (Kubler-Kielb et al., 2010). Pfs25 vaccine is currently in phase I clinical evaluations (Crompton et al., 2010). Gametocyte proteins such as Pfs230 and Pfs48/45 are other vaccine targets, which are immunogenic and under clinical testing (Saul, 2007; Targett and Greenwood, 2008).

6.2 Drugs

Antimalarial drugs are the first line of defense to combat and control malaria. These drugs target different stages of the parasite and are designed to inhibit enzymes that are critical to the parasite and to target essential functions of the parasite such as hemoglobin digestion/detoxification, invasion and metabolism (Bray et al., 2005; Kalanon and McFadden, 2010; Kappe et al., 2010). Some examples of stage-specific drugs are sporontocidal drugs that inhibit development of sporozoites in oocysts, gametocytocidal drugs which kill gametocytes, and blood schizontocidal compounds which kill the asexual blood stage parasites (Warrell, 2002). Three main uses for antimalarial drugs are for protection (prophylactic), therapeutic treatment, and preventing transmission via attacking gametocytes or inhibiting sporogony in infected mosquitoes.

Some of the commonly used drugs are quinolines, antifolates and artemisinin-combination therapies (ACTs). The oldest antimalarial drug group known as quinolines (e.g., quinine, chloroquinine, and mefloquine) are the first-choice for combating severe malaria and function to sequester heme or inhibit conversion of heme to hemozoin (Sadanand, 2010). Antifolate compounds, including pyrimethamine, proguanil and sulfadoxine, are known to inhibit *Plasmodium* growth by blocking enzymes involved in folate synthesis (Kappe et al., 2010). ACTs are artemisinin used in combination with a second antimalarial drug and are the most effective antimalarial drugs because they decrease parasite load earlier than other drugs and simultaneously kill *Plasmodium*

gametes in the mosquito (Hyde, 2002; Sadanand, 2010). The mechanism of action of artemisinin is unknown but it is speculated to target the ER, mitochondrian and digestive vacuoles and/or to inhibit hemozoin production (Sadanand, 2010; Tilley et al., 2011).

The emergence of parasite drug-resistance to commonly used drugs is, in part, due to the mutation rate of the parasite, fitness of the mutated parasite and improper drug exposure such as prescribing wrong drug dosages (Petersen et al., 2011). Many regions affected by drug-resistant parasites are in underdeveloped countries in Asia, Africa and South America, where continued drug use may lead to an increase in drug-resistant parasites (Petersen et al., 2011; WHO, 2011b). With the emergence of drug-resistant parasites, it is imperative that new antimalarial targets are discovered. Researchers have taken an interest in targeting the apicoplast organelle (it is unique to Apicomplexans) because of its essential functions in lipid, heme and isoprenoid synthesis (Ralph et al., 2004). Other targets are protease inhibitors of the asexual liver and blood stages that may inhibit parasite release from the schizont and proteases involved in hemoglobin degradation and invasion (Kappe et al., 2010; McKerrow et al., 2008). As the search for novel drug therapeutics continues, understanding parasite biology and identifying/characterizing new molecules could lead to the discovery of new drug targets and better treatments.

7. Dissertation

The purpose of this dissertation research was to identify and characterize a *Plasmodium* protein known as MAL13P1.319 and assess the role of MAL13P1.319 in the parasite. Since the initial effort was to discover and characterize novel sporozoite surface and secreted proteins, a data mining strategy using the *Plasmodium* genome database (PlasmoDB) was employed. PlasmoDB is a searchable database that contains genome information of various *Plasmodium* species in addition to transcriptome and proteome analyses via DNA microarray and protein mass spectrometry, respectively (Florens et al., 2002; Lasonder et al., 2002; Le Roch et al., 2003). Mining PlasmoDB allowed a specific selection of *Plasmodium* genes that have a signal peptide sequence, represent an unknown protein that is expressed in sporozoites, and are predicted to encode surface antigen or secreted protein. As a result of the data search, *P. falciparum* MAL13P1.319 (PfMAL13P1.319) was identified. The transcript expression profile of PfMAL13P1.319 shows that it is expressed in the intraerythrocytic stages, oocyst sporozoites and salivary gland sporozoites, as reported by Le Roch et al. (2003) via DNA microarray and reverse-transcriptase analyses described in this dissertation. For protein analyses of PfMAL13P1.319, two large-scale mass spectrometry studies were performed by Lasonder et al. (2002) and Florens et al. (2002). Lasonder et al. (2002) did not report any detection of PfMAL13P1.319 during the trophozoite, schizont, gametocyte and gamete stages while Florens et al. (2002) also demonstrated no protein

expression during the trophozoite and gametocyte stages in addition to the merozoite stage. Interestingly, Florens et al. (2002) reported PfMAL13P1.319 protein expression during the salivary gland sporozoite stage. However, further in depth characterization of protein expression that included stages not studied by Lasonder et al. (2002) and Florens et al. (2002) (i.e., rings, zygotes, ookinetes, hemolymph sporozoites and oocyst sporozoites) demonstrates the PfMAL13P1.319 protein to be present during the late intraerythrocytic stages with no detectable levels of the protein in salivary gland sporozoites. Overall, this dissertation focuses on analyzing MAL13P1.319 throughout the various parasite stages and assessing a potential role of the protein. This research was also extended to investigate the rodent malaria ortholog of MAL13P1.319 (PbMAL13P1.319) in order to compare the differences and/or similarities of MAL13P1.319 between both species. Collectively, this research analyzed the expression profile of MAL13P1.319 and its potential role in *Plasmodium* parasite biology.

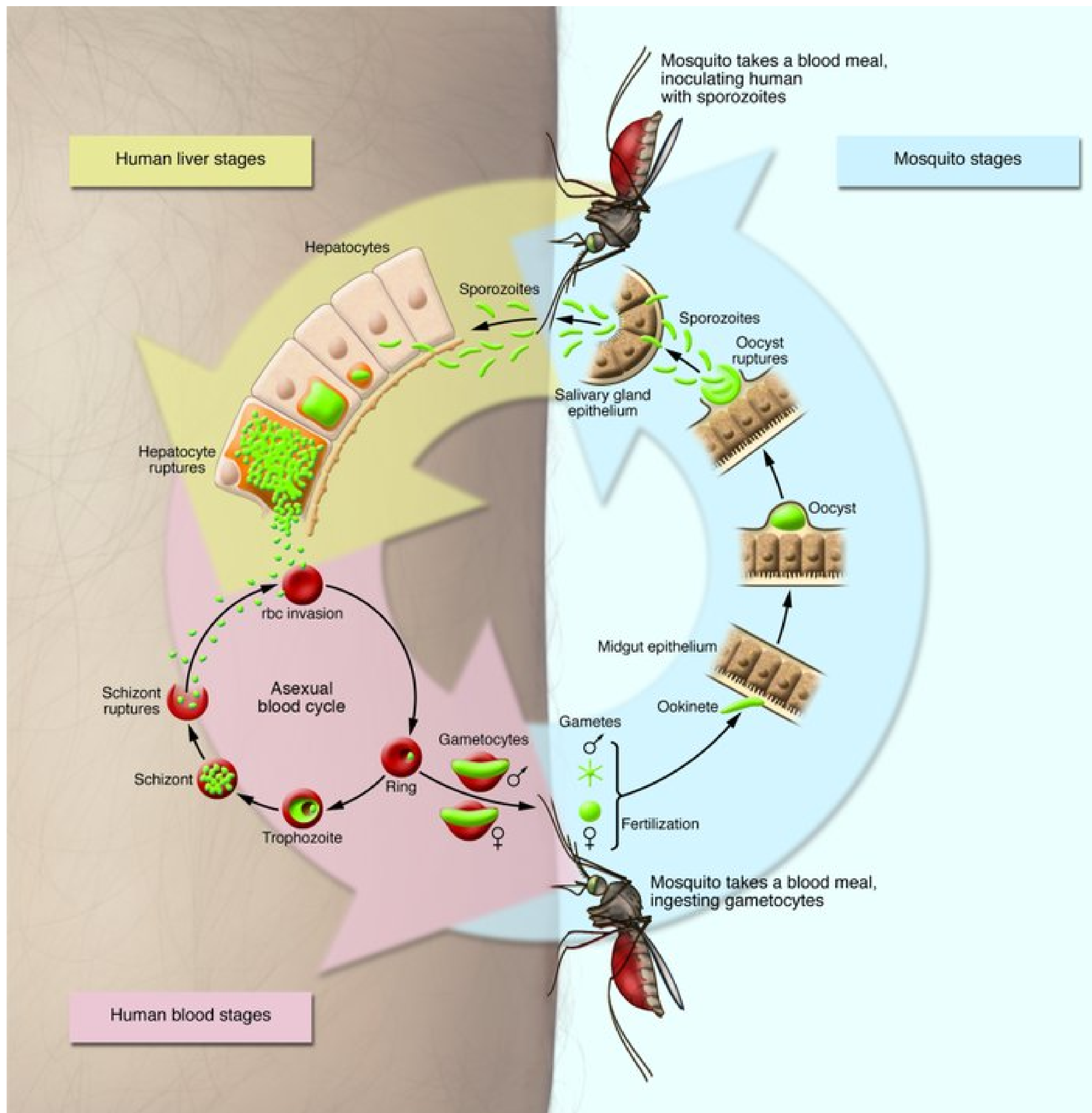

Figure 1.1. *Plasmodium falciparum* life cycle.

Once an infected *Anopheles* mosquito takes a blood meal, sporozoites are injected into the human dermis and can travel through the dermis to enter lymph vessels, the bloodstream and/or reside in the dermis for hours. After inoculation, sporozoites invade the liver cells and asexually reproduce, thereby resulting in tens of thousands of merozoites. These merozoites enter the bloodstream and

proceed through many rounds of asexual reproduction (i.e., where symptoms of malaria occur). While some parasites may invade more red blood cells, others may differentiate into sexual stage gametocytes which are the infectious stage for the mosquito. After gametocytes are taken up by a female mosquito during a blood meal, they can differentiate into female and male gametes which fertilize and develop into a zygote. Zygotes elongate and become motile developing into an ookinete, which will traverse the midgut wall and develop into an oocyst. The oocyst grows and produces thousands of sporozoites that burst out and travel through the hemolymph to the mosquito salivary glands. This illustration is from Crompton et al. (2010).

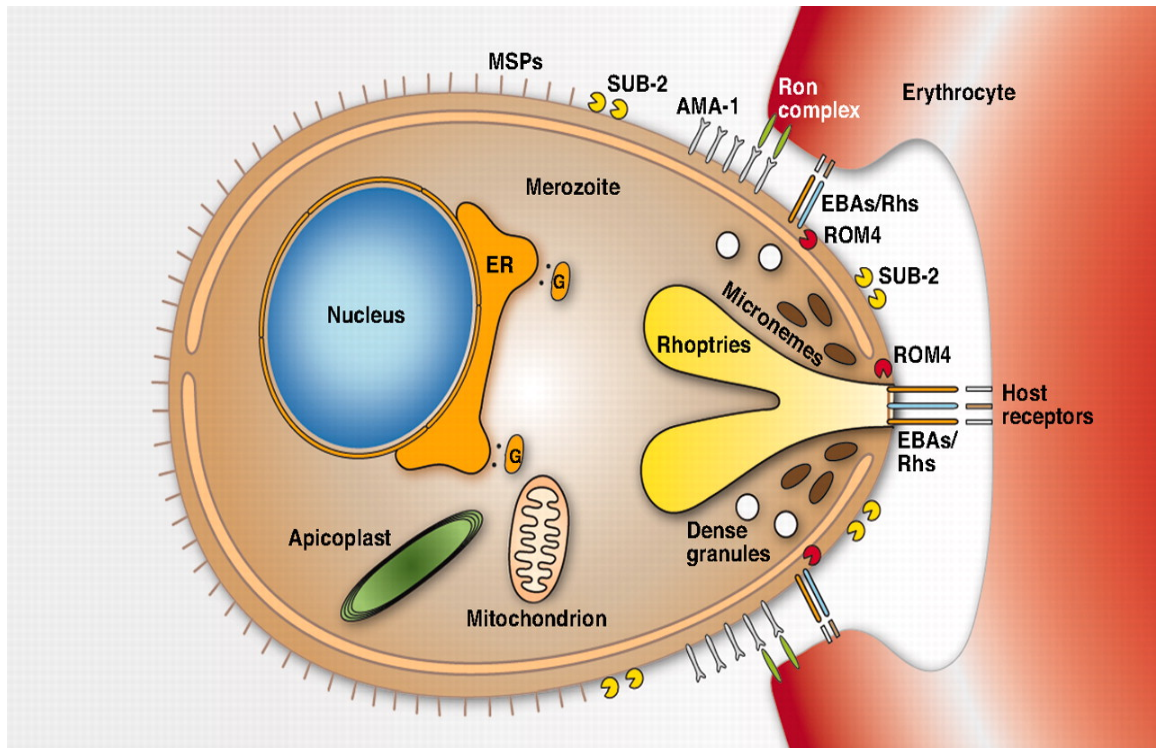

Figure 1.2. Apical complex proteins involved in merozoite invasion.

There are four major steps involved in merozoite invasion: 1) Merozoite binding between merozoite surface proteins and erythrocyte surface proteins 2) Reorientation of the merozoite so that the apical end of the parasite interacts with the erythrocyte membrane 3) Release of micronemal proteins for receptor-ligand interactions that will allow the merozoite to form a tight junction to the erythrocyte surface 4) Merozoite entry and formation of a parasitophorous vacuole (PVM). Some of the noted parasite ligands such as erythrocyte binding antigens (EBAs), merozoite surface proteins (MSPs), apical membrane antigen-1 (AMA-1), and rhoptry proteins (Rhs) are factors important for merozoite invasion. This image illustrates receptor-ligand interactions of parasite MSPs, AMA-1 and EBAs/Rhs with erythrocytes. PfAMA-1 forms an attachment with the rhoptry neck (Ron)

complex to establish the tight junction between the parasite and the cell. Some of these binding interactions are released during invasion by cleavage of rhomboid 4 (ROM4) and subtilisin 2 (SUB-2). This image is from Kappe et al. (2010).

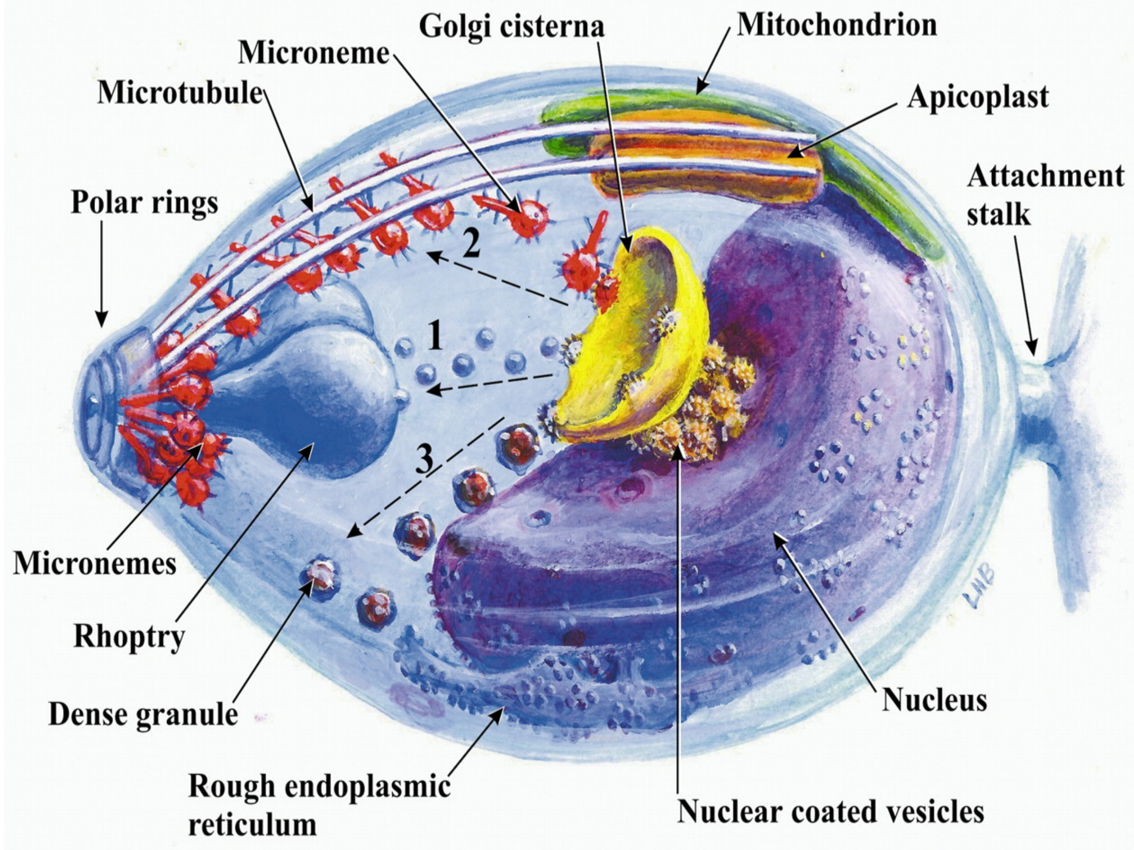

Figure 1.3. ***Plasmodium*** **merozoite secretory system.**

This illustration depicts three main trafficking routes from the apical complex. Proteins processed from the Golgi cisterna can proceed to the rhoptry i.e., via rhoptry-directed vesicles (1); micronemes i.e., targeted by microtubules (2); dense granules (3). This image is from Bannister et al. (2003).

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CHAPTER 2: DETERMINATION OF *PLASMODIUM FALCIPARUM* MAL13P1.319 PROTEIN PRESENCE THROUGHOUT VARIOUS PARASITE STAGES

Abstract

Plasmodium falciparum sporozoites present an effective target for control by vaccines, drug therapy, and/or novel mosquito control methods since they demonstrate a dual infectivity for the mosquito salivary glands and vertebrate tissue that is critical to the survival and development of the parasites. In an effort to discover molecules that are involved in sporozoite biology, a *P. falciparum* sporozoite gene known as MAL13P1.319 was identified by a search of the annotated *Plasmodium* genome database (PlasmoDB) using specific criteria, i.e., presence of a signal peptide sequence, expressed as a sporozoite protein as shown by mass spectrometry, and predicted to be a surface or secreted protein. PfMAL13P1.319 is a 790-amino acid protein that has orthologs in other *Plasmodium spp*. and appears to be unique to Apicomplexans. Although Florens et al. (2002) designated *P. falciparum* MAL13P1.319 solely as a sporozoite stage protein, molecular, biochemical and cellular characterization of PfMAL13P1.319 demonstrated protein expression during the intraerythrocytic stages.

3 Key Words: Malaria, Sporozoite, Gene Expression

1. Introduction

Human malaria is a mosquito-borne disease responsible for approximately 225 million human infections and an estimated 781,000 deaths annually, with most deaths occurring in African children. Primarily endemic in third world countries, efforts to control malaria are more demanding because of drug-resistant parasites, insecticide-resistant mosquitoes and economic instability. While there is a search for protein candidates that can serve as effective targets for vaccines, drug therapy, and/ or novel mosquito control methods, many have yet to be characterized.

In efforts to discover new targets, data mining of the *Plasmodium* genome database, PlasmoDB (Kissinger et al., 2002), was performed to specifically select *Plasmodium* genes that encode a signal peptide sequence, represent an unknown protein that is expressed in sporozoites, and is predicted to encode a surface antigen or secreted protein. A novel *Plasmodium falciparum* gene, PfMAL13P1.319, was identified as a 2.3-kb gene that encodes a 790-amino acid protein (i.e., 92.8 kDa with the signal peptide). According to Florens et al. (2002), PfMAL13P1.319 is expressed in sporozoites as determined by protein mass spectrometry, and it is predicted to be a surface or secreted protein according to two localization programs. PfMAL13P1.319 demonstrates orthologs with proteins of various *Plasmodium spp*. and appears to be unique to Apicomplexans since no orthologs of MAL13P1.319 were identified in higher eukaryotic organisms, thereby suggesting that this protein may be important to *Plasmodium* species.

This chapter addresses the molecular, cellular and biochemical characteristics of PfMAL13P1.319 and how data generated from a large scale protein mass spectrometry assessment in *P. falciparum* (Florens et al., 2002) compares to the studies reported herein.

2. Materials & Methods

2.1 Mosquito and *Plasmodium* parasite maintenance and transmission

Anopheles stephensi mosquitoes were reared according to the Malaria Research and Reference Reagent Resource Center (MR4) protocol and used for maintaining the *P. falciparum* life cycle. Briefly, mosquitoes were maintained at 27°C and approximately 80% humidity with a 16-hour light and 8-hour dark cycle and were supplied with a sucrose (0.3 M) source for feeding (Lacrue et al., 2005). Mosquitoes used for parasite transmission were 3-7 days old and also were maintained as mentioned previously. The *P. falciparum* NF54 strain and 3D7HT-GFP (a constitutively GFP-expressing parasite line) parasites also were maintained according to the MR4 protocol and used for mosquito-parasite infections. Briefly, *in vitro* cultures of NF54 erythrocytic stage parasites were maintained in a culture flask where fresh O+ sterile human red blood cells (RBCs) (Biochemed Services) were added at a 3% hematocrit in new RPMI 1640 (Gibco) culture medium containing 0.5% Albumax II (Gibco, Invitrogen Corporation) and 50 mg/L hypoxanthine (Sigma, USA) (media changes were every other day). Cultures were gassed with a 3% O_2, 3% CO_2 and 94% N_2 gas mixture and incubated at 37°C.

Gametocyte cultures used for mosquito parasite transmission and for transcript and protein analysis (i.e., enriched gametocytes) were established by setting NF54 cultures at a 0.5% parasitemia (i.e., by diluting with 50% washed O+ human RBCs/RPMI 1640 containing white male A+ human serum from Interstate Blood Bank, Inc). Culture medium was changed every other day and parasite cultures were used for mosquito infections 16 days later.

Mosquito parasite transmission studies were performed using naïve mosquitoes that were exposed to an infected blood meal containing day 16 gametocytes from a water-jacketed chamber covered with a thin membrane derived from pig intestine (Carter et al., 1993). Naïve mosquitoes (50-60) were placed in cartons and sucrose pads were removed overnight in order to starve the mosquitoes. On the day of infection, pig intestines were washed with milli-Q water three times and used to cover water-jacketed chambers that were continuously maintained at 37°C using a water-bath heater. Infected blood was centrifuged at 2,000 x g for 5 minutes and diluted to a final parasitemia of 0.5% using white male A+ human serum and 50% washed O+ human RBCs (i.e., half red blood cells and half human serum). Exflagellation of male gametocytes was observed by placing a drop of the blood meal on a glass slide which was covered with a Vaseline coated coverslip. After assessing exflagellation, naïve mosquitoes were exposed to infected blood for 30 minutes and cartons with >90% of blood-fed mosquitoes were used for subsequent experiments.

2.2 Identification and sequence analysis of PfMAL13P1.319

Initial efforts to identify novel sporozoite surface/secreted proteins that may be involved in sporozoite invasion of host tissues were performed by "data mining" or *in silico* screening of the *P. falciparum* database (PlasmoDB) (Kissinger et al., 2002). In utilizing this search, there were 1,025 genes that expressed protein during the sporozoite stages and of these 1,025 genes, 122 genes had a signal peptide which would indicate that a protein proceeds through the secretory pathway. In the event of obtaining an essential gene that is expressed during the erythrocytic stages, deleting this gene would be detrimental or lethal to the parasite survival and prevent future mosquito infections. Therefore, 34 out of the 122 genes were selected because they were found to be expressed only in sporozoites, based upon data available at the time of the mining approach. Fourteen genes of the 34 were unknown/hypothetical and were not previously characterized. After performing surface/secreted protein predictions using computer program analyses, 5 genes including PfMAL13P1.319 (also known as PF3D7_1363700) were identified. Since genomic and proteomic analyses of PfMAL13P1.319 were available in PlasmoDB, additional analyses were obtained using Expert Protein Analysis System (ExPASy) (www.expasy.org) proteomic tools such as: 1) TargetP and SignalP for signal peptide/anchor predictions; 2) PSORTII, TMAP, DGPI and Softberry (the latter was not an ExPASy tool; www.softberry.com) for localization, transmembrane and GPI anchor predictions; 3) Pfam for classifying family

proteins and domains and 4) Prosite for identifying families, functional sites, and other patterns/profiles (Appel et al., 1994). PfMAL13P1.319 protein sequence was subjected to Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) analysis to determine if it had orthologs in PlasmoDB (Kissinger et al., 2002), Sanger Institute (Gardner et al., 2002) and National Center for Biotechnology Information (NCBI) databases. Protein sequence alignments were done with Vector NTI Explorer ALignX tool (Invitrogen) in order to determine identical and similar amino acids.

2.3 *Plasmodium falciparum* parasite isolation

2.3A Sporozoite isolation

To obtain *Plasmodium* oocyst and salivary gland sporozoite stages, 3-7 day old mosquitoes maintained as previously described were exposed to an infected blood meal and dissected 8-10 days post-exposure (PE) for *P. falciparum* oocyst sporozoites and 14-17 days PE for *P. falciparum* salivary gland sporozoites. Isolation of oocyst sporozoites was performed by removing the mosquito midgut, transferring it to a slide with a drop of 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4), then rupturing the midgut oocysts by placing a coverslip onto the midgut to release mature sporozoites from the oocysts (LaCrue et al., 2005; MR4 protocol). *Plasmodium* salivary gland sporozoites were isolated by dissecting the salivary glands and placing them in a tube of 50 µl 1X PBS where sporozoites were released from the salivary glands by vigorous pipetting (LaCrue et al., 2005; MR4 protocol). Briefly,

sporozoites were centrifuged at 18,000 x g for 5 minutes at 4°C, homogenized using a silconized pipet tip, and centrifuged again to remove supernatant. Sporozoites then were washed one time with 1% fetal calf serum (HyClone) in 1X PBS and sporozoite numbers were determined by a hemocytometer.

2.3B Axenic liver stage isolation

P. falciparum axenic liver stage parasites (i.e., exoerythrocytic forms; EEFs) were obtained in the absence of host hepatocytes based on a Kappe et al. (2010) patent. Mosquitoes exposed to an infected blood meal were rinsed with 70% ethanol for at least 5 minutes and then salivary glands were dissected in sterile RPMI 1640 with penicillin/streptomycin [500U/ml] and isolated as previously described. Approximately 5 x 10^4 sporozoites were added to each well of a 48-well plate and maintained at 37°C in 5% CO_2 (Kappe, 2010). After 24 hours of sporozoite incubation, axenic liver stage parasites were used directly for RNA isolation and transcript analysis.

2.3C Obtaining parasite lysates for DNA and protein analysis

To obtain oocyst and salivary gland sporozoite lysates, sporozoites isolated from oocysts (n=~100 mosquitoes) or salivary glands (n=~100 mosquitoes) were either homogenized and used for RNA isolation (see Chapter 2 Section 2.3A) or resuspended with 1X reducing/loading buffer (i.e., 0.5% bromophenol blue, 4% SDS, 10% 2-β-mercaptoethanol) for protein analysis. Erythrocytic stage (i.e., a mixture of rings, trophozoites, schizonts and enriched gametocytes) lysates were prepared by centrifuging infected cells at 2,700 x g for

5 minutes, removing supernatant, adding saponin (Sigma)-RPMI1600 medium (i.e., for the lysis of RBCs to release parasites) to a final concentration of 0.05% and allowing the mixture to incubate for 2 minutes at room temperature. Saponin-treated cells were pelleted at 2,700 x g for 10 minutes and washed at least twice with RPMI 1640 medium. The resulting pellet either was resuspended in 1X PBS for genomic DNA isolation (Qiagen DNeasy Blood and Tissue Kit), 1X reducing buffer for protein analysis or TRIzol reagent (Invitrogen) for RNA isolation.

2.4 Synchronization of erythrocytic stages

Enrichment of erythrocytic stages (e.g. rings used for transfection/ electroporation) was obtained by synchronizing parasites using 5% D-sorbitol (Sigma) according to Lambros and Vanderberg (1979). Briefly, cells were pelleted at 2,000 x g for 5 minutes and resuspended with 5 pellet volumes of 5% D-sorbitol for 5 minutes at room temperature. Cells were centrifuged again at 2,000 x g for 5 minutes and washed three times with 10 pellet volumes of RPMI 1640. After removal of the final wash, pellets were resuspended in culture medium, gassed and placed back into a 37°C incubator until needed for analysis.

2.5 Reverse-transcription PCR (RT-PCR) and PCR analysis

To examine the expression of PfMAL13P1.319 in the oocyst sporozoite, salivary gland sporozoite, axenic liver stages and intraerythrocytic stages, total RNA was isolated using the TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. Total RNA was DNase treated (Ambion) to remove

residual genomic DNA and an 8 µl aliquot of RNA was used as a template for Superscript™ First-Strand Synthesis System RT-PCR (Invitrogen/Life Technologies) to make first-strand cDNA. PCR amplification of the cDNA was performed with gene specific forward (F) and reverse (R) primers:

PfMAL13P1.319-

F: 5'-GAGCACATCGATGGGGAGG-3'

R: 5'-TTAACTTGAGTCTAGACCTCTGG-3'

P. falciparum circumsporozoite gene (CS)-

F: 5'-CGCGGATCCATGATGAGAAAATTAGCTAT-3'

R: 5'-CCGCTCGAGCTAATTAAGGAACAAGAAG-3'

P. falciparum merozoite surface protein 1 (MSP1)-

F: 5'- AAACTAGAAGCTTTAGAAGATGCAG-3'

R: 5'-CCCTTCTTCATTATCTGCATTCTT-3')

Pfs230-

F: 5'- TATAACGCTGAAGAATCTATTCCTC-3'

R: 5'-GTACTAGGTTAAAGCTATCCGAGG-3'

PfHSP70-

F: 5'- AGGTATAGAAACTGTGGGTGG-3'

R: 5'-GATTGGTTGGCATACAGCTTC-3'

P. falciparum merozoite surface protein (PfMSP-1) control gene primers were used to detect transcripts for asexual blood stages; Pfs230 gene primers were used for gametocytes; *P. falciparum* circumsporozoite (PfCS) primers were

used to detect oocyst and salivary gland sporozoites; and *P. falciparum* heat shock protein 70 (PfHSP70) primers were used for liver stages. *P. falciparum* NF54 genomic DNA was a positive control template and the no reverse-transcriptase cDNA control was utilized to make certain that there was no genomic DNA contamination.

PCR analysis of cDNA was carried out using 1.5 mM MgCl2, 0.2 mM dNTP, 2.5 U Taq polymerase (Promega), 0.4 mM of each primer, and 8 µl of cDNA template to a total reaction volume of 50 µl. PCR amplification was performed as follows: 1) initial denaturation at 94°C for 5 minutes; 2) denaturation at 94°C for 30 seconds; 3) annealing at 50°C for 45 seconds; 4) extension at 62°C for 2 minutes and 5) final extension at 62°C for 10 minutes. Each amplification cycle was repeated 34 times. Resulting products were separated via 1% agarose gel electrophoresis and stained with ethidium bromide for visualization.

2.6 Generating recombinant protein for expression and purification

To produce antibodies for immunoblotting and localization studies, the signal peptide region was omitted and the remaining PfMAL13P1.319 gene was divided into thirds (i.e., fragments A-C). First, PCR amplification was performed using gene specific forward and reverse primers for these regions (BamHI and NotI restriction enzyme sites are italicized and underlined):

Fragment A: F: 5'-CG*<u>GGATCC</u>*TTGATCATAAAATATAATGTCAAG-3';

R: 5'-AGT*<u>GCGGCCGC</u>*TTATCCTATTTCATGTAATAACAAA-3'

Fragment B: F: 5'-CG*GGATCC*TTTGATGTATTAATAGTTAATATTGAT-3'

R: 5'-AGT*GCGGCCGC*TTACCCATCGATGTGCTCCTT-3'

Fragment C: F: 5'-CG*GGATCC*GAGGATATAACAAATGATGAAGCC-3'

R: 5'-AGT*GCGGCCGC*TTAACTTGAGTCTAGACCTCTGG-3'

Each fragment was cloned into the pCR®II-TOPO® vector (Invitrogen), sequenced, then digested and cloned into the pET-32a expression vector (Novagen). Recombinant DNA plasmids were transformed into Rosetti-gami cells (Novagen), which enhance disulfide bond formation, and the cells were induced with 1 mM IPTG. Insoluble and soluble fractions were obtained by sonicating harvested cells with 10-second bursts at a medium intensity setting using the Sonic Dismembrator (Fisher Scientific), freezing the lysate in liquid nitrogen for one minute, and thawing the lysate in a 42°C water bath. This rapid freeze thaw-sonication procedure was repeated at least three times. Removal of soluble protein was obtained by centrifugation at 25,000 x g for 20 minutes. The first third region of PfMAL13P1.319 (PfMAL13P1.319-A) was chosen for commercial antibody production because these antibodies would be able to detect truncated-protein production from gene-disrupted mutant parasites. Commercial rabbit polyclonal anti-PfMAL13P1.319-A antibodies were created by Sigma Genosys. The first immunization consisted of 200 µg of recombinant MAL13P1.319-A mixed with Freund's Complete Adjuvant while subsequent immunizations (i.e., a total of five injections) used 100 µg of MAL13P1.319-A plus Incomplete Freund's adjuvant. Recovered antibodies were titrated and tested for recombinant

PfMAL13P1.319-A detection. Preimmune serum also was obtained before immunizations.

All polyclonal anti-PfMAL13P1.319 antibodies were IgG-purified using the Melon Gel IgG Purification Kit (PIERCE). To further purify antibodies to reduce cross-reaction against tissues and pET-32a bacterial protein lysate, two purification methods were performed for either Western blot analysis or immunofluorescent assays (IFAs). For antibodies used in Western blot analyses, anti-PfMAL13P1.319 antibodies were diluted 1:5 with 1X PBS and incubated with pET-32a protein lysate bounded to nickel beads (Novagen His-Bind Resin) at 4°C on a rotating shaker for 4 hours (beads were changed every hour). The pET-32a protein lysates were made by inducing pET-32a-bacterial cells with 1 mM IPTG and lysing the cells to separate soluble and insoluble fractions. To bind pET-32a protein lysate to the beads, the soluble pET-32a lysates were incubated with 50% (w/v) His-Bind resin (Novagen) at 4°C on a rotating shaker overnight. After two washes with Urea buffer (8 M urea, 2 M thiourea, 1% CHAPS, 20 mM DTT) followed by three washes of 1X PBS, the 50% (w/v) beads in 1X PBS were aliquoted and subsequently used for antibody purification.

Antibodies for IFAs were purified using a preabsorption method which consisted of preabsorbing the antibodies against protein lysates of mosquito midgut, mosquito salivary glands, human RBCs and pET-32a bacterial protein lysates (Lacrue et al., 2005). Protein lysates were made by homogenizing mosquito midguts (n=10) or salivary glands (n=20) using a pestle homogenizer

and for RBCs, cells were saponin-lysed/washed. All lysates were resuspended with 1X reducing buffer, denatured at 95°C for 10 minutes and separated on a short, 10% SDS-PAGE mini-gel (approximately 2.5 cm). Proteins were transferred to Optitran nitrocellulose. Each lane was cut into nitrocellulose strips and individually incubated in a 1.5 ml eppendorf tube with 1 ml of 1:10 diluted anti-PfMAL13P1.319 antibodies or 1:10 diluted preimmune serum for at least one hour on a rotator at 4°C. Anti-PfMAL13P1.319 antibodies and preimmune sera were each incubated with three strips of proteins from midguts, salivary glands, RBCs, and pET-32a protein lysates.

2.7 Western blot analysis

Erythrocytic stage parasite lysates were prepared by saponin-treating parasite-infected RBCs and resuspending the pellet with a 1:1 dilution of 1X reducing buffer and 1X PBS. Lysates were separated on a small 10 or 12% SDS-PAGE gel and transferred onto a nitroceullose membrane. Membranes were blocked with 5% non-fat dry milk-Tris buffered saline (TBS; 0.15 M NaCl, 50 mM Tris) for approximately 1 hour at room temperature, washed three times in 1X TBS, then incubated with a 1:100 dilution of purified preimmune serum (i.e., used a negative control to show no cross-reacting antibodies) or rabbit-anti-PfMAL13P1.319 antibodies in TBST (TBS/0.05% Tween 20) overnight at 4°C on a shaker. Recombinant PfMAL13P1.319-A protein was used as a positive control. After overnight incubation, the membrane was washed three times with 1X TBST, and incubated with a 1:20,000 dilution of secondary antibody (i.e.,

peroxidase-conjugated anti-rabbit IgG; Sigma) and visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). There were at least three biological replicates performed for Western blot analyses.

2.8 Immunofluorescence assays (IFAs) and confocal microscopy

For immunofluorescence analysis of the PfMAL13P1.319 protein during the intraerythrocytic stages, parasite-infected blood smears (i.e., the same parasites as were used for transcriptional analysis) of mixed intraerythrocytic stages or mixed gametocyte stages were made on glass slides, fixed with 100% ice-cold methanol and 100% acetone at -20°C, washed three times with 1X PBS and blocked with 1X PBS/10% fetal bovine serum (FBS) for 4 hours. Slides were then incubated with either preabsorbed control preimmune serum or preabsorbed anti-PfMAL13P1.319 antibodies in 1X PBS/10% FBS (1:50) overnight at 4°C. Smears were washed three times with 1X PBS then incubated with control primary antibodies (1:200) (i.e., anti-merozoite surface protein 1 (MSP1) monoclonal antibody for asexual stages or anti-Pfs230 monoclonal antibody for gametocytes) (MR4, 2011). Lastly, smears were washed with 1X PBS and double-labeled with TO-PRO-3 nuclear stain (1:50) (Molecular Probes) and secondary antibodies Alexa-488 and -586 (1:200) (Molecular Probes) for 2 hours at room temperature. After three washes, one drop of ProLong® Gold (Invitrogen) antifade reagent was added to the slide followed by a coverslip. Parasites were analyzed using a Zeiss Laser Scanning Microscope (LSM) 510 META NLO two-photon point-scanning confocal microscope with a 100X oil

immersion objective. An estimated 300 parasites were examined for each of the three biological replicates of mixed erythrocytic stages, while approximately 100 gametocytes (i.e., mixed gametocyte stages) were examined for each of the two biological replicates.

Oocyst and salivary gland sporozoite slides were made according to LaCrue et al., (2005). Briefly, isolated sporozoites were resuspended in 4% paraformaldehyde (alcohol-free) in 1XPBS/0.075% Triton-X (Fisher Scientific) for 30-60 minutes and then 1,000-10,000 sporozoites were spotted onto ringed slides coated with 3-aminopropyltriethoxysilane (Sigma). After overnight air-drying, slides were washed 3-4 times with 1XPBS/0.1% TritonX-100 by placing 40-100 µl of wash solution on the ring and allowing the solution to incubate for 5 minutes. After washing, slides were blocked with 40-100 µl of 2% goat serum (Jackson ImmunoResearch) plus 2% bovine serum albumin (BSA; USBiological) for 4 hours at room temperature. Preabsorbed preimmune or anti-PfMAL13P1.319 antibodies were diluted (1:25) in 1% goat serum plus 1% BSA and a volume of 40-100 µl incubated on the slides overnight at 4°C. Slides were washed with 1XPBS/0.1% TritonX-100 and incubated with mouse anti-*P. falciparum* circumsporozoite antibodies (anti-PfCS; a positive control that detects the circumsporozoite protein on the surface of the parasite; 1:200) for 4 hours at room temperature (MR4, 2011). After three washes, Alexa-labeled anti-rabbit (Alexa-488; 1:200), anti-mouse IgG (Alexa-586; 1:200), and TOPRO3 nuclear stain (1:50) were combined and incubated on the slides for at least 2 hours at

room temperature with no light exposure. Three to four washes using 1XPBS/0.1% TritonX-100 were completed and one drop of ProLong® Gold antifade reagent was added to the slide followed by a coverslip. Parasites were examined using a Zeiss 510 LSM. Two biological replicates of oocyst sporozoites were performed and 150 oocyst sporozoites/ replicate were counted and analyzed for PfMAL13P1.319 expression. At least two biological replicates of salivary gland sporozoites at days 14, 15, 16 and 20 PE were performed with100-150 sporozoites/day/replicate observed. Additionally, one biological replicate of sporozoites at days 17, 18, 19, and 21 PE with a minimum of 20 sporozoites for each time point were observed.

2.9 PfMAL13P1.319-GFP construct design

PfMAL13P1.319 was PCR-amplified from a NF54 parasite strain genomic DNA template using forward primer and reverse primers:

F: 5'-CCG*CTCGAG*GGAGCACATCGATGGGGAG-3'

R: 5'-*CCTAGG*ACTTGAGTCTAGACCTCTGGA-3'

(XhoI and AvrII restriction enzymes are italicized and underlined). The resulting product was cloned into the pPM2GT vector (Klemba et al., 2004) to generate the plasmid designated as MpPM2GT. MpPM2GT allows the green fluorescent protein (GFP) to be controlled under the endogenous promoter of PfMAL13P1.319 and selected using the drug-resistance cassette containing human dihydrofolate reductase (DHFR) sequence (Duraisingh et al., 2002). This

MpPM2GT plasmid was sequenced to verify correct nucleotides and to ensure that the sequence was in frame, thereby allowing expression of GFP.

2.10 Isolating clonal populations of PfMAL13P1.319-GFP parasites and genotype analysis via PCR and Southern blot analysis

In order to transfect MpPM2GT into NF54 parasites, sorbitol-synchronized ring stage parasites at a 2-3% parasitemia were electroporated with 50-100 μg of MpPM2GT plasmid DNA using low voltage conditions (BTX 600; 0.2 cm cuvette, 0.31 kV, 950 μF) (Fidock and Wellems, 1997; Lambros and Vanderberg, 1979). Selection for parasite integrants (i.e., plasmid integration into the genome or carried within the cell) was performed using 5 nM WR99210 (Jacobus Pharmaceutical Company Inc.) with three drug selection cycles (i.e., each WR99210 drug cycle = 4-6 weeks with drug and 3-4 weeks of drug removal).

To assess the genotype of the parasite and detect if the parasites had successfully integrated the construct, a PCR analysis was performed using:

Wild-type control primers-

F: 5’-CCGCTCGAGGGAGCACATCGATGGGGAG-3’

R: 5’-CCTAGGACTTGAGTCTAGACCTCTGGA-3’

Episome control primers (hDHFR drug cassette)-

F: 5’-ATGGTTGGTTCGCTAAACTGC-3’

R: 5’-TTAATCATTCTTCTCATATACTTC-3’

Integration primers-

F: 5’-TGATAAAAATGGACAAACAGGG-3’

R: 5’-TCCGTATGTTGCATCACC-3’

PCR conditions were performed using the same conditions described earlier in Chapter 2 Section 2.5, however in a 20 µl reaction volume.

To further confirm the genotype, Southern blot analyses were done using 3-5 µg of DNA (i.e., either from *P. falciparum* NF54 genomic DNA, plasmid DNA or MpPM2GT parasite culture DNA) that was digested with 20 units of AleI and BamHI restriction enzymes for 4 hours in a 37°C water bath. Digested DNA was separated by a 0.8% electrophoretic agarose gel and transferred to a nylon membrane (Osmonics). Membranes were UV crosslinked and pre-hybridized at 65°C in Church buffer (0.17% Phosphoric acid, 1% BSA, 1 mM EDTA, 7% SDS, 3.5% sodium phosphate dibasic) for 2 hours. After pre-hybridization, the membrane was hybridized with PfMAL13P1.319-specific probes which were amplified using the following primer sets and digoxigenin (DIG; Roche Applied Science)-labeled:

F: 5’-CCGCTCGAGGGAGCACATCGATGGGGAG-3’

R: 5’-CCTAGGACTTGAGTCTAGACCTCTGGA-3’

Membrane and probe incubations were performed at 50°C in Church buffer overnight and washed twice with 2X SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0 using 1 M HCL) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 minutes and twice with 0.5X SSC containing 0.1% SDS at 65°C for 15 minutes. Detection of DNA was achieved using the DIG Kit (Roche Applied Science) according to manufacturer’s instructions.

CSPD (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.13,7] decan-4-yl) phenyl phosphate; Roche Applied Science) was used for chemiluminescence detection and membranes were exposed to X-ray film.

After the genotype was confirmed, clonal populations were obtained by performing limiting dilutions (Maher et al., 2008). Limiting dilutions were done by diluting parasites with culture medium to 0.5 and/or 0.25 parasites/well in two 96-well plates. Each well consisted of 200 µl of a 2.5% hematocrit (i.e., the ratio of blood to RPMI 1640 culture medium containing 0.5% Albumax II and hypoxanthine [50 mg/L]). Plates were placed in a gassed (3% O_2, 3% CO_2 and 94% N_2) sealed Modular Incubator Chamber (Billups-Rothenberg Incorporated) at 37°C. To increase humidity, a small tissue culture plate with RPMI 1640 medium was placed inside the chamber. Chambers were gassed every second day, while culture medium was changed once a week with a fresh 0.5% hematocrit. On days 20-24, smears were made using parasites from random wells to determine if parasites were present. Wells containing parasites were expanded in 12-well plates at a 3% hematocrit in 2 mL of culture medium. Once the parasitemia increased to approximately 2%, half the culture was cryopreserved in Glycerolyte 57 (Baxter) and stored in liquid nitrogen while the other half was expanded into a T25 flask (Corning Incorporated) at a 3% hematocrit in 10 mL of culture medium. PCR and Southern blot analysis were performed to identify MpPM2GT clones.

2.11 Fluorescence microscopy for GFP trafficking studies

Stable transgenic *P. falciparum* MpPM2GT parasite cultures (i.e., PfMAL13P1.319 and GFP expression controlled under the endogenous promoter) were sorbitol-synchronized to allow specific intraerythrocytic stage parasites to be analyzed. Enriched gametocyte stages were established as mentioned in Chapter 2 Section 2.1 and also were analyzed. Infected red blood cells were resuspended with 5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and incubated at room temperature for 5 minutes. Cells were centrifuged at 18,000 x g for 3 minutes, washed with 1X PBS, and resuspended with a 1:2 volume of 1X PBS. To analyze zygotes and ookinetes for GFP expression, 5-10 midguts of mosquitoes exposed to an infected blood meal were removed 32-36 hours post-infection (PI) and placed in 10 μl of 5 μg/ml of DAPI in 1XPBS on a glass slide. At days 8-10 PI, oocysts were analyzed by the same procedure. Hemolymph sporozoites were isolated by perfusing the mosquito body cavity with 1X PBS and collecting the perfusate (LaCrue et al., 2006). Hemolymph sporozoites were centrifuged at 18,000 x g for 5 minutes and supernatant was removed and the pellet was resuspended with 15 μl of DAPI solution. Sporozoites were placed onto a slide followed with a coverslip. Salivary gland sporozoites were obtained by isolating 5 pairs of infected salivary glands in 5 μg/ml DAPI solution on a glass slide, and then covered with a coverslip for visualization of sporozoites. All slides were examined for DAPI and green fluorescence using an Olympus BX51 fluorescence microscope.

Controls used for the GFP trafficking studies were *P. falciparum* NF54 wild-type parasites (i.e., the negative control) and *P. falciparum* 3D7HT-GFP (i.e., *a P. falciparum* parasite line that constitutively expresses the GFP protein in all parasite stages; the positive control) (Talman et al., 2010). There were three independent biological replicates performed for each of the seven parasite stages analyzed. For each biological replicate, the numbers for each stage analyzed were: 50-100 trophozoites/schizonts/gametocytes, 2-5 zygotes and ookinetes, 5-15 oocysts with sporozoites, 2 hemolymph sporozoites, and ~20 salivary gland sporozoites for each day between day 14-20 PE.

3. Results and Discussion

3.1 Sequence analyses of *P. falciparum* MAL13P1.319

As a result of the *P. falciparum* genome sequencing project and large scale microarray and proteomic studies, there are new opportunities to discover novel drugs and vaccine candidates. Ideal targets to prevent malaria transmission are usually surface antigens and secreted proteins involved in parasite invasion of the mosquito and human hosts (Gordon et al., 1995; Kubler-Kielb et al., 2010; Malkin et al., 2008). In an effort to discover molecules involved in parasite invasion, the *P. falciparum* gene PfMAL13P1.319 (also known as PF3D7_1363700) was selected, via *in silico* screening of the *Plasmodium* proteome database, as a sporozoite gene candidate based upon its signal peptide prediction, expression during the sporozoite stage parasite and surface/secreted antigen prediction. PfMAL13P1.319 is a 790-amino acid protein

that encodes a 92.8-kDa protein with the signal peptide and 89.1-kDa protein without the signal peptide. Signal peptide predictions according to SignalP and TargetP programs demonstrated that PfMAL13P1.319 has a signal peptide, and therefore should enter the secretory pathway. In support of this prediction, Softberry and PSORTII are two localization programs that predicted PfMAL13P1.319 to be either a secreted or plasma membrane protein. Based on topology predictions, there is one transmembrane domain located within the signal peptide region, which would suggest that PfMAL13P1.319 may be a secreted protein once the signal peptide is cleaved. The ScanProsite program identified many potential enzyme modification sites such as amidation, N-glycosylation and N-myristolation which would suggest that PfMAL13P1.319 may be processed for protein function.

PfMAL13P1.319 was predicted to have a putative indole-3-glycerol phosphate synthase (IGPS) domain according to the PlasmoDB malaria database (Kissinger et al., 2002), GeneDB (Hertz-Fowler et al., 2004), and the Pfam pattern profile program, thereby suggesting that PfMAL13P.319 may function in parasite metabolism. IGPS is an enzyme that catalyzes the fourth step in the tryptophan biosynthetic pathway (i.e., indole ring-closure reaction) where the substrate 1-(o-carboxyphenylamino)-1deoxyribulose-5’-phosphate (CdRP) is converted to indole 3-glycerol phosphate (Creighton and Yanofsky, 1966). PfMAL13P1.319 protein alignment with IGPS displayed a 10.1% amino acid homology (specifically 5.0% identical amino acids) and no functional catalytic

residues were present. Based on this result, PfMAL13P1.319 would not seem likely to function as an IGPS.

BLAST searches of *P. falciparum* MAL13P1.319 performed on PlasmoDB and NCBI showed significant homology with a protein in the human malaria parasites *P. vivax* (60.8%) and *P. knowlesi* (62.5%); chicken malaria parasite *P. gallinaceum* (53%); rodent malaria parasites *P. berghei* (60.3%), *P. chabaudi* (63%) and *P. yoelii* (61.6%) and the non-human primate parasite *P. reichenowi* (81%) (Figure 2.1). In addition, BLAST searches demonstrated lower protein homology with other Apicomplexan proteins such as *Toxoplasma gondii* (15.4%); *Neospora caninum* (14.6%); *Theileria annulata* (30%); and *Babesia bovis* (26%). There were no orthologs in other higher eukaryotic organisms suggesting that MAL13P1.319 appears to be unique to *Plasmodium* and has identical regions and amino acids in proteins from the phylum Apicomplexa. Therefore, MAL13P1.319 could be an attractive candidate for drug and vaccine targets.

In addition, the alignment of the MAL13P1.319 protein and its orthologs identified conserved regions of unknown function. These conserved regions were analyzed by BLAST analysis, using the short, nearly exact match program from the NCBI database (Altschul et al., 1997); however, only the indole-3-glycerol phosphate synthase domain was identified from this search. Interestingly, a repeat sequence (repeated four times) of aspartic acid, isoleucine, asparagine, and histidine (D-I-N-H) is specific only to the MAL13P1.319 ortholog in *P. falciparum*. Studies have shown that repeat regions

have a role in binding to host receptors and immune evasion, which may be a potential role for the repeat region of PfMAL13P1.319 (Anders et al., 1988; Anders et al., 1993; Cowman et al., 1985; Magowan et al., 2000; Waller et al., 1999). Since repeats are common in *Plasmodium* proteins located on the merozoite surface and have roles as antibody targets, in trafficking, and interact with actin/myosin, this repeat region of PfMAL13P1.319 also may have a functional and/or structural role that may be essential to the parasite (Daher et al., 2010; Mills et al., 2002; Polley et al., 2003; Wickham et al., 2001).

3.2 *P. falciparum* MAL13P1.319 transcript is present in intraerythrocytic stage parasites, oocyst sporozoites and salivary gland sporozoites

To determine the transcript expression profile of the intron-less 2.3-kb PfMAL13P1.319 gene throughout various parasite stages, cDNAs from mixed asexual blood stages, enriched gametocyte cultures, day 8-10 PE oocyst sporozoites, day 14-17 PE salivary gland sporozoites and axenic liver stages were analyzed via reverse-transcriptase PCR (RT-PCR) using PfMAL13P1.319 gene specific primers. PfMAL13P1.319 transcripts were present throughout all the tested stages except axenic liver stages, suggesting that PfMAL13P1.319 transcript expression is stage-specific (Figure 2.2). *P. falciparum* genomic DNA was used as a positive control while a no reverse transcriptase reaction showed that there was no genomic DNA contamination.

Le Roch et al. (2003) used a high density oligonucleotide array to determine the transcript expression profile of *P. falciparum* where they

specifically examined the transcriptome of mosquito salivary gland sporozoites, asexual blood stages (i.e., rings, trophozoites, schizonts and merozoites) and gametocytes. Although these authors did not analyze transcript expression in oocyst sporozoites and axenic liver stages, their study complemented the RT-PCR data performed earlier, supporting that PfMAL13P1.319 transcript was present during the mosquito oocyst and salivary gland sporozoite stages and intraerythrocytic stages (i.e. a mixture of trophozoites, schizonts, and gametocytes) (Table 2.1). More specifically, the high density oligonucleotide array data showed a lower level of PfMAL13P1.319 transcript expression during the salivary gland sporozoites and ring stage parasites than trophozoites, schizonts and gametocytes. Since the RT-PCR study did not distinctly observe asexual stage time points, it is not certain what specific parasite stage may contribute more to PfMAL13P1.319 transcript expression. In addition, Siau et al. (2008) performed a transcriptome analysis via DNA microarray of salivary gland sporozoites and sporozoites incubated for one hour at 37°C with hepatocytes in order to assess if there were molecular differences between inactive salivary gland sporozoites and activated (a state of "readiness" for hepatocyte invasion) *P. falciparum* salivary gland sporozoites. The authors reported the PfMAL13P1.319 transcript to be upregulated 3-fold, suggesting that PfMAL13P1.319 may prepare or help activate the sporozoite for successful infection of the host tissue. Overall, RT-PCR and DNA microarray transcript

results showed a diverse transcript expression profile where PfMAL13P1.319 was present in many parasite stages.

3.3 Expression of recombinant PfMAL13P1.319-A and antibody specificity

To create anti-PfMAL13P1.319 antibodies that would be used in future protein localization studies, the PfMAL13P1.319-A fragment was cloned into the pET-32a vector and protein was induced in Rosetta-gami cells. Polyclonal antibodies were raised against PfMAL13P1.319 inclusion bodies in rabbits and purified according to the methods described earlier. Anti-PfMAL13P1.319 antibodies detected recombinant PfMAL13P1.319 (49 kDa) while purified preimmmune antiserum (i.e., the negative control) did not recognize this protein (Figure 2.3). In addition, purified anti-PfMAL13P1.319 antibodies did not recognize pET-32a-tag proteins, suggesting that these antibodies were specific and had minimal to no antibodies that would recognize the pET32a bacterial protein lysates.

3.4 Analysis of *P. falciparum* MAL13P1.319 protein presence in the intraerythrocytic stages

To assess if there were a correlation between PfMAL13P1.319 transcript and protein presence in the erythrocytic stages, *P. falciparum* asynchronous erythrocytic stage lysates were immunoblotted with purified anti-MAL13P1.319 antibodies. By Western blot analysis, MAL13P1.319 was detected in mixed *P. falciparum* erythrocytic parasites and enriched gametocyte lysates, suggesting that the PfMAL13P1.319 protein is present during the erythrocytic stages (Figure

2.3). The non-infected RBC control demonstrated no cross-reactivity with the antibodies. Anti-PfMAL13P1.319 antibodies did not react against non-infected red blood cells thereby demonstrating no protein cross-reactivity, while preimmune control serum exhibited no cross-reacting antibodies against recombinant PfMAL13P1.319-A and RBCs.

To ascertain PfMAL13P1.319 protein presence and localization on a single-cell level, IFAs were performed using purified anti-PfMAL13P1.319 antibodies with blood smears of mixed *P. falciparum* erythrocytic stages (i.e., rings, trophozoites, schizonts and gametocytes) and mixed gametocyte stages (i.e., I-V). PfMAL13P1.319 was shown to be present during the trophozoite, schizont and gametocyte stages (Figure 2.4). There was minimal to no detection of PfMAL13P1.319 protein during the early and late ring stages, suggesting that PfMAL13P1.319 expression is time and stage-specific. Using MSP-1 antibodies, PfMAL13P1.319 co-localized with the surface protein MSP-1, however due to low resolution, it has yet to be determined if PfMAL13P1.319 is a true surface protein or if it specifically localizes to other areas of the parasite.

In addition to IFAs, a GFP-trafficking approach using the endogenous PfMAL13P1.319 promoter to identify stage-specific expression was performed to investigate the localization/presence of PfMAL13P1.319 throughout the mosquito and human host parasite stages. The 3'-end of PfMAL13P1.319 was cloned in-frame with GFP in the pPM2GT vector (Klemba et al., 2004) yielding MpPM2GT. Following MpPM2GT transfection and drug selection, three clonal populations

(i.e., stable transgenic MpPM2GT parasite cultures) were isolated and showed the presence of integration which is represented by a 4.2 kb band (Figure 2.6A). Episome was also present in clonal populations 1 and 4 (i.e., a 5.1 kb band), but no wild-type allele was detected in any of the three clonal populations. These populations were also analyzed for MpPM2GT protein expression using anti-GFP antibodies. MpPM2GT clones displayed PfMAL13P1.319 protein fused to GFP (~110 kDa), thereby demonstrating and confirming PfMAL13P1.319 expression during the erythrocytic stages (Figure 2.6B).

One line of the transgenic parasites (i.e., MpPM2GT-Clone 81) was analyzed for the localization of PfMAL13P1.319-GFP fusion protein on a single-celled level. Similar to IFA results, green fluorescence was observed in trophozoites and schizonts while early and late ring stages displayed minimal to no expression (Figure 2.7). However, one discrepancy with the IFA results was the lack of GFP fluorescence during the gametocyte stages. This result could be attributed to the nature of GFP fluorescence: 1) GFP expression may be less sensitive than immunocytochemistry using Alexa-conjugated secondary antibodies that gives a stronger signal (Hutter, 2006); 2) Polyclonal antibodies could be cross-reacting against an epitope shared by multiple proteins or targets (Dr. Tom Phillips, personal correspondence); 3) PfMAL13P1.319-GFP protein could be misfolded or processed differently than wild-type PfMAL13P1.319 protein (Cabantous et al., 2005).

High-accuracy mass spectrometry of the *P. falciparum* proteome has been used to identify proteins that could be potential drug and vaccine targets. A study performed by Lasonder et al. (2002) identified 1,289 proteins in the parasite line NF54 from trophozoites, schizonts, gametocytes, and gametes using a large-scale, high accuracy mass spectrometric proteome approach. Florens et al. (2002) also utilized a similiar approach using the parasite line 3D7 and identified over 2400 proteins from salivary gland sporozoites, merozoites, trophozoites and gametocytes. Unlike the previous results using the IFA and GFP trafficking studies, Lasonder et al. (2002) and Florens et al. (2002) did not report the presence of the PfMAL13P1.319 protein in any of the observed intraerythrocytic stages (i.e., trophozoites, schizonts, merozoites, and gametocytes) (Table 2.1). Lasonder et al. (2002) stated that some peptides were not sequenced due to the complexity or stage specificity of the sample (e.g. sexual stages) which caused changes in protein abundance between the stages. In addition, these authors reported that a known asexual stage specific protein (i.e., PfEMP1), surprisingly did not exhibit high-scoring peptides, which they stated could be due to parasite strain specificity (i.e., expressed at low levels in the NF54 strain) or poor extraction of the proteins. Therefore, these reasons may explain why PfMAL13P1.319 protein was not detected in the erythrocytic stages by these authors. Another explanation for the conflicting data is the type of technique used to assess protein expression. Lasonder et al. (2002) and Florens et al. (2002) both used a global profile, large-scale mass spectrometry approach

while the IFA and GFP studies were performed on a specific single-cell level. Unlike the IFAs and GFP studies performed earlier, both sets of authors synchronized the asexual blood stages and collected these parasites at specific time points which may have prevented the analysis of proteins that may have been expressed before the collection time point and therefore they did not detect the PfMAL13P1.319 protein.

3.5 Analysis of *P. falciparum* MAL13P1.319 protein expression in the mosquito parasite stages

To determine if PfMAL13P1.319 was present within the mosquito stages (i.e., zygotes, ookinetes, oocyst sporozoites, hemolymph sporozoites and salivary gland sporozoites), IFAs and GFP trafficking studies were performed. The IFA studies examined day 10PE oocyst sporozoites and day 14-21PE salivary gland sporozoites and the GFP studies also analyzed these same stages as well as zygotes, ookinetes and hemolymph sporozoites. By IFAs, anti-PfMAL13P1.319 antibodies did not detect PfMAL13P1.319 protein in day 10PE oocyst sporozoites and salivary gland sporozoites and preabsorbed preimmune (i.e., the negative control) displayed minimal to no cross-reactivity with the sporozoites (Figure 2.5). GFP trafficking studies also did not detect PfMAL13P1.319 protein in any of the stages analyzed, although 3D7HT-GFP parasites (i.e., the positive control) were observed in all stages, thereby suggesting no technical issues in the method used (Figure 2.7). Hence, PfMAL13P1.319 was either expressed in undetectable amounts or not expressed

during these mosquito parasite stages. As mentioned earlier, Florens et al. (2002) performed a high-throughput protein mass spectrometry study to identify proteins isolated from day 14PE salivary gland sporozoites. In this study, the PfMAL13P1.319 protein was reported to be expressed during day 14PE sporozoites (Table 2.1) and cross-correlation scores measured by SEQUEST for peptide/spectrum matches demonstrated that PfMAL13P1.319 protein expression (i.e., a spectrum of 1) was approximately ten times less than the dominant surface sporozoite protein, circumsporozoite (spectra of 10). Therefore, the results would suggest that PfMAL13P1.319 protein expression is extremely low during the sporozoite stage, which was consistent with the single cell IFA and GFP results that showed little to no expression in this stage. Unlike the IFA and GFP studies, Florens et al. (2002) did not examine proteins from zygotes, ookinetes, oocyst sporozoites, hemolymph sporozoites, and "older" salivary gland sporozoites, therefore no analysis of PfMAL13P1.319 presence in these mosquito parasite stages can be elucidated from Florens et al. (2002). Overall, these results support that PfMAL13P1.319 presence during the intraerythrocytic stages may serve an important purpose.

4. Chapter Summary

This chapter discusses an *in silico* approach to identify a novel protein known as *P. falciparum* MAL13P1.319, and the molecular and cellular characterization of PfMAL13P1.319. Previous studies performed by Florens et al. (2002) demonstrated that the PfMAL13P1.319 protein was expressed during the

sporozoite stage and not during the trophozoite, merozoite, and gametocyte stages. Additionally, Lasonder et al. (2002) did not detect the PfMAL13P1.319 protein during the trophozoite, schizont, gametocyte and gamete stages. In comparison to these results, protein expression profiles of PfMAL13P1.319 via IFAs and GFP studies, reported in this chapter, showed PfMAL13P1.319 to be present during the trophozoite, schizont and gametocyte stages and had minimal to no detection during the salivary gland sporozoite stages. In addition, the IFA and GFP studies examined other parasite stages not investigated via protein mass spectrometry, such as rings, zygotes, ookinetes, oocyst sporozoites, and hemolymph sporozoites. As a result, the PfMAL13P1.319 protein was not detected in any of these stages. PfMAL13P1.319 appears to be stage-specific and may have a role during the intraerythrocytic stages. Other interesting features of PfMAL13P1.319 were the well-conserved orthologs in many other *Plasmodium spp*. and presence of a unique D-I-N-H repeat sequence, which could serve a specific purpose for PfMAL13P1.319 function or structure.

Plasmodium MAL13P1.319 Transcriptome Data

	Rings	Trophozoites	Schizonts	Merozoites	Gametocytes	Gametes, Zygote, Ookinete	Sporozoite	Sporozoites incubated 1 hr at 37°C with hepatocytes	Liver Stages
Expression	[1] No, *P. falciparum*	[1] Yes, *P. falciparum*	[1] Yes, *P. falciparum*	[1] Yes, *P. falciparum*	[1] No, *P. falciparum*	N/A	[1] Yes, *P. falciparum* [2] Yes, *P. falciparum*	[1] Yes, *P. falciparum*	N/A
Author	[1] Le Roch et al., Science. 2003 Sep 12;301(5639):1503-8	[1] Le Roch et al., Science. 2003 Sep 12;301(5639):1503-8	[1] Le Roch et al., Science. 2003 Sep 12;301(5639):1503-8	[1] Le Roch et al., Science. 2003 Sep 12;301(5639):1503-8	[1] Le Roch et al., Science. 2003 Sep 12;301(5639):1503-8	N/A	[1] Le Roch et al., Science. 2003 Sep 12;301(5639):1503-8. [2] Siau et al., PLoS Pathog. 2008 Aug 8;4(8)	[1] Siau et al., PLoS Pathog. 2008 Aug 8;4(8)	N/A

Plasmodium MAL13P1.319 Proteome Data

	Rings	Trophozoites	Schizonts	Merozoites	Gametocytes	Gametes, Zygote, Ookinete	Sporozoite	Liver Stages
Expression	N/A	[1] No, *P. falciparum* [2] No, *P. falciparum*	[1] No, *P. falciparum*	[1] No, *P. berghei, P. chabaudi adami* and *P. yoelii* (17XL) merozoite [2] No, *P. falciparum*	[1] No, *P. falciparum* [2] No, *P. falciparum* [3] No, *P. berghei*	[1] No , *P. falciparum* gametes	[1] Yes, *P. falciparum*	[1] No, *P. berghei* and *P. yoelii*
Author	N/A	[1] Florens et al., Nature. 2002 Oct 3;419(6906):520-6 [2] Lasonder et al., Nature. 2002 Oct 3;419(6906):537-42	[1] Lasonder et al., Nature. 2002 Oct 3;419(6906):537-42	[1] Sam-Yellowe et al., J Proteome Res. 2004 Sep-Oct;3(5):995-1001 [2] Florens et al., Nature. 2002 Oct 3;419(6906):520-6	[1] Florens et al., Nature. 2002 Oct 3;419(6906):520-6 [2] Lasonder et al., Nature. 2002 Oct 3;419(6906):537-42 [3] Khan et al., Cell. 2005 Jun 3;121(5):659-60	[1] Lasonder et al., Nature. 2002 Oct 3;419(6906):537-42	[1] Florens et al., Nature. 2002 Oct 3;419(6906):520	[1] Tarun et al., Proc Natl Acad Sci U S A. 2008 Jan 8;105(1):305-10. Epub 2008 Jan 2

N/A= data not available

Table 2.1. *Plasmodium* transcriptome and proteome data from literature sources.

```
                  1                                                                                                                       125
PfMal Orf   (1)   -------------MIARIYKYIITLFYFLLYIFILFHCLNSLIIKYNVKVEGLRLVSRMK--RGKILKGCFPKDGILHNHYISTLFIKNDGEIYNKYIGGKKEKVRKRKNDIGGRVRFRRYGKDS
PbMal Orf   (1)   -------------MPRIKLIFLLIICTIRYVFSTKVTKITKVTNRSLGMPKLAYCGNINGDRKYYIQTCDFAKKRRRKNENPSLHVKKK----------------------KNIYIKIYGRDH
PcMal Orf   (1)   ------------WKMLRINLIFLLIIYTIGYVFS----AKITKTNRSLGMPKVEYYGNINGDRRYYIQTCDIAKSRRRKNENPSLHVKKN----------------------KIKYIKLYGRDH
PgMal Orf   (1)   --------------------------------------------------------------------------------------------------------------------------
PkMal Orf   (1)   -----------------MRRFALIIIITVVVITTC-----IYSVKRTSPRQNLQILEKR--------KKISFVRYTQTKDHIRSSILKRN----------------------EKPNLRIYGKDH
PvMal Orf   (1)   ------------METPKMGRFPLAIILTVAVLTMC-----AYSVKRAAPRQGLHILGRR--------KKCHFVRHPQRKDHMRSAIPKGK----------------------ANVQLRIYGKDH
PyMal Orf   (1)   -------------MLRINLIFLLIIYTIRYVFS---IKITKTTNKSLGVSKLGYCGNINGDRKYYIQMCDFAKNRRRNNENPSLHVKKN----------------------KKKYIKIYGRDH
PrMal Orf   (1)   HNLYSIRYDETKNMIARIYKFIITLFYFLLCIFILFHCLNTLIIKYNVKVEGLRLVSRMK--RGKILKGCFPKDGILNKHYISTLFIKNDGEIYNKYIGGKKGTVRKRKNDIGGSVSFRRYGKDS
                  126                                                                                                                     250
PfMal Orf   (111) RNPHLAYEYINKIIENKKYEITKLLEENCDENNPLQIRMKYLQHTMNNKLSESLKRSNNDEKHRLSLIADMKRKIFCSIEKEKKKEKDHKTKEGLYNNINVGYDNLQDEKYIYEDILKNEKEFDD
PbMal Orf   (89)  RNPYLAYEHINRIIENKKYEVTKLLEENADENSPLQIRLKYLQHTMNNKLSESLKIIHSDEKHRLSMVADIKRKVFCSTNKADEN-------------------YDLKKNINNYEIENERKKNE
PcMal Orf   (87)  RNPYLAYEHINRIIENKKYEVTKLLEENADENSPLQIRLKYLQHTMNNKLSESLKRIHSDEKHRLSMVADIKRKVFCSTNTNDEN-------------------LDLKKNIDNYEIENERKQNE
PgMal Orf   (1)   ----------------------IIRRNCDENNPLQIRMKYLQHTMNNKLTENLKRVNSDEKHRLSLIADIKRKVFCSSNWKKGDN---------------------NLMDG---MGKNEKKEH
PkMal Orf   (73)  RNPHLAYEYVNKIIENKKYEVSKLLEENFEEDNPLQIRLKYLQHTMNNKLSESLKRANPDEKHRLSMVADMKRKIFCSVSREKEGS-----------------HSTVDNVINEEGMSPMEESSG
PvMal Orf   (78)  RNPHLAYEYVNKIIENKKYEVSKLLEENFEEDNPLQIRMKYLQHTMNNKLSESLKRTSPDEKHRLSMVADMKRKIFCSVSRKEEGS-----------------HHTADEVISEEGVSQTEAVTA
PyMal Orf   (86)  RNPYLAYEHINRIIENKKYEVTKLLEENADENSPLQIRLKYLQHTMNNKLSESLKRIHSDEKHRLSMVADIKRKIFCSTNKNDEN-------------------YDLQENINNYEIENERKKNE
PrMal Orf   (124) RNPHLAYEYINKIIENKKYEVTKLLEENCDENNPLQIRMNYLQHTMNNKLSESLKRSSNDEKHRLSLIADMKRKIFGTIEKEKKKEKEHKTKEGLYKNISVGYNNLQNEKYTYEDILKNEKELHD
                  251                                                                                                                     375
PfMal Orf   (236) DDIMKNNEEKKK-NINNNYIYQHNFLNLSNPGNVSLLLHEIGFDVLIVNIDELSTQGNINDLKDIIKSTRTLPRNKRPAIVVDDIIIHPIQIALAVENQADGVILNLSYLKNDMEEMLQYCVNVG
PbMal Orf   (194) NMINNS----K----NEKYVYQNNFLNLTNPGEASLMLHKIGFDVLIVNIDSLSAQGTLNDLSDVIKSTRTLTRNSRPAVVVDDVIIHPIQIALAVENKADGVILNLSYLKNDLEDMLNYCVNLG
PcMal Orf   (192) KMVNSS----N----NEKYVYQHNFLNLTNPGEASLMLHKIGFDVLIVNIDSLSTQGTLNDLSDVIKSTRTLTRNSRPAVVVDDVIIHPIQIALAVENKADGVILNLSYLRNDLEDMLNYCVNLG
PgMal Orf   (78)  NFSQNP-----------NFVYQNSFLNLADPGNVSLLLHQIGFDVLIVNTDNLSTDGMLNDLSEVIKSTRTLPRKVRPAIVVDDVIIHPIQIALAVENKADGVILNLSYLKNDLEDMLNYCVNLG
PkMal Orf   (180) KNTEKRTRVWK----EENYMYQNNFLNLANPGNVSLMLHEIGFDVLIVNIDNLSTQGNLNDLSDVVRSSRKFSRNVRPAVVVDDVIIHPIQIALAVENKADGVILNLSYLRNDLEDMLNYCVNLG
PvMal Orf   (185) KDTQKGRRIWK----EENYMYQNNFLNLTNPGKVSLLMHEIGFDVLIVNIDNLSAQGNLNDLCDVVRSVRKLTRNVRPAVVVDDVIIHPIQIALAVENKADGVILNLSYLKNDLEDMLNYCVNLG
PyMal Orf   (191) NMINNS----K----NEKYVYQNNFLNLTNPGEASLMLHKIGFDVLIVNIDSLSTQGTLNDLSDVIKSTRTLTRNSRPAVVVDDVIIHPIQIALAVENKADGVILNLSYLKSDLEDMLNYCVNLG
PrMal Orf   (249) VDIMKNNEEKKKKNINNHYIYQHNFLNLSNPGKVSLLLHEIGFDVLIVNIDELSTQGNINDLKDIIKSTRTLPRNKRPAIVVD-----------------------------------------
                  376                                                                                                                     500
PfMal Orf   (360) TQAIVEVHDYNDIYFATQCGAYILMINEYDFYNNIYEYNHAIKAINYTIPEIITIAKVNTNEVNYIEKLGSLGYDSICLEKKLIDDDLQQFVTSCKGWSAPHKTLLYLNRNNYLKEFLTYDKNGQ
PbMal Orf   (311) TQAIVEVHDYNDIYYATQCGSYILMINEFDFINNRYEYNHAIKAISYSIPELITIAKINVSDINYVEKLGSLGYDSICLEKKLIDEDLEVFVQSCKNWKAPHKTLLYLNRNNYLKNFLTYKNN-S
PcMal Orf   (309) TQAIVEVHDYNDIYYATQCGSYILMINEFDFINNRYEYNHAIKAISYSIPELITIAKINVSDVNYVEKLGSLGYDSICLEKKLIDDDLETFVQSCKNWKAPHKTLLYLNRNNYLKDFLTYKNN-S
PgMal Orf   (192) TQAIVEVHDYNDIYFATKCGSYIIMINEFDFLNNQYEYNHAVKAINYTIPEIITIAKINISDINYVEKLGSLGYDSICLEKKLIDDDLENFVHLCKSWNAPHKTLLYLNRNSYLKEFLTYKNE--
PkMal Orf   (301) TQAIVEVHDYNDIYFATQCGSYILMINEFDFLNNEYEYNHAIKAISYTIPELITIAKINVDDFNYAHKLGSLGYDSICLEKKLVDNDLPNFVEACKSWSAPHKTLLYMNRNNYLKNFLTYTNN-P
PvMal Orf   (306) TQAIVEVHDYNDIYFATQCGSYILMINEFDFLNNQYEYNHAIKAISYTIPELITIAKINVDDLNYATKLGSLGYDSICLERKLVDGDLPNFVQACKSWNAPHKTLLYMNRNSYLKNFLTCTNDPS
PyMal Orf   (308) TQAIVEVHDYNDIYYATQCGSYILMINEFDFINNRYEYNHAIKAISYSIPELITIAKINVSDINYVEKLGSIGYDSICLEKKLIDEDLEVFVQSCKNWKAPHKTLLYLNRNNYLKDFLTYKNN-S
PrMal Orf   (332) -----------------------------------HAIKAINYTIP-IITIAKVNTNEVNYIEKLGSLGYDSICLEKKLIDDDLQQFVTSCKAWSAPHKTLLYLNRNNYLKEFLTYDKNGQ
                  501                                                                                                                     625
PfMal Orf   (485) TGENHKDITENLKNLYDDKNISNNYSHTLLKRYEKEHIDGEDITNDEAMNGAINHDISQDINHDINHDISKDINHDISQDINHDIKRYNVLNKDNPMKYKTEGNKNNYAEKKESFENKCIDNKNI
PbMal Orf   (435) PGENYKDTTKNLEKMYDDKNLENKYSEQMLKDYEKEFIN-------------------------------------------------QINQKSENVSQDN-------------------
PcMal Orf   (433) TDENYKDTTKNLEKMYDDKNLENNYSEKMLKDYEKDFIDVEDAKTNEEII-----------------------------KKNETTQ---DPQINQKNENVKQDS-------------------
PgMal Orf   (315) SEEKHKEITDNLKNIYDDKNIGNKYSEKLLKNYERDYIDIEDEKKEEKKE-----------------------------EKKEEKK---DEKKDEKKDEINKRK-------------------
PkMal Orf   (425) SGETHKDITENLKKMYDDKNLTNRNSHELLKNYERDFIDAVEEGALAQSA-----------------------------DPQGTQQGNIDPLPTGSKQNGNGPI------A-------E-----
PvMal Orf   (431) GGETHQDITDNLKKIYDDKNLQNRTSHELLKNYERDFIDAVEEGPPGQSS-----------------------------EPQQTQQGGRDPQETQQGGRDPQETQQGGIDPRQTGDKPNGSNPM
PyMal Orf   (432) PDENHKDTTKNLEKMYDDRNLENKYSEKMLKDYEKDFIDVEDVKKNEEII-----------------------------RKNETTP---ISQINQKSENINQDN-------------------
PrMal Orf   (417) TRDNHKEITENLKNLYDDKNISNNYSHTLLKRYEKEHIDGEDITNDEAINGGIN-----------------------QDISQDIKRYNILNKDNPMKYKIQGNKNNYAEKKESFENKCIDNKNM
                  626                                                                                                                     750
PfMal Orf   (610) KHTNMNKKEEILSKEEKQIVQNFKNEKKRELMLLSQMKEIIKEVDDQCKNYDTNTDEQNQKKEEKLESLLENFTKLDKNFLKGFFSDEEINNIENTMKNAVEQKNKIQSGEINIDHKNLTGYPMG
PbMal Orf   (487) --------SSLLTNDEKKIINNFKQERKNEIMLLNQMKDIIKEVDNQCKNYEHFSEEESIKKEEKLESLLENFTKLDKSFLKQFFSDEEINNIENSIKNAVHQKKKIQDGEINIENT-----NFT
PcMal Orf   (505) --------SSLLTNDEKKIINNFKQERKKEITLLNQMKDIIKEVDDQCKNYDNFSEEESIKKEEKLESLLENFTKLDKNFLKQFFSDEEINNIENSIKNAVQQKKKIQNGEINVDSN-----NFT
PgMal Orf   (387) ---DESTKDNLLTKEENHIIKNFKEEKKKELILLNEMKNIIKEVDEQCKNYEAYSDYENKEEKLESLLENFTKLDKNYLRGFFSDEEIKNIEGTIKQTLLQKKNIEQGNFHFNEKNLT-NDIT
PkMal Orf   (502) --EGEKVKDNLLTSDEKKIVQNFKREKKREIYLLNQMRDIIKQVDDQCKNYESFSDDENKKKEEKLETLLENFTKMDKNFLRGFFSDEEIKNIENSIRKSIQQKEKIEKGEINIDENSVA-RDIY
PvMal Orf   (526) AGEADKTKDNLLTSDEKKIVQNFKREKKRELFLLNQMKDIIKQVDDQCKNYDSFSEEENKKKEEKLETLLENFTKMDKKFLRGFFSDEEIKNIENSIKKSVQQKEKIEKGEMNIDDNPVA-RDMY
PyMal Orf   (504) --------SSLLTNDEKKIINNFKQERKKEITLLNQMKDIIKEVDDQCKNYDNFSEEESIKKEEKLESLLENFTKLDKNFLKQFFSDEEINNIENSIKNAVQQKKKIQDGEINIENN-----NFT
PrMal Orf   (518) KHTNMNKKEEILSKEEKQIVQNFKNEKKRELMLLSQMKEIIKEVDDQCKNYHTNTDEENQKKEEKLESLLENFTKLDKNFLKGFFSDEEINNMENPMKNAVEQKNKIQSGEINIDHKNLTGYPMA
                  751                                     807
PfMal Orf   (735) TLQNSPMNNLDIYNLTKEYFGETQNS-DAHTENIFDSDQNKLLDESTEDSSRGLDSS
PbMal Orf   (599) GFPSNQMNDFDINRLTKEFFDTNGSG------NNHKIDNKLLDDYSDDSSSRGLSSQ
PcMal Orf   (617) GFPSSQMNDFDINRLTKEFFDTNDSG------NGHKIDNKLLDDYSDDSSSRGLDSQ
PgMal Orf   (508) NIENYQNEDIDIHKLTKEFFDNKNSE------NNDTIDNSS---NDLLDDSRGLSSQ
PkMal Orf   (624) GFPANQGENFDITKLTKEFFDNDSGGEKQSDEESHKLGLDSTGDSSDGASSRGVDSQ
PvMal Orf   (650) GIPTTQGDDFDISKLTQEFFDNGSGGETRLDEGSQKLGGDSPSEESPGESSRGVDSQ
PyMal Orf   (616) GFPSNQMNGFDINRLTKEFFDTNDSG------NNHKIDNKLLDDYSDDSSSRGLNSQ
PrMal Orf   (643) TLQNTHMNNLDIYNLTKQYFGETQNS-DGHTENILDSDQNNLLDESSEDSSRGLGSS
```

Figure 2.1. Alignment of the *P. falciparum* MAL13P1.319 (PfMal) protein sequence with its orthologs. PfMAL13P1.319 shares conserved regions with *P. berghei* (PbMal orf= PB000510.01.0 and Pb_5105-4-3059-2532 in PlasmoDB), *P. chabaudi* (PcMal orf= Pc_3576-5-2829-829 in PlasmoDB), *P. gallinaceum* (PgMal orf= Pg_c000129384.Contig1-2-1079-2746), *P. knowlesi* (PkMal orf=PK13_6510w in Sanger Institute), *P. vivax* (PvMal= Pv115140), *P. yoelii* (PyMal orf=chrPyl_00226-5-31074-29071 and PY00839 in PlasmoDB), and *P. reichenowi* (PrMal=reich142b09.plk, reich382d09.qlk, reich915f08.plk, reich838g12.plk). Identical residues are shaded in yellow while similiar amino acids are shown in blue. The signal peptide is shaded green and the sequence used for antibody production is indicated by the horizontal bar. The D-I-N-H repeat sequence is highlighted in red.

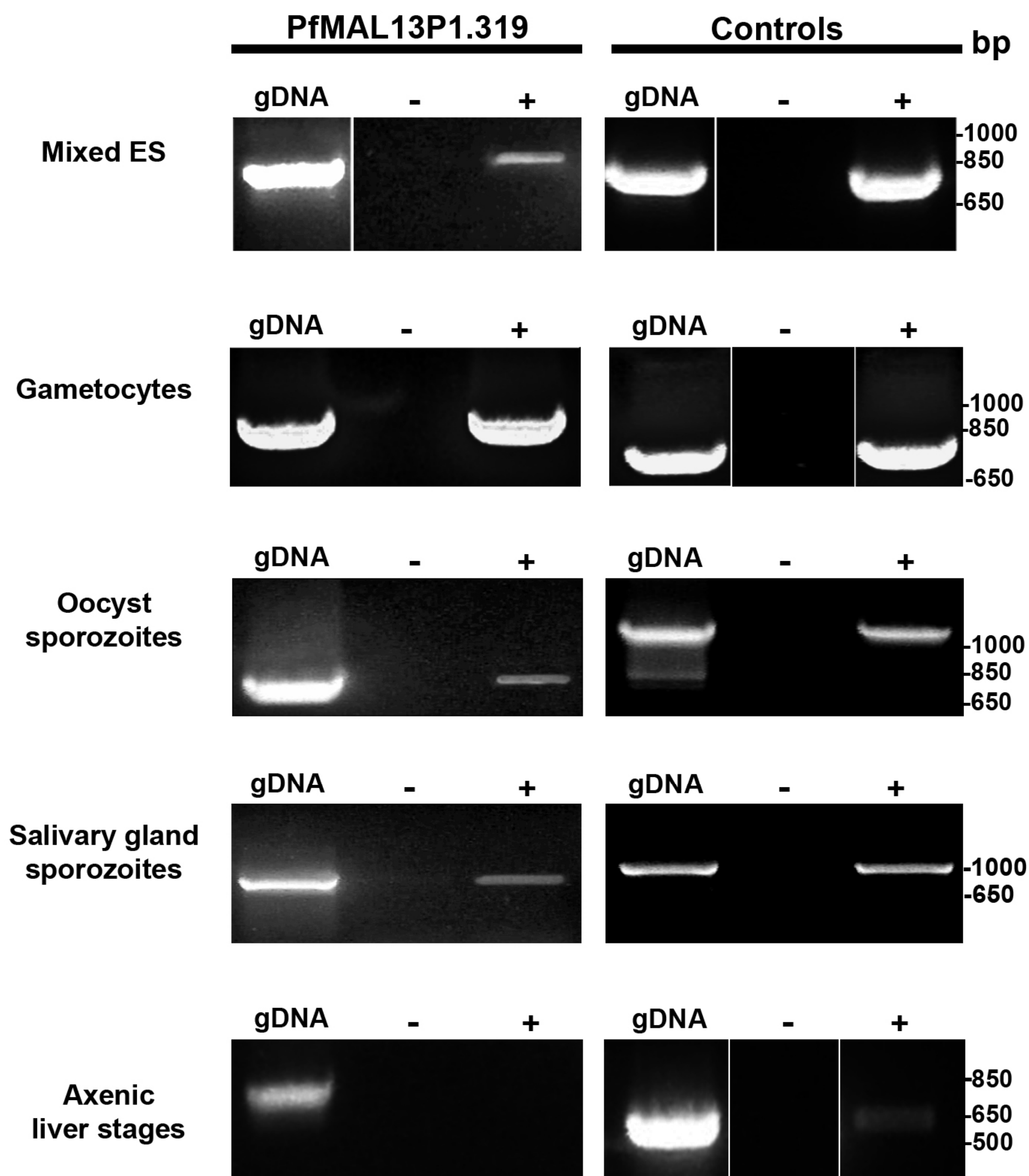

PfMAL13P1.319
Controls
bp
gDNA
-
+
Mixed ES
Gametocytes
Oocyst sporozoites
Salivary gland sporozoites
Axenic liver stages
1000
850
650
500

Figure 2.2. PfMAL13P1.319 transcripts are present in various stages of *P. falciparum*.

MAL13P1.319 transcripts are present in asexual blood stages, gametocytes, oocyst sporozoites, and salivary gland sporozoites as determined by reverse-transcription PCR (RT-PCR). cDNA was produced from RNA isolated from mixed erythrocytic stages, enriched gametocytes, salivary gland sporozoites, oocyst sporozoites, and axenic liver stages. PCR amplifications were performed using PfMAL13P1.319 specific primers, *P. falciparum* merozoite surface protein 1 (MSP1) control primers for blood stage parasites, Pfs230 control primers for gametocytes, *P. falciparum* circumsporozoite (CS) for salivary gland and oocyst sporozoites and *P. falciparum* heat shock protein 70 (PfHSP70) for axenic liver stages. *P. falciparum* genomic DNA (gDNA+) was used as a positive control while a no reverse transcriptase (RT-) reaction showed that there was no genomic DNA contamination. The arrow denotes the 815 bp MAL13P1.319 RT-PCR product. DNA size markers in base pairs (bp) are shown to the right of the figure.

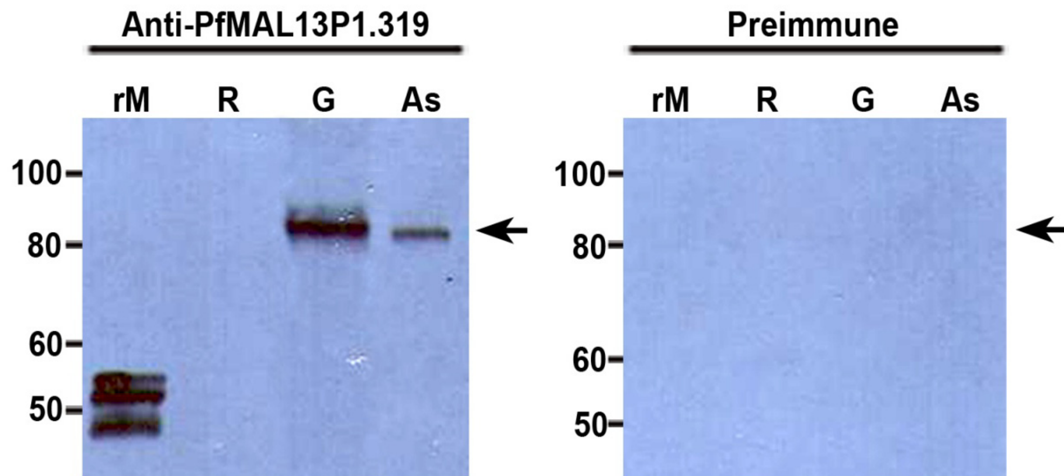


Figure 2.3. PfMAL13P1.319 protein is present during intraerythrocytic stages via Western blot analysis.

PfMAL13P1.319 protein was recognized by anti-PfMAL13P1.319 antibodies in the *P. falciparum* asynchronous asexual blood stages (As) and enriched gametocyte (G) lysates via Western blot analysis (as denoted by the arrow). The preimmune antibody did not detect this protein. The recombinant PfMAL13P1.319-A protein was used as a positive control and the non-infected RBCs (RBC) were used as a negative control. Molecular weight markers in kilodaltons are shown to the left of each figure. Three independent biological replicates were performed and representative images are shown.

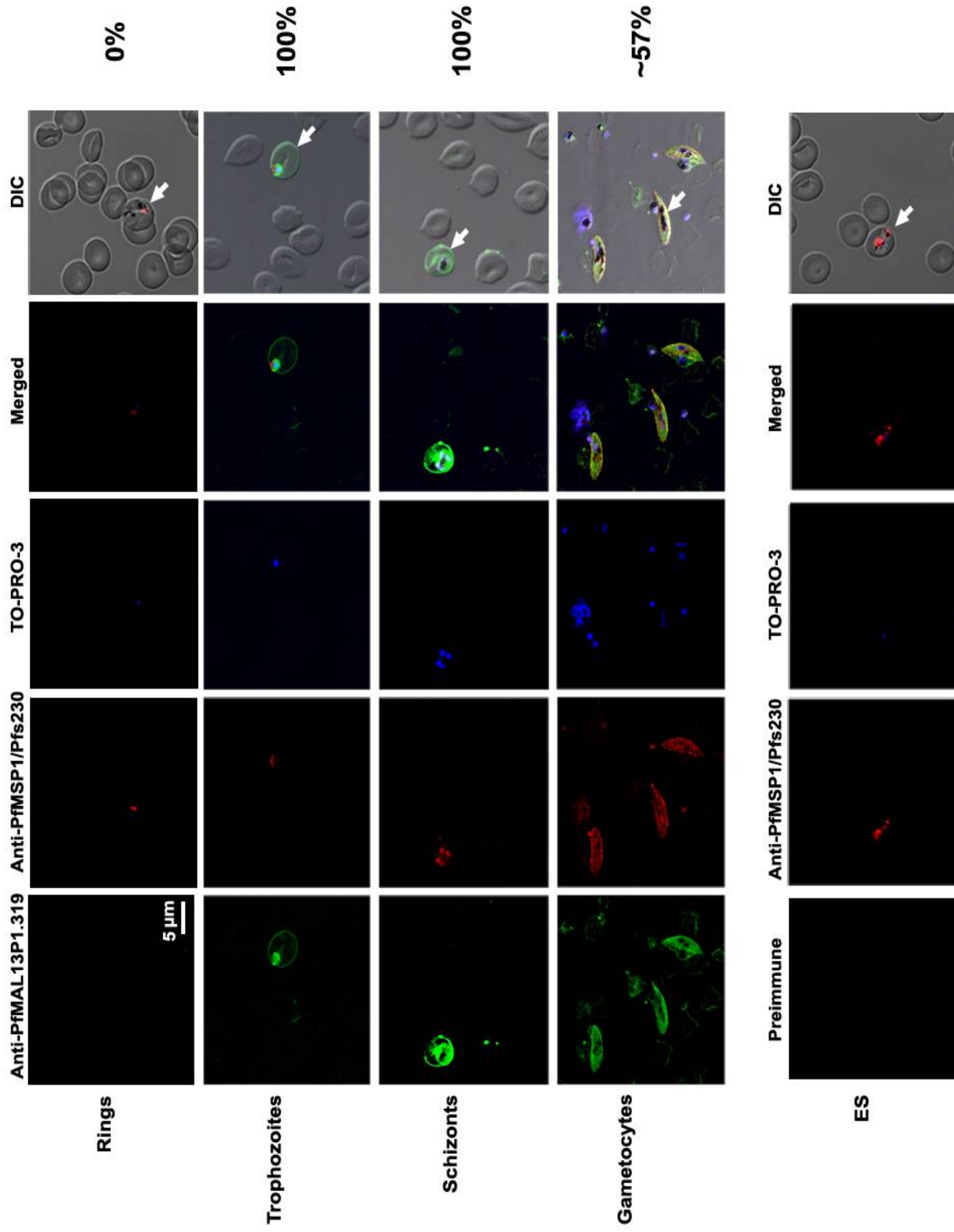

0%
100%
100%
~57%
DIC
Merged
TO-PRO-3
Anti-PfMSP1/Pfs230
Anti-PfMAL13P1.319
5 µm
Preimmune
Rings
Trophozoites
Schizonts
Gametocytes
ES

Figure 2.4. PfMAL13P1.319 protein appears to be present during late intraerythrocytic stages as shown by immuno-localization studies using confocal microscopy. Parasite-infected blood smears were triple-labeled with anti-PfMAL13P1.319 polyclonal antibody (PfMAL13P1.319), either anti-merozoite surface protein 1 (MSP1) monoclonal antibody for asexual stages or anti-Pfs230 monoclonal antibody for gametocytes (used as positive controls), and TO-PRO-3 (nuclear stain). Triplicate biological replicates of parasite-infected blood smears consistently show PfMAL13P1.319 expression during the trophozoite (100%), schizont (100%), and gametocyte stages (~57%) and the protein appears to co-localize with known surface proteins (shown by the yellow color). Approximately 100-300 stage-specific parasites/replicate were observed to calculate the average percentage of PfMAL13P1.319-expressing parasites. The control pre-immune serum displayed minimal cross-reactivity with erythrocytic parasite proteins. Representative images are shown with a scale bar, 5 μm.

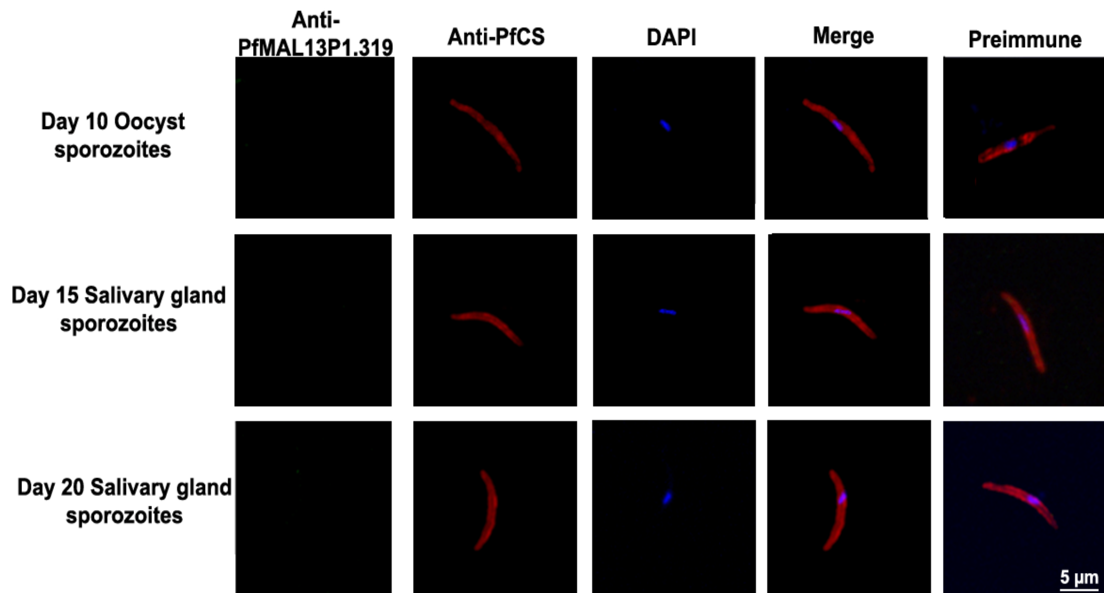
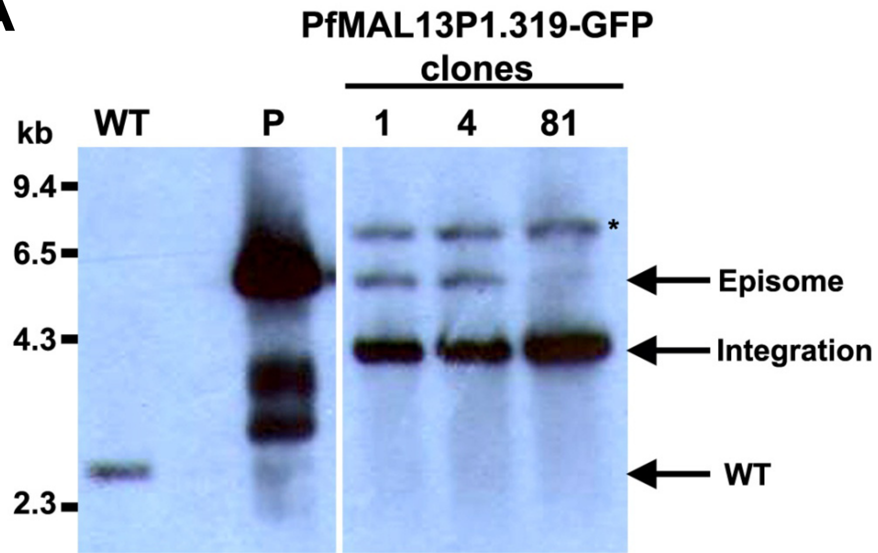


Figure 2.5. PfMAL13P1.319 is not present during the oocyst and salivary gland sporozoite stages via immunofluorescence assays/confocal microscopy. Oocyst and salivary gland sporozoites were triple-labeled with anti-PfMAL13P1.319 polyclonal antibody (PfMAL13P1.319), anti-*P. falciparum* circumsporozoite (PfCS) antibody (used as a positive control), and TO-PRO-3 (nuclear stain). The preimmune serum (i.e., the negative control) shows no cross-reactivity with sporozoites. Additional sporozoite time points (days 14 and16 PE) were examined for PfMAL13P1.319 expression; however there was no detection of the protein (data not shown). Two or three biological replicates were done with 100-150 sporozoites/replicate for each time point observed. Representative images are shown with a scale bar, 5 µm.


A
PfMAL13P1.319-GFP
clones
kb
WT
P
1
4
81
9.4
6.5
4.3
2.3
*
Episome
Integration
WT

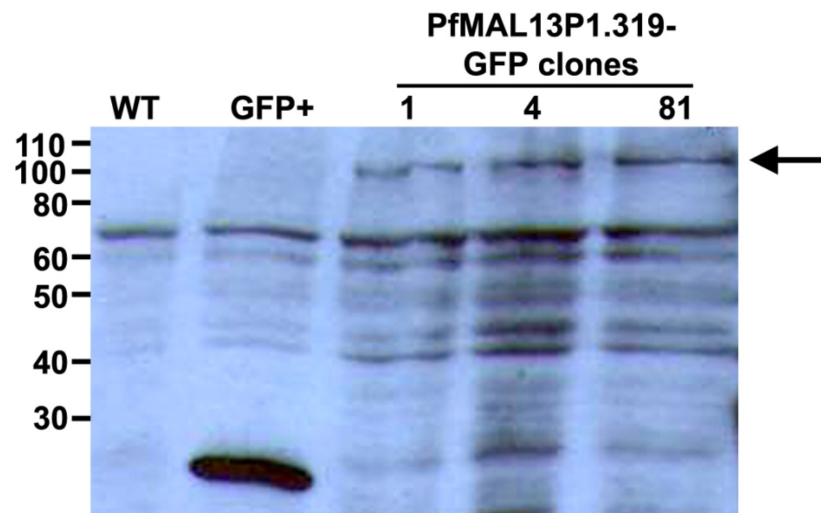

B
PfMAL13P1.319-
GFP clones
WT
GFP+
1
4
81
110
100
80
60
50
40
30

Figure 2.6A-B. PfMAL13P1.319-GFP clonal populations have stable integration of MpPM2GT plasmid DNA that appears to express PfMAL13P1.319-GFP during the erythrocytic stages. A stable integration event (i.e., the PfMAL13P1.319 gene fused to GFP and regulated by the PfMAL13P1.319 endogenous promoter) into the PfMAL13P1.319 chromosomal locus was detected via PCR (data not shown) and Southern blot. (A) Integration was detected by the presence of a 4.2-kb band size in clonal populations 1, 4, and 81 while wild-type with episome was represented by a 2.7-kb and 5.1-kb band. The asterisk denotes an unknown band which could be due to plasmid rearrangement after transfection. Genomic DNA from wild-type *P. falciparum* NF54 parasites (WT) and transfected episome (P) were used as controls in the Southern blot experiments and a digoxigenin-labeled MAL13P1.319 DNA probe was used for detection. (B) PfMAL13P1.319-GFP fusion protein (~110 kDa) appears to be present using anti-GFP antibodies for detection in all three clonal populations. Wild-type (WT) and 3D7HT-GFP (GFP+) parasites were used as negative and positive controls, respectively, in the Western blot.

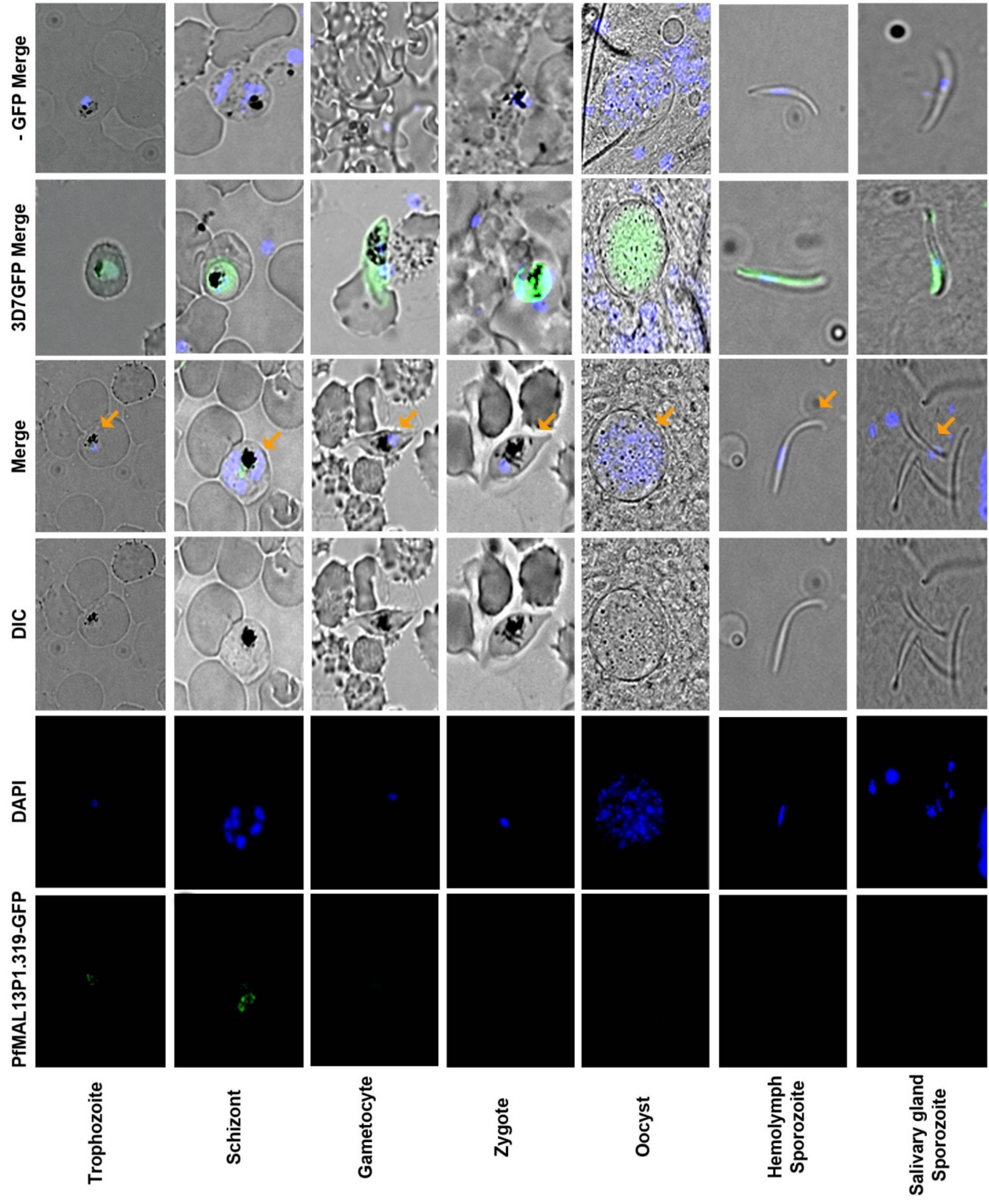

- GFP Merge
3D7GFP Merge
Merge
DIC
DAPI
PfMAL13P1.319-GFP
Trophozoite
Schizont
Gametocyte
Zygote
Oocyst
Hemolymph
Sporozoite
Salivary gland
Sporozoite

Figure 2.7. PfMAL13P1.319-GFP expression in various transgenic parasite stages. The PfMAL13P1.319-GFP fusion protein (i.e., using clonal population 81) was expressed only during the late asexual blood stages such as the trophozoites and schizonts. No expression was observed during the gametocyte stage and mosquito parasite stages (i.e., zygote, oocysts, hemolymph sporozoite, and salivary gland sporozoite). Expression of the fusion protein was under the control of the PfMAL13P1.319 promoter. DAPI was used for parasite nuclei staining. Fluorescent images shown above are representative of PfMAL13P1.319 expression during that specific stage and three biological replicates were performed for each stage observed.

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CHAPTER 3: *PLASMODIUM FALCIPARUM* MAL13P1.319 ATTEMPTED FUNCTIONAL STUDIES

Abstract

In addition to the previous characterization of PfMAL13P.319, functional studies were performed to assess if PfMAL13P1.319 acts in parasite development and/or invasion. The first approach to obtaining PfMAL13P1.319-disrupted parasites was by the classical single cross-over homologous recombination method using positive selection. Integration of the disruption construct was shown via PCR and Southern blot analysis; however a clonal population was not obtained even after multiple attempts. Due to the inability of isolating a clonal population, results would suggest that PfMAL13P1.319 may be essential to parasite survival and disrupting the gene is fatal to the parasite. In addition to the single crossover disruption, gene deletion via double homologous recombination was performed to assess the role of PfMAL13P1.319 during the erythrocytic stage parasites and determine if the gene is indispensable for parasite survival.

Key Words: Functional Analysis, Single and Double Crossovers, PfMAL13P1.319

1. Introduction

Functional analyses of *Plasmodium falciparum* genes have recently paved a way to understanding the role of many proteins in parasite invasion, development, metabolism and motility. With the entire *Plasmodium falciparum* genome (i.e., 14 chromosomes and ~25 megabases encoding ~5,000 genes) being sequenced and the creation of the *Plasmodium* Genome Database (PlasmoDB) (Kissinger et al., 2002), new drug/vaccine targets have been identified and characterized by informative program analyses. To understand the biological role and mechanism of these genes, gene disruption/deletion, stable expression of transgenes, and allelic exchange are some common manipulation methods performed by researchers (Crabb et al., 1997; Duraisingh et al., 2002; Triglia et al., 1998; Wu et al., 1996). Since *Plasmodium* lacks functional and bioinformatic evidence of RNAi machinery, RNAi silencing is not a recommended method to manipulate genes (Baum et al., 2009; Xue et al., 2008).

Unfortunately, altering the *Plasmodium* genome is very difficult since its A/T-rich genes are often unstable in the *Escherichia coli* system, which complicates the generation of transfection constructs (Carvalho and Menard, 2005). In addition, constructs need to traverse four membranes to reach the nucleus, thereby resulting in a long period of time to obtain transgenic parasites. Studies performed by Wu et al. (1995) showed that plasmids transfected into malaria parasites were stable and transiently expressed. This result paved the way for other researchers to discover targeted integration into chromosomal DNA

using drug selectable markers such as human or *Toxoplasma gondii* dihydrofolate reductase-thymidylate synthase (DHFR) (Crabb and Cowman, 1996; Fidock and Wellems, 1997; Menard and Janse, 1997; Wu et al., 1996; Wu et al., 1995).

To achieve *Plasmodium* gene disruptions or deletions, gene targeting requires homologous recombination between the chromosomal DNA and plasmid DNA during the haploid stage of the parasite. Single recombination/crossover is the typical method that allows a truncated portion of the gene of interest to remain, while the other part of the gene is interrupted by vector sequence and shifted further downstream (Figure 3.1A). Single recombination events that positively select for mutant parasites are a common functional method in the *P. falciparum* field and require parasites to be cultured for long periods of time in order to select for integrants. When using the single recombination approach, there are issues, however, with the length of culture time (i.e., due primarily to the replication of circular episomal plasmids with the selectable marker), loss of gametocyte infectivity, wild-type reversion, and inability to create deleterious-phenotype mutants (i.e., due to the parasites with the replicating episomes outgrowing the parasites with integration) (Maier et al., 2006; Trager and Jensen, 1997).

To prevent many of these issues, Duraisingh et al. (2002) recently developed a targeted-gene deletion method using both negative (i.e., herpes simplex virus thymidine kinase that is susceptible to ganciclovir) and positive

selection. Gene deletions are performed using double recombination/crossover, where the plasmid containing the positive drug selectable marker is flanked by two regions (i.e., the untranslated region; UTR) of the gene of interest. After homologous recombination of these two regions, the selectable maker replaces the chromosomal gene (Duraisingh et al., 2002). While both methods assess the functional characteristic of a gene of interest, the gene deletion method avoids single-crossover limitations and improves the ability to isolate mutant parasites that potentially can be deleterious to parasite growth by avoiding the out-competition of wild-type parasites with episome (Crabb, 2002). A limitation of the deletion approach is the initial single crossover event before the double crossover which could result in the selection of disrupted parasites and therefore experience some of issues associated with single crossovers (Duraisingh et al., 2002; Maier et al., 2006; Taylor et al., 2001). One cause for single recombination events in the deletion strategy is because the negative selectable marker expresses insufficient amounts of the enzyme, which produces a toxin needed to kill parasites carrying an episome (Maier et al., 2006).

Many *Plasmodium* genes are unable to be targeted due to the essential nature of the gene. Since targeting and selecting of mutant parasites occurs during the erythrocytic stages, many of these stage-specific genes initially are affected. For example, apical membrane associated protein (AMA-1) was targeted for gene disruption however, the parasites did not develop suggesting that the gene was essential for the survival of the parasite (Triglia et al., 2000).

Unlike AMA-1, disruption of the falcipain-3 (FP3) gene (i.e., a papain-family cysteine protease) was established, however always in the presence of a wild-type gene. The authors suggested that this result was due to a second intact gene copy that allowed the parasites to survive (Sijwali et al., 2006). In addition, Russo et al., (2009) demonstrated that the *P. falciparum* calpain (Pcalp) gene was essential after multiple and various attempts to control calpain expression (Russo et al., 2009). After six attempts of double crossovers using the pHHT-tk vector (Duraisingh et al., 2002) and four attempts of single crossover using the pPM2GT vector (Klemba et al., 2004), Russo et al. (2009) were not able to establish parasites with integration (Russo et al., 2009). The merozoite surface protein (MSP-1_{19}) also demonstrated an importance to erythrocytic stage survival. Parasites were able to integrate the disruption plasmid during the first round of drug selection; however, after the subsequent drug cycles, the integrants did not reappear or survive due to the loss of MSP-1_{19} (O'Donnell et al., 2000). Other methods can be employed when limitations such as deleterious growth phenotypes are present. Alternative methods that have been successful at analyzing protein function are 1) antibody blocking assays (i.e., where antibodies generated against the protein of interest hinder protein interaction to other parasite or host proteins); 2) conditional mutagenesis whereby gene expression is controlled by inducible promoters that can create a conditional “on” and “off” state; and 3) allelic replacement which is used to replace an

endogenous allele with a new allele that has desired mutations (Carvalho and Menard, 2005; Pandey et al., 2006; Russo et al., 2009).

This chapter will address the multiple trials to disrupt PfMAL13P1.319 and discuss initial attempts at gene deletion using pCC-1 (Maier et al., 2006) and pHHT-tk (Duraisingh et al., 2002) deletion constructs.

2. Materials and Methods

2.1 Generating transgenic PfMAL13P1.319 parasites using a single crossover vector

2.1A Single crossover construct design

Gene disruption constructs were designed by cloning a truncated version of PfMAL13P1.319 (i.e., either 441 bp or 710 bp of the 5'-end of the MAL13P1.319 gene) into the pCAM-BSD vector (Mamoun et al., 1999), which utilizes the blasticidin S deaminase (BSD) gene of *Aspergillus terreus* to select for blasticidin-resistant parasites. In addition, a pHD22Y vector (Fidock and Wellems, 1997) that uses the human dihydrofolate reductase (DHFR) to select for WR99210-resistant parasites was also used for PfMAL13P1.319 disruption cloning. Forward (F) and reverse (R) primers used to amplify the PfMAL13P1.319 truncations are shown below (restriction enzymes are italicized and underlined):

PfMAL13P1.319-441-bp insert-

F: 5'-CG*GGATCC*TGGAAGGATTAAGATTAGTTAGTC-3'

R: 5'-AGT*GCGGCCGC*TTCCTCTTCATATCAGCAATTAATG-3'

PfMAL13P1.319-718-bp insert-

F: 5'-CG*GGATCC*TGGAAGGATTAAGATTAGTTAGTC-3'

R: 5'-AGT*GCGGCCGC*TCCTATTTCATGTAATAACAAAC-3'

Purified products that were amplified using PCR conditions described in Chapter 2 section 2.5 were cloned into the pCR®II-TOPOI vector (Invitrogen) and DNA sequenced in order to confirm that the targeting sequence was correct. Then products were digested using BamHI and NotI enzymes and ligated into the double-digested/gel purified pCAM-BSD vector, thereby generating M441pCAM and M710pCAM.

2.1B Transfection and obtaining a clonal population of mutant PfMAL13P1.319 disrupted parasites

P. falciparum NF54 parasites were maintained and synchronized as previously mentioned in Chapter 2 section 2.1 and 2.4, respectively (Lambros and Vanderberg, 1979). Ring stage parasites of 2-3% parasitemia were transfected via electroporation with 50-100 µg of purified and sterile plasmid DNA in Cytomix (120 mM KCL, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes pH 7.6) and maintained in RPMI 1640 culture medium. After 48 hours, parasites were cultured with medium with 2.5 µg/ml of blasticidin-HCl (Invitrogen) until parasites reappeared (i.e., 4-6 weeks later). To increase the number of parasites that had integrated the construct into the genome, parasites were subjected to 2-4 rounds of drug selection and cycled on/off drug. Episome-carrying parasites and parasites that had integrated the

construct into their genome were tested via PCR and Southern blot analysis. For both PCR and Southern blot analysis, genomic DNA was extracted using a Miniprep kit (Qiagen) according to manufacturer's instructions. Specific PCR primer combinations were used to detect the parasite genotype:

Integration-

F: 5'-ACGCATGACACAATTTATATTCC-3'

R: 5'-GGAAACAGCTATGACCATG-3'

Episome-

Vector F: 5'-GCTTTTAATATTTTTATTCCTAATCATG-3'

Vector R: 5'-GTGTGGAATTGTGAGCGGATAAC-3'

Or

BSD F: 5'-CCTTTGTCTCAAGAAGAATC-3'

BSD R: 5'-GCCCTCCCACACATAAC-3'

Wild-type-

(M441pCAM)

F: 5'-CGGGATCCTGGAAGGATTAAGATTAGTTAGTC-3'

R: 5'-AGTGCGGCCGCTTCCTCTTCATATCAGCAATTAATG-3'

(M710pCAM)

F: 5'-CGGGATCCTGGAAGGATTAAGATTAGTTAGTC-3'

R: 5'-AGTGCGGCCGCTCCTATTTCATGTAATAACAAAC-3'

PCR conditions were performed as mentioned in Chapter 2 section 2.5. In order to obtain a clonal population, a limiting dilution protocol used by Dr. David

Fidock's laboratory protocol was performed (Adjalley et al., 2010). Dilutions were made in a 96-well plate where each well contained a final dilution of 0.5 or 0.25 infected red blood cell (IRBC)/well (i.e., also 1 IRBC/2 wells or 1 IRBC/4wells). For M441pCAM-transfected cultures four 0.5- and four 0.25-IRBC/well were used for screening clonal populations (using one 98-well plates for each limiting dilution). M710pCAM-transfected cultures were analyzed using a 0.25 IRBC/well, 0.5 IRBC/well, 1.0 IRBC/well and 2.0 IRBC/well. Plates were placed in a gassed-sealed Modular Incubator Chamber (Billups-Rothenberg Incorporated) at 37°C in order to maintain parasite growth and development. Cultures were replaced with fresh medium and 50% human RBCs every 7th day, while not disturbing the cell monolayer. On day 21, parasites were analyzed for integration via PCR. When extracting parasite genomic DNA from the 96-well plate cultures, a duplicate 96-well plate was created to maintain the parasite culture. The remaining culture was saponin-treated, washed, and resuspended in DSP (20 mM Tris-HCl [pH 7.6], 50 mM KCl, 2.5 mM $MgCl_2$, proteinase K [1 g/L], and 0.5% Tween 20) for direct use in PCR reactions (Adjalley et al., 2010). To detect a clonal population, integration, episome and wild-type primer combinations, as previously mentioned, were used for PCR. All positive-tested clonal populations were expanded, cryopreserved, and genotypically tested using Southern blot analysis.

Southern blot analyses generally were performed as discussed in Chapter 2 section 2.10, however we used BamHI and XbaI restriction enzymes and PfMAL13P1.319-441-bp gene-specific and BSD gene probes. Briefly, 3-5 µg of

DNA (i.e., wild-type, pCAM plasmid and transfected parasite DNA) was digested with restriction endonucleases, separated on an 0.8% agarose gel, transferred to a nylon membrane and pre-hybridized in Church buffer to prevent non-specific binding. The membrane was probed with either a PfMAL13P1.319-DIG probe (i.e., specific to the 441-bp-targeting region of PfMAL13P1.319) or BSD-DIG probe, which was generated using the PCR DIG Probe Synthesis Kit (Roche). Detection of bands was achieved using the DIG Kit (Roche Applied Science) and the chemiluminescent substrate, CSPD (Roche).

2.2 Generating transgenic PfMAL13P1.319 parasites using double crossover vector

2.2A PfMAL13P1.319 deletion-construct design

PfMAL13P1.319 5'- and 3'-ends were PCR amplified, double-digested and cloned directly into pHHT-TK (Duraisingh et al., 2002) and pCC-1 (Maier et al., 2006) double crossover vectors. Using NF54 parasite genomic DNA, PCR amplicons of the 5'-untranslated region (UTR) and 100 bp of the 5'-end of PfMAL13P1.319 and 3'-UTR and the 100 bp of 3'-end of the gene were generated using the primers listed below (restriction enzymes are italicized and underlined):

5'UTR-

F: 5'-CATG*CCATGG*GCTGTAGTATGTATATGATGGTGG-3'

R: 5'-CG*GAATTC*AATCTTAATCCTTCCACCTTGAC-3'

3'UTR-

F: 5'-GG*ACTAGT*CGACAGAAGATTCATCCAGAGG-3'

R: 5'-TCC*CCGCGG*TGGGAATGCTTAAAAAATAACCAG-3'

After PCR (PCR conditions were performed according to Chapter 2 Section 2.5), products were digested with either NcoI/EcoRI (5'UTR) or SpeI/SacII (3'UTR) and purified using a PCR Purification Kit (Qiagen). This purified fragment was ligated into the double-digested/gel purified pHHT-TK and pCC-1 vectors, thereby resulting in the PfMALpHHT-TK and PfMALpCC-1 constructs.

2.2B Selection of transgenic PfMAL13P1.319 deletion mutant

Constructs were prepared and transfected into NF54 parasites as mentioned previously in Chapter 3 Section 2.1B. Two days post-transfection, 5 nM WR99210 was added to select for parasites with the human DHFR gene and maintained with fresh drug medium until parasites appeared. An aliquot of infected red blood cells was cryopreserved and the rest were negatively selected using either 4 μm of ganciclovir (i.e., for pHHT-TK; Sigma) or 1 μm of 5-fluorocytosine (5-FC for pCC-1; gift from Dr, David Fidock) until parasites disappeared.

3. Results and Discussion

3.1 Successful production and transfection of a PfMAL13P1.319-disruption construct

In order to disrupt PfMAL13P1.319 via single recombination, 441- and 710-bp of the 5'-end of PfMAL13P1.319 (i.e., designated M441pCAM and

M710pCAM, respectively) were cloned into pCAM-BSD and transfected into *P. falciparum* NF54 parasites. In addition, 710 bp of the 5'-end of PfMAL13P1.319 were also cloned into pHD22Y and transfected into NF54 parasites, however parasites never reappeared after several weeks of initial drug selection. In contrast to the results with the pHD22Y vector, M441pCAM cultures exposed to two on/off drug cycles yielded drug-resistant parasites that had integrated the plasmids or carried the plasmid episomally. Also, the M710pCAM cultures had drug-resistant parasites after three drug cycles. To specifically detect integration of the PfMAL13P1.319 disruption construct in the PfMAL13P1.319 chromosomal locus, PCR was performed using chromosomal and vector reverse primers and DNA isolated from transfection cultures. An expected product of integration was detected for both transfection cultures using the transfected parasite genomic DNA as a template during the second drug cycles (Figure 3.1B). While most integration events increase with each drug cycle (O'Donnell et al., 2002; Wu et al., 1996), M441pCAM integrants did not appear to increase during the third and fourth drug cycle via PCR and displayed a low level of PCR product. The M710pCAM population also displayed a constant level of integrants during the third drug selection via PCR, however unlike M441pCAM, integration was not subsequently detected during the fourth round of drug selection (data not shown). Although both transfected cultures had parasites with the gene disruption construct successfully integrated into the correct location of the parasite's genome, they also contained wild-type parasites with episomal DNA.

The presence of wild-type parasites with episomal DNA would suggest that wild-type parasites with episomes were outgrowing PfMAL13P1.319-disrupted parasites because mutants had a deleterious growth phenotype (Gardiner et al., 2003; Sijwali et al., 2006). The growth competition between wild-type with episomes and mutant parasites also may explain the constant low level of M441pCAM integrants and disappearance of integration in M710pCAM parasites.

Southern blot analysis was also performed to detect parasites with integration in M441pCAM and M710pCAM cultures. Genomic DNA (3-5 μg) was digested, and electrophoretically separated on a 1.0% agarose gel, transferred to a nylon membrane, and hybridized with specific probes. During the 3rd and 4th drug cycle of the M441pCAM cultures, parasites with plasmid integration (i.e., represented with a 2.2 kb band) were detected. In addition, PfMALP1.319 probe-hybridization of M441pCAM- and M710pCAM- parasite DNA revealed a 2.6 kb band that corresponds to the size of an intact PfMAL13P1.319 gene in wild-type cultures, while a 4.9 kb/5.2 kb band represented episomal DNA in M441pCAM and M710pCAM populations, respectively (Figure 3.1C). In general, results showed that M441pCAM cultures were mixed with integrants and wild-type parasites carrying the episome. Unlike M441pCAM, the M710pCAM transfection culture did not demonstrate integrants via Southern blot during the 3rd drug selection, although PCR analysis of M710pCAM transfected cultures did show integration (i.e., only during the 3rd drug cycle). The failure to detect integration in

the M710pCAM culture using Southern blot could potentially be due to the insensitivity of the Southern blot method in comparison to PCR or due to the rare integration event that may not increase because PfMAL13P1.319 is needed for erythrocytic stage parasite survival. Overall, the results discussed in this section would suggest a deleterious growth phenotype of PfMAL13P1.319 since wild-type parasites with episomes appeared to out-grow the parasites with integration in order to compensate for possible loss-of-function of PfMAL13P1.319 (Maier et al., 2006; Sanders et al., 2006; Trager and Jensen, 1997).

3.2 Multiple attempts at isolating mutant parasite

Since the M441- and M710-pCAM vectors were able to recombine into the PfMAL13P1.319 locus resulting in a mixed population of wild-type and parasites with integration, a limiting dilution was performed to isolate a clonal population of mutant parasites. With the knowledge that the parasites with integration may be rare to detect (i.e., due to the low number of integrants), many limiting dilutions were attempted (Table 1). For M441pCAM transfected cultures, four 96-well plates of 0.5 IRBC/well and four of 0.25 IRBC/well were screened for a clonal population. Seven clones out of 784 wells displayed both parasites with integration and wild-type parasites with episomes. M710pCAM transfected cultures were screened using one 0.25, 0.5, 1.0, and 2.0 IRBC/well, resulting in four mixed clonal populations out of 392 wells. Therefore, no population with only parasites with integration was detected. The inability to separate the integrants from wild-type parasites with the episome could be due to wild-type

parasites with the replicating episome outgrowing the integrants because of the lethal nature of the PfMAL13P1.319 disruption (Crabb, 2002). The studies of Sijwali et al. (2006) demonstrated similar disruption characteristics for *P. falciparum* Falcipain-3 (FP3), where integrants appeared only in the presence of wild-type parasites. With this result, the authors attempted a complete deletion of the gene; however a clonal population of deletion mutants failed to develop. Therefore Sijwali et al. (2006) concluded that FP3 was critical for erythrocytic stage parasite survival.

3.3 Current status of PfMAL13P1.319 deletion mutant parasites

Because the single recombination approach was not successful in obtaining a clonal population, a deletion/double crossover strategy was attempted. The gene deletion system positively selects for those parasites that have integrated the DHFR cassette via double-crossover recombination and negatively selects for parasites carrying the episome (i.e., using either a thymidine kinase or cytosine deaminase gene). Two gene deletion constructs were created by cloning the 5'- and 3'-untranslated regions (UTR) of the gene into the pHHT-TK or pCC-1 plasmids which both contain the selectable marker dihydrofolate reductase (DHFR) for positive selection and thymidine kinase and cytosine deaminase for negative selection, respectively (Duraisingh et al., 2002; Maier et al., 2006).

After 3-4 weeks of WR99210 positive selection, resistant parasites were obtained and exposed to either ganciclovir or 5-FC for negative selection.

Negative selection was applied to cultures for approximately 4 weeks, however parasites did not display any effects to the drug. This result would suggest that either the culture population may contain parasites that have successfully integrated the plasmid into the genome or the negative drug selectable marker may not be functional (i.e., not expressing the gene that converts a normal metabolite into a toxic metabolite), thereby resulting in the proliferation of parasites. These predictions have yet to be tested.

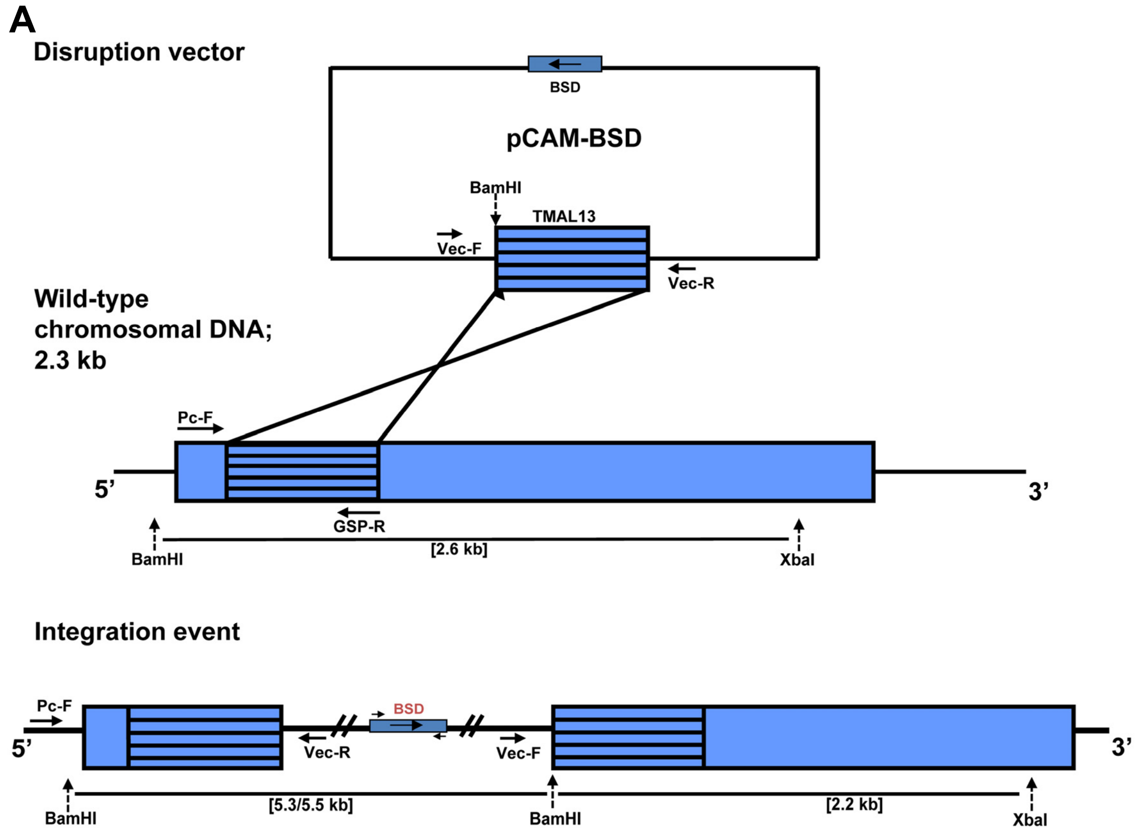
4. Chapter Summary

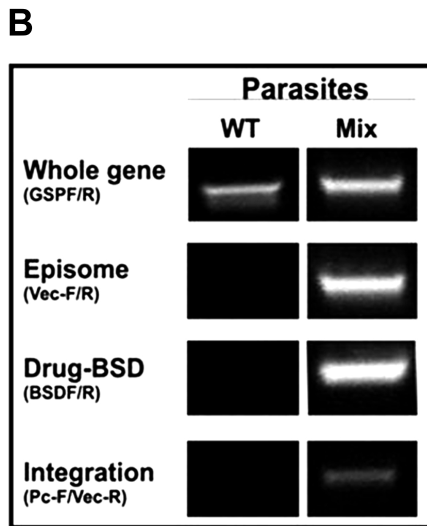
In summary, PfMAL13P1.319 was expressed during the late erythrocytic stages and these functional studies were attempted to assess the role of PfMAL13P1.319 during that parasite stage. The single-crossover disruption plasmids (M441pCAM and M710pCAM) were able to successfully integrate into the correct chromosomal location via homologous recombination. By PCR analysis, M441pCAM parasites displayed a rare integration event that did not increase after the second drug cycle while M710pCAM parasites also displayed a rare integration after the second drug cycle, however integrants disappeared during the fourth drug cycle. Multiple attempts to isolate a clonal population of the PfMAL13P1.319-disrupted parasites via limiting dilutions were performed and each potential clonal population always had wild-type parasites with episomes present. Again, this supports that wild-type parasites with episomes out-compete integrants which could be due to the lethal nature of PfMAL13P1.319 disruptions (Crabb, 2002; Gardiner et al., 2003). Many erythrocytic stage proteins that are

essential for parasite survival have resulted in failed disruption and deletion attempts (O'Donnell et al., 2000; Russo et al., 2009; Sanders et al., 2006; Sijwali et al., 2006; Triglia et al., 2000). Therefore, these results would support a potential role of PfMAL13P1.319 that is critical to the parasite and as such would be of interest for antimalarial drug discoveries.

Type of Gene Manipulation	Number of Wells Analyzed via Limiting Dilutions	Number of wells with integrants and wild-type parasites with episome	Number of Clonal Populations with Integration ONLY
M441pCAM Single crossover; Disruption	784	20	0
M710pCAM Single crossover; Disruption	392	4	0

Table 3.1. Gene manipulation attempts for PfMAL13P1.319. M441pCAM transfected cultures were established by a single crossover event with 441 bp of PfMAL13P1.319 while M710pCAM utilized 710 bp of PfMAL13P1.319 for homologous recombination. This table displays the total number of wells analyzed and of the total, the number of wells that displayed parasites with integration and wild-type parasites with episome.


A
Disruption vector
BSD
pCAM-BSD
BamHI
TMAL13
Vec-F
Vec-R
Wild-type
chromosomal DNA;
2.3 kb
Pc-F
5'
3'
GSP-R
BamHI
[2.6 kb]
XbaI
Integration event
Pc-F
BSD
5'
Vec-R
Vec-F
3'
BamHI
[5.3/5.5 kb]
BamHI
[2.2 kb]
XbaI


B
Parasites
WT
Mix
Whole gene
(GSPF/R)
Episome
(Vec-F/R)
Drug-BSD
(BSDF/R)
Integration
(Pc-F/Vec-R)

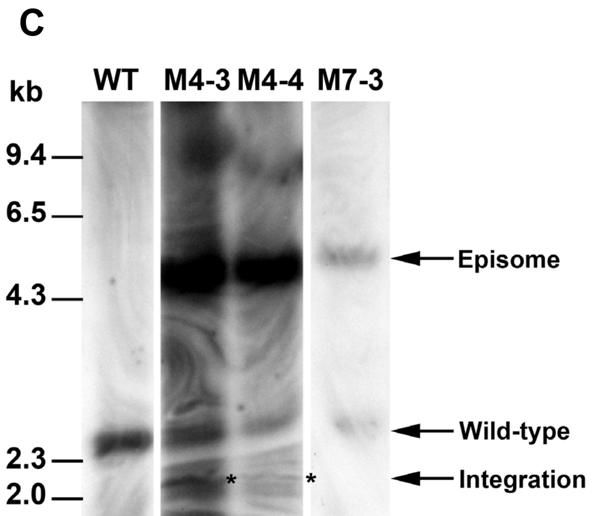

C
kb
WT
M4-3
M4-4
M7-3
9.4
6.5
4.3
2.3
2.0
Episome
Wild-type
Integration

Figure 3.1.

A) An illustration of disruption-plasmid integration into the chromosomal DNA via single recombination/crossover. The schematic depicts the *P. falciparum* transfection vector, pCAM-BSD, with a truncated PfMAL13P1.319 (TMAL13=441 bp) fragment that integrates into the wild-type PfMal13P1.319 locus via single crossover (note this is not drawn to scale). Integration was detected using the chromosomal forward primer (Pc-F) and vector reverse primer (Vec-R). Wild-type DNA was detected using Pc-F primer and gene specific primer reverse (GSP-R). Blasticidin S deaminase (BSD) gene primers and vector primers (Vec-F/R) were used to detect episomal DNA.

B) Transgenic parasites were generated by integration of the M441pCAM disruption vector into chromosomal PfMAL13P1.319 via single homologous recombination. PCR-amplified products indicated the presence of wild-type parasites, episome DNA and parasites that have the disruption construct integrated into the PfMAL13P1.319 chromosomal locus. A genomic DNA template from wild-type parasites (WT) was used as a control, while M441pCAM transfected culture DNA (Mix) was the experimental template. PCR primer combinations listed to the left of the figure were used to amplify: 1) wild-type parasites: gene specific forward and reverse (GSPF/R); 2) episomal DNA: Vector F/R and BSD gene F/R; 3) integration: chromosomal forward (Pc-F) and vector R.

C) Mixed populations of wild-type and integrants were observed in M441pCAM and M710pCAM transfection cultures via Southern blot analysis. Genomic DNA from M441pCAM and M710pCAM was extracted at different drug cycles and digested with BamHI and XbaI. Probes specific to the 441 bp-targeting region of PfMAL13P1.319 were hybridized to BamHI/XbaI-digested genomic DNAs from wild-type parasites, M441pCAM- and M710pCAM-disrupted parasites and pCAM plasmid DNA. Detection of a 2.6 kb band corresponded to wild-type parasites and bands at 4.9 kb for M441pCAM and 5.2 kb for M710pCAM represented episomal DNA (also see Figure 3.1A for depiction of band sizes). The presence of integration corresponded to a 2.2 kb band (as denoted by the astericks) for M441pCAM and M710pCAM, since the other band of integration at 5.3/5.5 kb (i.e., for M441- and M710-pCAM, respectively) can not be discerned due to its close proximity to the episomal band. Genomic DNA templates used were: 1) *P. falciparum* NF54 wild-type (WT); 2) M441pCAM during the 3rd drug cycle (M4-3); 3) M441pCAM during the 4th drug cycle (M4-4); and 4) M710pCAM during the 3rd drug cycle (M7-3). Arrows to the right of the figure denote bands that represent wild-type, episome and integration.

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CHAPTER 4: COMPARATIVE STUDIES OF A MAL13P1.319 ORTHOLOG IN *PLASMODIUM BERGHEI*, A RODENT MALARIA MODEL SYSTEM

Abstract

In the previous chapters, *Plasmodium falciparum* MAL13P1.319 has been described as a conserved erythrocytic stage gene that may play an essential role to the parasite. Since functional studies of PfMAL13P1.319 have been complicated due to the long duration of obtaining a rare integration event, the rodent malaria model system can serve as a comparative study. The *Plasmodium berghei* ortholog of MAL13P1.319 (PbMAL13P1.319) demonstrates ~60% similarity to PfMAL13P1.319 and is predicted to encode either an extracellular or plasma membrane protein with a signal peptide, according to software localization programs. Like PfMAL13P1.319, PbMAL13P1.319 transcripts are expressed during the erythrocytic stages, but unlike the *P. falciparum* ortholog, transcripts were not present in the mosquito parasite stages that were examined. Herein, this chapter reports the current characterization of PbMAL13P1.319 and the attempts made to assess an *in vivo* function of PbMAL13P1.319 during various parasite stages. Similar methods of *P. falciparum* MAL13P1.319 characterization (e.g., immunofluorescent assays, GFP-trafficking studies and gene deletions) were used in an attempt to elucidate the transcript and protein expression profile and assess if the role of PbMAL13P1.319 is similar to or different from PfMAL13P1.319.

Key Words: Comparative Approach, Function, *Plasmodium berghei*

1. Introduction

The rodent malaria system has been a convenient model system for studying mammalian malaria biology *in vivo*, parasite-host interactions and vaccine and drug development. The genome organization/synteny between the rodent malaria and human malaria is conserved and the molecular biology of each organism is similar (Carlton et al., 2001; Janse et al., 1994; Waters, 2002). The studies performed in this chapter used *Plasmodium berghei* parasites, which are one of the four rodent parasite species that infect African murine rodents. *P. berghei* parasites are relatively easy to handle/maintain and are also transmitted by *Anopheles stephensi*, which is a commonly-reared mosquito in the laboratory. A partial shotgun sequence at 3X coverage of the *P. berghei* strain ANKA 15cy1 has been made available by the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk). The entire genome has 14 chromosomes with an estimated size of 18-20 Mb (i.e., comparable to *P. falciparum*). Like *P. falciparum*, *P. berghei* has a high A/T content (i.e., >77%) with 69.9-95.5% identity to DNA sequences in *P. falciparum* (Carlton et al., 2001; Pfander et al., 2011; Yap et al., 1997). Comparative mapping of genes has shown high similarity of gene content/order and organization and many of the vaccine candidates such as the circumsporozoite protein (CSP), P25/P28 ookinete proteins, apical membrane antigen-1 (AMA-1) and merozoite surface protein-1 (PbMSP-1_{19}) are present within this model organism thereby allowing better

characterization methods and vaccine/drug testing (Janse et al., 1994; Waters, 2002). Although there are many similarities between *P. falciparum* and *P. berghei,* there are differences at the molecular level such as different proteins involved in erythrocyte recognition and attachment and different surface proteins on the infected erythrocyte (Chitnis and Blackman, 2000; Cowman et al., 2000; Pinder et al., 2000; Preiser et al., 2000).

P. berghei is a convenient model system for investigating parasite development and parasite-host interactions in both the mosquito and rodent host because the genome sequence and organization information are readily available. This rodent malaria model can provide a large amount of different life cycle stages that are not easily accessible in *P. falciparum* (e.g., mosquito and liver stages) and can avoid issues with gametocyte infectivity, which are often associated with *P. falciparum* transfection cultures. *P. berghei* infections are relatively more lethal to laboratory mice, in contrast to other rodent malarias, and provide a good model system for the study of severe malaria and immunity (Vincke, 1948). Also, technologies used for manipulating parasite genes such as linearized plasmid-transfections with short homologous regions and the Nucleofector® technology, are more time-efficient than with *P. falciparum* (Janse et al., 2006a).

Another convenience of the *P. berghei* model system is the well-conserved life cycle in both the mosquito and vertebrate host that is shared with *P. falciparum*. There are differences in the morphology/size and the duration of

development in comparison to *P. falciparum.* Some examples are *P. berghei* gametocytes having a shorter development time of 26-30 hours post infection with a round/oval morphology and oocysts being relatively smaller than *P. falciparum*. In addition, pre-erythrocytic and erythrocytic cycles are shorter in *P. berghei,* thereby allowing results and reagents to be obtained in lesser time.

As a comparative model system of *P. falciparum,* the *P. berghei* rodent malaria system was used to assess the role of MAL13P1.319 in sporozoite biology. In this chapter, similar methods of *P. falciparum* MAL13P1.319 characterization (e.g., IFAs, GFP-trafficking studies and gene deletions) were performed for the *P. berghei* ortholog of MAL13P1.319 (PbMAL13P1.319), in order to determine if the role of PbMAL13P1.319 is similar to or different from PfMAL13P1.319.

2. Materials and Methods

2.1 Parasite maintenance and erythrocytic stage genomic DNA isolation

Plasmodium berghei ANKA cl15cy1 strain was used for all studies and *P. berghei* ANKA 507m6cl1 was used for GFP trafficking studies. Both parasite strains were propagated in Swiss Webster mice (ages 4-5 weeks). Maintenance of *P. berghei* parasites in the rodent host was achieved by an infected blood passage into new mice via intraperitoneal injections (MR4, 2011; Sinden et al., 2002). Briefly, 100 µl of *P. berghei*-infected blood diluted with saline buffer (Hospira; 0.9% sodium chlorine inj., usp) was injected intraperitoneally into a mouse. Approximately three days after infection, parasitemia was analyzed by a

tail vein bleed. Once the parasitemia reached at least 3%, parasites were cryopreserved with Glycerolyte 57 (Baxter) and/or blood passaged to a new mouse. Blood passaging consisted of injecting 200 µl of approximately $1x10^5$ infected erythrocytes per mouse, intraperitoneally.

Plasmodium berghei ANKA cl15cy1 strain genomic DNA was obtained as mentioned in Chapter 2 Section 2.3. Briefly, infected blood was pelleted by centrifugation and then saponin-RPMI1640 medium was added. Saponin-treated cells were pelleted, washed and either resuspended in 1X PBS for genomic DNA isolation (Qiagen DNeasy Blood and Tissue Kit) or in TRIzol reagent (Invitrogen) for RNA isolation.

2.2 Sequence analyses of PbMAL13P1.319

PlasmoDB and NCBI BLAST analyses of the MAL13P1.319 protein identified two *P. berghei* open reading frames (ORFs) (i.e., accession number Pb000510.01.00 and Pb000082.00.00) (Altschul et al., 1997; Kissinger et al., 2002). Protein alignment with these two ORFs showed that Pb000510.01.00 and Pb000082.00.00 had significant homology to the first and last half of the PfMAL13P1.319 protein, respectively. To confirm that these two ORFs represented halves of the same gene, the Pathogen Genomics department of the Wellcome Trust Sanger Institute was contacted. Arnab Pain and Ulrike Boehme of the Sanger Institute analyzed the MAL13P1.319 sequence with relevant *P. berghei* contigs in their current assembly and also compared the sequence synteny with the *P. chabaudi* contigs (*P. chabaudi* is a closely related parasite to

P. berghei that also causes rodent malaria and its genome is partially sequenced). Based on synteny with *P. chabaudi*, they discovered and confirmed that the Pb00510.01.00 and Pb000082.00.00 sequences were one gene, designated as PbMAL13P1.319. PbMAL13P1.319 sequence analyses were performed as described in Chapter 2 section 2.2. Briefly, signal peptides were predicted using the Signal P and Target P programs and localization predictions were made by Softberry and PSORTII.

2.3 Transcript expression analysis via RT-PCR

RNA was isolated as mentioned previously in Chapter 2 section 2.3 from infected RBCs, Day 10 PE oocyst and Day 21 PE salivary gland sporozoites (infected mosquitoes were supplied by MR4). The gene expression primers that were designed and used to amplify PbMAL13P1.319 and controls were the following;

PbMAL13P1.319-

F: 5'-TAAGCCAAGACAATTCTTCTC-3'

R: 5'-TCATTGCGAACTCAGTCCTC-3'

P. berghei circumsporozoite gene (CS)-

F: 5'-CTTGCCGATGCTCCCGAAGG-3'

R: 5'-CGCGCTTGGGATATAAGAATCG-3'

P. berghei merozoite surface protein-1 gene (PbMSP-1_{19})-

F: 5'-GAACTACTGGTGCAGAAGCAG-3'

R: 5'-CAAGTGTTGAAAGTGGTACATC-3'

RT-PCR and PCR analysis of cDNA was carried out using 1.5 mM MgCl2, 0.2 mM dNTP, 2.5 U Taq polymerase (Promega), 0.4 mM of each primer, and 8 µl of cDNA template to a total reaction volume of 50 µl. PCR amplification was performed as follows: 1) initial denaturation at 94°C for 5 minutes; 2) denaturation at 94°C for 45 seconds; 3) annealing at 57°C for 45 seconds; 4) extension at 62°C for 3 minutes and 5) final extension at 62°C for 10 minutes. Each amplification cycle was repeated 34 times. Resulting products were separated via 1% agarose gel electrophoresis and stained with ethidium bromide for visualization. For controls, *P. berghei* merozoite surface protein-1 (PbMSP-1_{19}) gene primers were used to detect asexual blood stages and *P. berghei* circumsporozoite (PbCS) gene primers were used to detect oocyst and salivary gland sporozoites. *Plasmodium berghei* ANKA cl15cy1 strain genomic DNA was a positive control template and the no reverse-transcriptase cDNA control was utilized to make certain that there was no genomic DNA contamination.

2.4 Construction of PbMAL13P1.319-GFP and –deletion vectors

For green fluorescent protein (GFP)-reporter expression that is under the control of the endogenous promoter, the pL0031 vector that is available from MR4 (Kooij et al., 2005; MR4, 2011; Schmidt-Christensen et al., 2008) was used to clone ~1000 bp of the 3'-end of PbMAL13P1.319 (i.e., without the stop codon). This fragment was PCR-amplified using the gene specific primers (restriction enzyme sites are underlined and italicized) listed below:

F: 5'-AGT*GCGGCCGC*GCAGTTGAAAATAAAGCTGATGG-3'

R: 5’-CG*GGATCC*TTGCGAACTCAGTCCTCTTGAAG-3’

PCR conditions were the same as Chapter 2 Section 2.5. The PCR products were digested with BamHI and NotI enzymes and ligated into a double-digested pL0031 to yield the GFP-trafficking plasmid, PbMALpL0031.

To generate PbMAL13P1.319-deletion mutants, a deletion vector known as pL0001 was used (Mair et al., 2006; MR4, 2011). This vector has a *Toxoplasma gondii* dihydrofolate reductase (TgDHFR) gene used for drug selection and two cloning sites for double homologous recombination located upstream and downstream of the selectable marker. Approximately 1000 bp of the 5’- and 3’- flanking regions of PbMAL13P1.319 were PCR amplified (PCR conditions described in Chapter 2 Section 2.5) using *Plasmodium berghei* ANKA cl15cy1 strain genomic DNA and gene specific primers (i.e., shown below with restriction enzymes underlined and italicized) and cloned up- and down-stream of the selection marker:

5’UTR

F: 5’-AGT*GCGGCCGC*GAACACGATAAACATAAAAGTCCC-3’

R: 5’-CG*GGATCC*CTGCTACCATTGATAGTCTATGC-3’

3’UTR

F: 5’-CCC*AAGCTT*CGAAATATGCATGAATAATATCCCC-3’

R: 5’-GG*GGTACC*AAAGCGAAAATGTAAGCCAAGAC-3’

PCR products yielding the 5'-UTR were digested with NotI and BamHI and 3'UTR products were digested with KpnI and HindIII. Both products were ligated into pL0001 to yield the deletion plasmid, PbMALpL0001.

2.5 Plasmid transfection and genotyping

PbMAL13P13.19-GFP and –deletion vectors (i.e., PbMALpL0031 and PbMALpL0001, respectively) were introduced into wild-type *P. berghei* ANKA reference clone cl15cyl via an AMAXA Nucleofector II® device (program U-33) (Janse et al., 2006b). First, plasmids were linearized by digesting with NotI or NotI/KpnI (i.e., for PbMALpL0031 and PbMaLpL0001, respectively), separated on a 1% electrophoresis agarose, excised from the gel and purified via a Gel Purification Kit (Qiagen). *P. berghei* schizonts were collected from infected Swiss Webster mice at a 1-3% parasitemia and cultured with complete RPMI1640 culture medium (RPMI1640 with HEPES, 0.2 M NaHCO3, neomycin-sulfate [0.1mg/ml], 20% fetal bovine serum) *in vitro* at 37°C with slight shaking overnight. The culture suspension was mixed with 52% Accudenz® (Accurate Chemical & Scientific Corporation) and centrifuged at 450 x g for 20 minutes at room temperature with no brake. Enriched schizonts were collected at the interphase between the two suspensions and were electroporated with 5-10 µg of plasmid DNA using Amaxa Nucleofector device program U33, and 150 µl injected into the tail vein of the mouse. Pyrimethamine water [0.07 mg/ml] was provided for the mice for 7-10 days (i.e., until drug-resistant parasites appeared at a high parasitemia) before blood collections via cardiac puncture.

All blood collections were cryopreserved in liquid nitrogen at a 1:2 dilution with Glycerolyte 57 solution (Baxter) and genotyped. For genotype analysis, blood was saponin-lysed and DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit. PCR (i.e., same conditions in Chapter 2 section 2.5) was performed to show specific plasmid integration, wild-type parasites, and episomal DNA (i.e., using the TgDHFR selectable marker). 100 ng of wild-type, plasmid DNA and transfected culture DNA (i.e., either PbMALpL0031 or PbMALpL0001) were used as templates and specific primers were used to detect integration, wild-type and episomal DNA:

PbMALpL0031 integration primers-

F: 5'-CATTGATAGCTTATCAGCACAAGG-3'

R: 5'-GTAGTGGAAGTGGGAGAGG-3'

PbMALpL0031wild-type primers-

F: 5'-CCCAAGCTTAAAGCGAAAATGTAAGCCAAGAC-3'

R: 5'-GGGGTACCCGAAATATGCATGAATAATATCCCC-3'

PbMALpL0031 episomal primers-

F: 5'-CGCTAATTCAACCCATTGCG-3'

R: 5'-GTAGTGGAAGTGGGAGAGG-3'

PbMALpL0001 5' integration primers-

F: 5'-CATTGAAGAGCATAAACTGTGG-3'

R: 5'-GACAATATTAATACAACAGAGAAG-3'

PbMALpL0001 3' integration primers-

F: 5'-CAATAATAATATATAAAAATATAGTTAC-3'

R: 5'-AATAACTGCTATAAATATCGAG-3'

PbMALpL0001 wild-type primers-

F: 5'-CATTGATAGCTTATCAGCACAAGG-3'

R: 5'-GTATCTTTGTAGTTTTCACC-3'

PbMALpL0001 episomal primers-

F: 5'-GCATCAACAACGGCCTCCC-3'

R: 5'-TCTGGATTCGTCCGTGCGGG-3'

To confirm PbMALpL0031 integration, Southern blotting was performed as described in Chapter 2 section 2.10. Briefly, 3-5 µg of genomic DNA from wild-type, plasmid and transfected culture DNA were digested, separated on a 1% agarose gel and transferred to a nylon membrane. Both TgDHFR and PbMALpL0031 hybridization probes were labeled with digoxigenin (DIG) and used to detect integration, wild-type and episomal DNA.

2.6 Obtaining clonal populations via limiting dilution

After the genotype was confirmed, clonal populations were obtained by performing limiting dilutions according to a *P. berghei* transfection protocol by Janse et al. (2004). Infected blood from mice with a 0.3-1% parasitemia was diluted with culture medium (RPMI1640 with 25 mM HEPES and 25% fetal bovine serum) to a final concentration of 2 parasites/0.2 ml culture medium (Janse, 2004). Mice (10-20) were injected with 0.2 ml of the diluted parasite media into the tail vein. After 8 days, the parasitemia of all mice was assessed

and blood was collected from parasite-positive mice for cryopreservation and DNA analyses.

2.7 Fluorescence microscopy for GFP trafficking studies

Fluorescence microscopy was performed as described in Chapter 2 section 2.11 using an aliquot of *P. berghei*-infected mouse blood.

3. Results and Discussion

3.1 *P. berghei* MAL13P1.319 sequence analyses

In an effort to determine if PbMAL13P1.319 had similar or different sequence characteristics, sequence analyses mentioned in Chapter 2 Section 2.2 were performed. Results demonstrated that PbMAL13P1.319 is a 2010 bp gene that encodes a 669-amino acid protein (i.e., 78 kDa with the signal peptide) which shares ~60% identity with PfMAL13P1.319 (Figure 4.1). Like PfMAL13P1.319, PbMAL13P1.319 is predicted to encode a signal peptide as determined by Target P and Signal P and could be either a secreted or surface protein that localizes to the endoplasmic reticulum or plasma membrane according to PSORTII and Softberry localization programs. Based on topology predictions, there were two predicted transmembrane domains located within the protein, which would suggest that PbMAL13P1.319 may be an integral protein once the signal peptide is cleaved. Similar to PfMAL13P1.319, PbMAL13P1.319 also was predicted to have a putative indole-3-glycerol phosphate synthase (IGPS) domain according to PlasmoDB (Kissinger et al., 2002); however, protein alignment with IGPS showed the same results as PfMAL13P1.319 and therefore

would suggest that PbMAL13P1.319 does not function as an IGPS. Unlike PfMAL13P1.319, PbMAL13P1.319 did not have the aspartic acid, isoleucine, asparagine, and histidine (D-I-N-H) repeat sequences which could indicate a different function for the *P. berghei* ortholog of MAL13P1.319.

3.2 Transcript analyses of PbMAL13P1.319 during the intraerythrocytic, oocyst sporozoite, and salivary gland sporozoite stages

PbMAL13P1.319 transcript was determined by RT-PCR using cDNA templates that were prepared from erythrocytic stages, oocyst sporozoites and salivary gland sporozoites. Transcription of PbMAL13P1.319 was only detected during the erythrocytic stages and unlike PfMAL13P1.319, no transcript was detected during the oocyst and salivary gland sporozoite stages (Figure 4.2). The presence of PbMAL13P1.319 transcript during erythrocytic stages may suggest that PbMAL13P1.319 has a role during the erythrocytic stage parasite, however without protein expression data, no conclusions can be made. Controls used to demonstrate the presence of these specific parasite stages in the samples were PbCS for sporozoites and PbMSP-1_{19} for erythrocytic stages.

3.3 PbMALpL0031 GFP vector integrated into chromosomal DNA

In order to determine PbMAL13P1.319 protein expression throughout various parasite stages, the pL0031 vector was used to construct a GFP-fused PbMAL13P1.319 protein where expression would be controlled by the endogenous promoter. Purified *P. berghei* schizonts were transfected with a linearized PbMALpL0031 plasmid and then these schizonts were injected into the

mouse tail vein. *P. berghei* parasites were selected with pyrimethamine to screen for parasites that have integrated the plasmid into the genome by a single crossover event and to screen for parasites carrying the episome.

Successful integration of drug-resistant parasites was first demonstrated by PCR (Figure 4.3). Drug-resistant parasites were subjected to four limiting dilutions with 12-15 mice per GFP-limiting dilution (i.e., a total of 48-60 mice) in attempts to obtain a clonal population. Twenty of the total mice developed drug-resistant parasites which were all analyzed by PCR. PCR results displayed multiple products, rather than a single product, using primers that detect integration, which may be due to the primer combinations. In addition, Janse et al. (2004) states that PCR analyses of limiting dilution cultures may give confusing PCR results which prevent the detection of correct integration. Hence, this may be a standard issue when using transfected *P. berghei* genomic DNA. Therefore parasites were further analyzed by Southern blot analyses to confirm integration. Transfected-parasite DNA was hybridized with a PbMAL13P1.319 probe, which showed bands corresponding to integration and wild-type with episomes (Figure 4.4). Specifically with population PbMALpL0031-Green (G), both bands representing integration appeared in addition to a faint band that represents the presence of wild-type carrying the episome. This result would suggest that the PbMALpL0031-G population may have more integrated parasites than the other population (i.e., Blue/Red; B/R) and would be a better candidate for analyzing GFP-expression.

Although the PbMALpL0031 population was mixed with wild-type parasites with episomes and integrants, a preliminary analysis of GFP fluorescence was attempted for the erythrocytic stages using the PbMALpL0031-G population, a constitutively-expressed GFP parasite line (i.e., *P. berghei* ANKA 507m6cl1; used as a positive control) and *P. berghei* ANKA cl15cy1 wild-type parasites as a negative control. Results showed no detection of GFP which could be due to the presence of more wild-type parasites carrying an episome (data not shown). On the hand, it is possible that PbMAL13P1.319 may not express protein during the other erythrocytic stages. Nevertheless, obtaining a clonal population would be essential before making this claim.

3.4 The PbMALpL0001 construct showed no integration into chromosomal DNA after multiple transfection and limiting dilution attempts

In an attempt to assess the functional role of PbMAL13P1.319 in parasite biology, we performed a targeted gene deletion of PbMAL13P1.319. The PbMAL13P1.319 deletion vector was constructed by ligating approximately 1.0 kb of the upstream and downstream UTR of the PbMAL13P1.319 gene into the pL0001 transfection vector (Mair et al., 2006; MR4, 2011). The linearized PbMALpL0001 deletion construct was transfected into purified mature schizonts of the *P. berghei* ANKA cl15cy1 strain using an Amaxa Nucleofector device (Janse et al., 2006a). After drug selections, since integration was verified via PCR (data not shown), two limiting dilutions were performed using 15 mice (i.e., a total of 30 mice). However, a clonal population was never detected. This

result would suggest that either PbMALpL0001 mutant parasites are rare to detect and/or the gene may be essential for parasite survival.

4. Chapter Summary

Since PbMAL13P1.319 has approximately 60% amino acid identity to PfMAL13P1.319 and likewise was predicted to encode a signal peptide and be a secreted or surface protein, we hypothesized that its transcript and protein expression profiles would be similar to PfMAL13P1.319. Preliminary characterization of PbMAL13P1.319 transcript demonstrated intraerythrocytic stage expression and no detectable levels of transcript were present in oocyst and salivary gland sporozoites. This preliminary result would support a stage-specific transcript expression; however other parasite stages (e.g., sexual stages) would need to be evaluated before making this claim. To evaluate protein localization throughout all the parasite stages, GFP was fused to PbMAL13P1.319 in a plasmid and this construct integrated into the host chromosomal DNA via single homologous recombination. Preliminary analysis of a mixed population (i.e., wild-type with episomes and parasites with integration) demonstrated no detection of GFP fluorescence which could be due to more wild-type parasites being present or no PbMAL13P1.319 protein production during the erythrocytic stages. To investigate the functional role of PbMAL13P1.319, 5'- and 3' UTRs were cloned into the pL0001 deletion vector and transfected into *P. berghei* parasites. Unfortunately after performing limiting dilution on 30 mice, a clonal population of mutant parasites was not able to be

obtained. This result could be due to a rare integration event or the necessity of PbMAL13P1.319 protein expression for parasite survival. Overall, the results in this chapter demonstrated similarities and differences between the *P. falciparum and P. berghei* ortholog of MAL13P1.319 and provided a more detailed study of MAL13P1.319.

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                   1                                                                              80
PfMAL13      (1)   MIARIYKYIITLFYFLLYIFILFHCLNSLIIKYNVKVEGLRLVSRMKRGKILKGCFPKDGILHNHYISTLFIKNDGEIYN
PbMAL13      (1)   ------------MPRIKLIFLLIICT--IRYVFSTKVTKITKVTNRSLGMPKLAYCGNINGDRKYYIQTCDFAKKRRRKN
                   81                                                                            160
PfMAL13     (81)   KYIGGKKEKVRKRKNDIGGRVRFRRYGKDSRNPHLAYEYINKIIENKKYEITKLLEENCDENNPLQIRMKYLQHTMNNKL
PbMAL13     (67)   ENPS---LHVKKKK-----NIYIKIYGRDHRNPYLAYEHINRIIENKKYEVTKLLEENADENSPLQIRLKYLQHTMNNKL
                   161                                                                           240
PfMAL13    (161)   SESLKRSNNDEKHRLSLIADMKRKIFCSIEKEKKKEKDHKTKEGLYNNINVGYDNLQDEKYIYEDILKNEKEFDDDDIMK
PbMAL13    (139)   SESLKIIHSDEKHRLSMVADIKRKVFCSTNKADEN---------------------------YDLKKNINNYEIENERK
                   241                                                                           320
PfMAL13    (241)   NNEEKKKNINNNYIYQHNFLNLSNPGN-VSLLLHEIGFDVLIVNIDELSTQGNINDLKDIIKSTRTLPRNKRPAIVVDDI
PbMAL13    (191)   KNENMINNSNEKYVYQNNFLNLTNPGEKASLMLHKIGFDVLIVNIDSLSAQGTLNDLSDVIKSTRTLTRNSRPAVVVDDV
                   321                                                                           400
PfMAL13    (320)   IIHPIQIALAVENQADGVILNLSYLKNDMEEMLQYCVNVGTQAIVEVHDYNDIYFATQCGAYILMINEYDFYNNIYEYNH
PbMAL13    (271)   IIHPIQIALAVENKADGVILNLSYLKNDLEDMLNYCVNLGTQAIVEVHDYNDIYYATQCGSYILMINEFDFINNRYEYNH
                   401                                                                           480
PfMAL13    (400)   AIKAINYTIPEIITIAKVNTNEVNYIEKLGSLGYDSICLEKKLIDDDLQQFVTSCKGWSAPHKTLLYLNRNNYLKEFLTY
PbMAL13    (351)   AIKAISYSIPELITIAKINVSDINYVEKLGSLGYDSICLEKKLIDEDLEVFVQSCKNWKAPHKTLLYLNRNNYLKNFLTY
                   481                                                                           560
PfMAL13    (480)   DKNGQTGENHKDITENLKNLYDDKNISNNYSHTLLKRYEKEHIDGEDITNDEAMNGAINHDISQDINHDINHDISKDINH
PbMAL13    (431)   KNNS-PGENYKDTTKNLEKMYDDKNLENKYSEQMLKDYEKEFINVEDSK-------------------------------
                   561                                                                           640
PfMAL13    (560)   DISQDINHDIKRYNVLNKDNPMKYKTEGNKNNYAEKKESFENKCIDNKNIKHTNMNKKEEILSKEEKQIVQNFKNEKKRE
PbMAL13    (479)   -----KNEEVVRKNETAKISQINQKSE---------------------NVSQDNSS----LLTNDEKKIINNFKQERKNE
                   641                                                                           720
PfMAL13    (640)   LMLLSQMKEIIKEVDDQCKNYDTNTDEQNQKKEEKLESLLENFTKLDKNFLKGFFSDEEINNIENTMKNAVEQKNKIQSG
PbMAL13    (529)   IMLLNQMKDIIKEVDNQCKNYEHFSEEESIKKEEKLESLLENFTKLDKSFLKQFFSDEEINNIENSIKNAVHQKKKIQDG
                   721                                                                    792
PfMAL13    (720)   EINIDHKNLTGYPMGTLQNSPMNNLDIYNLTKEYFGETQNSDAHTENIFDSDQNKLLDESTEDSS-RGLDSS
PbMAL13    (609)   EINIENTNFTGFPS-----NQMNDFDINRLTKEFFDTNGSGNNH------KIDNKLLDDYSDDSSSRGLSSQ
```

Figure 4.1. The *Plasmodium berghei* ortholog of MAL13P1.319 displays ~60% similarity to its counterpart in *Plasmodium falciparum*. The predicted signal peptide for PfMAL13P1.319 (PfMAL13) and PbMAL13P1.319 (PbMAL13) is highlighted in blue and identical and similar amino acids are highlighted in yellow and green, respectively.

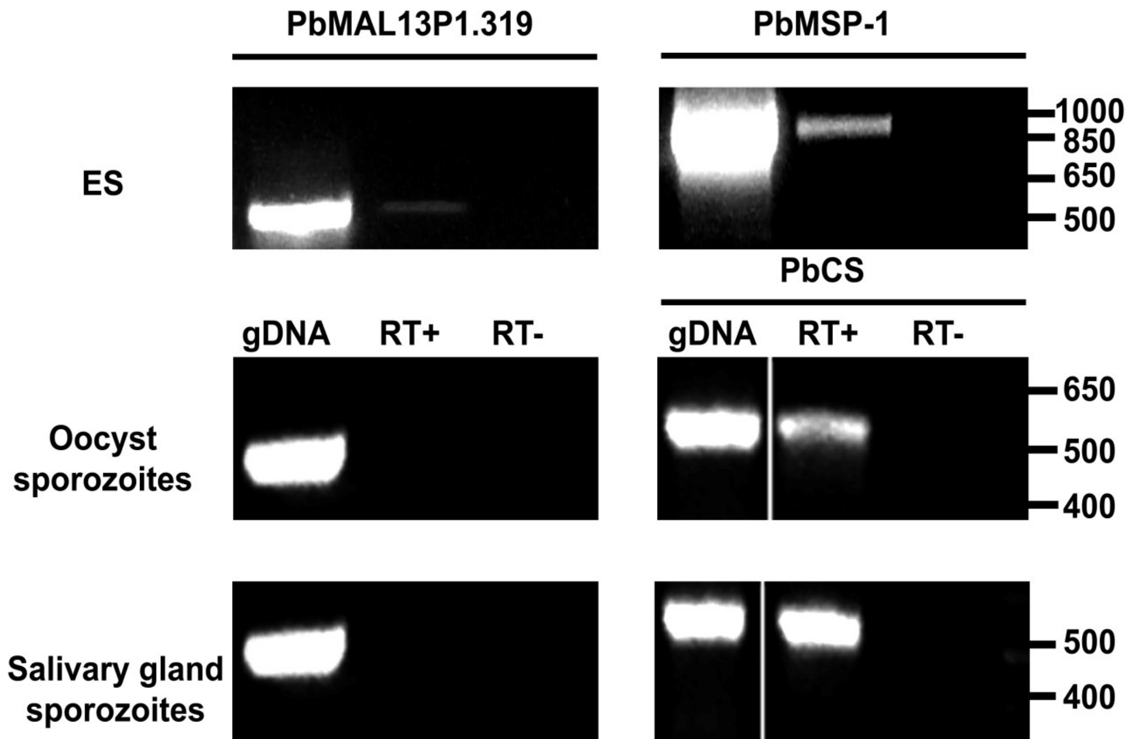

Figure 4.2. PbMAL13P1.319 transcript is only present during *P. berghei* intraerythrocytic stages and not in oocyst and salivary gland sporozoites. Total RNA was isolated from *P. berghei* infected red blood cells (ES), Day 10PE oocyst sporozoites and Day 21PE salivary gland sporozoites and subjected to RT-PCR. PbMAL13P1.319 gene specific primers were used to amplify a product that corresponds to PbMAL13P1.319 transcript expression. *P. berghei* ANKA cl15cy1 parasite genomic DNA (gDNA) was used a positive control template. No reverse-transcriptase controls (RT-) were used as a negative control to detect genomic DNA contamination. Control primers used in this study were PbCS for oocyst and salivary gland sporozoites and PbMSP-1_{19} for erythrocytic stages. Two biological replicates were performed for the erythrocytic stages and one biological replicate was performed for oocyst and salivary gland sporozoites.

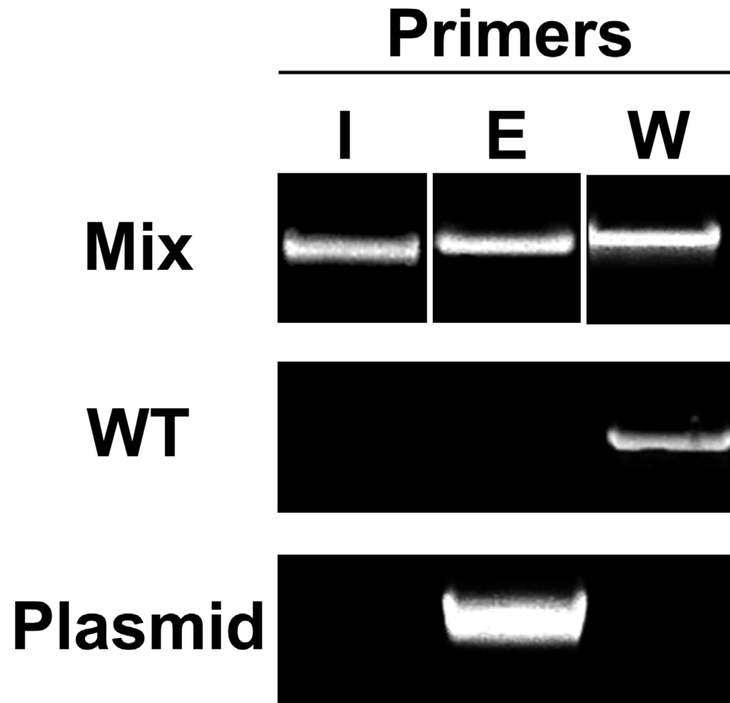

Figure 4.3. Transgenic parasites (i.e., the pre-clonal population) were generated by integration of the PbMALpL0031 vector into chromosomal PbMAL13P1.319 via single homologous recombination. PCR-amplified products indicated the presence of: 1) PbMALpL0031 construct integrated into the PfMAL13P1.319 chromosomal locus using integration-specific primers (I); 2) episomal DNA using vector primers (E) and 3) wild-type parasites carrying episomes using wild-type primers (W) in the PbMALpL0031-transfected parasite cultures (Mix). *P. berghei* ANKA cl15cy1 parasite genomic DNA (WT) and PbMALpL0031 transfected plasmid DNA (Plasmid) were used as positive control templates. All templates are listed to the left of the image.

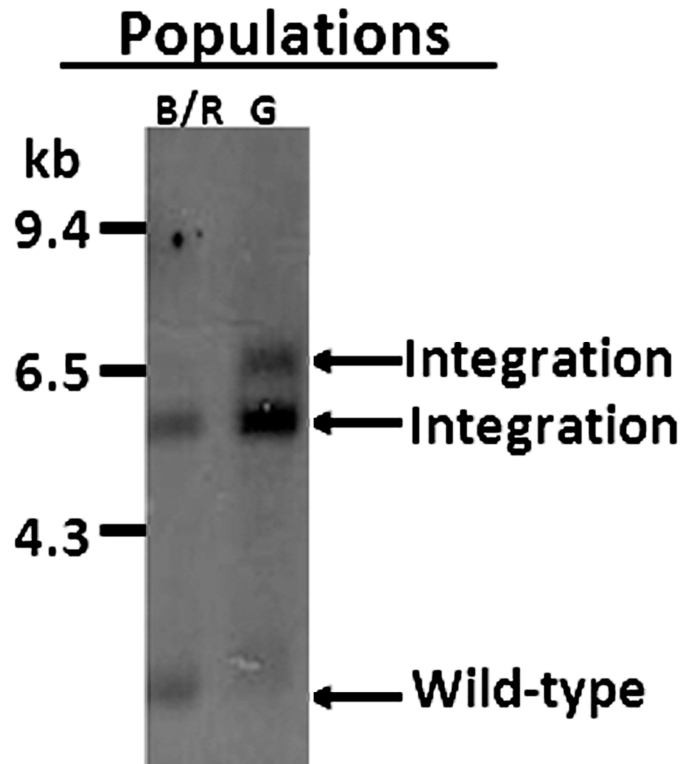

Figure 4.4. Mixed clonal populations of PbMALpL0031 demonstrate parasites with integration via Southern blot analysis. Digested DNA from two PbMALpL0031 mixed clonal populations was used for Southern blot analysis and probed by a PbMAL13P1.319-specific DNA probe. Genomic DNA from Blue/Red (B/R) and Green (G) PbMALpL0031-populations was extracted and digested with HgaI and SapI restriction enzymes. Detection of a 2.8-kb band represented wild-type while the 6.1-kb and 7.5-kb bands represented plasmid-integration into parasite chromosomal DNA, as denoted by the arrows.

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CHAPTER 5: DISCUSSION

Identifying and characterizing gene candidates involved in host-parasite interactions, development, and metabolism throughout the entire life cycle is a major asset to understanding the overall biology of malaria parasites and discovering interesting candidates for vaccine/drug intervention. This dissertation characterizes a once unknown *Plasmodium* gene, MAL13P1.319, which was identified by data mining of the *Plasmodium* genome database (PlasmoDB) and predicted to encode a sporozoite surface antigen or secreted protein. MAL13P1.319 has an N-terminal signal sequence and was discovered as a well conserved protein in many *Plasmodium* species and was present in other Apicomplexans. *P. falciparum* MAL13P1.319 (PfMAL13P1.319) has a unique repeat sequence of asparatic acid, isoleucine, asparagine and histidine (D-I-N-H). Studies have shown that repeat regions have a role in protein-protein interaction and act as a distraction to hinder immune responses to important molecule sequences, which may indicate a potential role for PfMAL13P1.319 and its repeat region (Anders et al., 1988; Anders et al., 1993; Cowman et al., 1985; Magowan et al., 2000; Waller et al., 1999). As compared to other *Plasmodium* spp. (that were analyzed in the protein alignment), this repeat sequence was only identified in PfMAL13P1.319 suggesting a possible region that is important to the parasite's survival in humans.

Indole-3-glycerol phosphate synthase (IGPS) is an enzyme that catalyzes the fourth step in the tryptophan biosynthetic pathway (i.e., indole ring-closure reaction) where the substrate 1-(o-carboxyphenylamino)-1deoxyribulose-5'-phosphate (CdRP) is converted to indole 3-glycerol phosphate (Creighton and Yanofsky, 1966). MAL13P1.319 was predicted to have a putative domain of IGPS which may have a potential role in parasite metabolism. However, since sequence analysis showed a 10.1% identical and similar amino acid homology (specifically 5.0% identical amino acids) with IGPS and no functional catalytic residues, these results do not support MAL13P1.319 as a metabolic enzyme involved in tryptophan biosynthesis.

The transcript expression profile of PfMAL13P1.319 from the large scale DNA microarray studies performed by Le Roch et al. (2003) and Siau et al. (2008), and RT-PCR analyses performed in Chapter 2 of this dissertation demonstrated transcript presence in oocyst and salivary gland sporozoites of the mosquito host and intraerythrocytic stages of the human host. However, these results did not correlate with protein expression which was only detected during the intraerythrocytic stages and had minimal to no detection during mosquito parasite stages via IFAs and GFP studies. The protein expression profile of PfMAL13P1.319 also conflicted with high-throughput protein mass spectrometry studies performed by Lasonder et al. (2002) and Florens et al. (2002). Unlike the IFA and GFP studies, these authors reported no detection of PfMAL13P1.319 during the intraerythrocytic stages. Explanations for this conflicting data could be

due to: 1) the global profile of proteins using a large scale mass spectrometry approach versus proteins analyzed on a single-cell level; 2) the synchronization of parasites preventing the detection of proteins expressed before the collection time point; 3) different parasite strains expressing different proteins and at varying levels and 4) the poor extraction of erythrocytic stage proteins for protein mass spectrometry. In addition, the large-scale mass spectrometry study by Florens et al. (2002) showed PfMAL13P1.319 protein expression only during the salivary gland sporozoite stage, while the IFA and GFP studies displayed minimal to no detectable protein expression in salivary gland sporozoites. The authors detected a low spectrum number of 1 for PfMAL13P1.319 in comparison to other significantly-expressed sporozoite proteins like the circumsporozoite protein with a spectra number of 10, thereby suggesting that PfMAL13P1.319 is indeed expressed at very low levels. Since Florens et al. (2002) specifically utilized a "shotgun proteomics" approach known as multidimensional protein identification technology (MudPIT), this method would provide a large-scale global view of the sporozoite proteome that may detect low-abundance proteins in a largely unbiased manner (Washburn et al., 2001; Wu and MacCoss, 2002) and therefore could result in the low spectra number for the PfMAL13P1.319 protein. On the other hand, when performing antibody-labeling and GFP trafficking studies of salivary gland sporozoites, these methods may not be sensitive enough to detect low abundance proteins even on a single cell level.

These results may explain the undetectable PfMAL13P1.319 levels in sporozoites observed by the IFA and GFP trafficking studies.

Localization of PfMAL13P1.319 during the late erythrocytic stages would suggest an array of functions such as parasite metabolism, development and invasion. Therefore, functional studies were attempted to identify a specific role of PfMAL13P1.319. Evidence of wild-type parasites with the episome out-competing integrants was supported by: 1) a rare integration event never increasing with more drug cycles; 2) numerous failed attempts to isolate a clonal population of the PfMAL13P1.319-disrupted parasites via limiting dilutions and 3) always detecting wild-type parasites with the episome in the transfected cultures. One reason for this possible out-competition could be due to the lethal nature of the gene disruption (Crabb, 2002; Gardiner et al., 2003). Since many essential erythrocytic stage proteins have resulted in failed disruption and deletion attempts (O'Donnell et al., 2000; Russo et al., 2009; Sanders et al., 2006; Sijwali et al., 2006; Triglia et al., 2000), this would suggest that PfMAL13P1.319 may participate in a mechanism essential for parasite survival.

To better assess a possible function of PfMAL13P1.319, studying PfMAL13P1.319 protein and its association with other *P. falciparum* parasite or host proteins could lead to identifying a direct or indirect role of PfMAL13P1.319 during the erythrocytic stages. Also, other methods that could be performed to support if PfMAL13P1.319 is critical for the survival of intraerythrocytic stage parasites are allelic replacement, conditional mutagenesis, and antibody-blocking

studies (Muralidharan et al., 2011; Pandey et al., 2006; Reed et al., 2000; Russo et al., 2009; Triglia et al., 1998). Utilizing a truncated form of the PfMAL13P1.319 gene with specific mutations introduced into the endogenous gene by allelic replacement would provide a possible mutant phenotype that could lead to a better understanding of PfMAL13P1.319 function. However, if PfMAL13P1.319 demonstrated a deleterious phenotype, the function of PfMAL13P1.319 still would remain a mystery. Therefore, regulating PfMAL13P1.319 expression via conditional mutagenesis would be another option to assess function. PfMAL13P1.319 antibody-blocking studies are another method that could be utilized in order to assess if anti-PfMAL13P1.319 antibodies would block any potential protein interactions to other parasite or host proteins important for parasite survival.

As a comparative model, *P. berghei* rodent malaria model was used to characterize the *P. berghei* ortholog of MAL13P1.319 (PbMAL13P1.319). There were many similarities between PbMAL13P1.319 and PfMAL13P1.319 such as 1) presence of a signal peptide 2) surface antigen and/or secreted protein predictions 3) erythrocytic stage transcript expression and 4) inability to isolate MAL13P1.319 mutant parasites. Unlike PfMAL13P1.319, PbMAL13P1.319 did not display transcript expression in oocyst and salivary gland sporozoites suggesting that PbMAL13P.1319 may function during the erythrocytic stages where transcripts were detected. However, without a protein expression profile of PbMAL13P1.319, no conclusions can be made.

In summary, studies performed in this dissertation demonstrated PfMAL13P1.319 to be a well-conserved intraerythrocytic stage protein unique to Apicomplexans which could possibly be essential for erythrocytic stage parasite survival. Our attempts to elucidate the molecular, biochemical and cellular role of PfMAL13P1.319 have provided insight into its transcript and protein expression profiles and potential importance during the erythrocytic stages. Overall, these studies support MAL13P1.319 as a potential vaccine/drug target in the erythrocytic stages.

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VITA

Renee Nicole Roberts was born to Edward and Samai Pollard in Langley AFB, Virginia and was raised in three different states since her father was in the military. Renee graduated with honors from Hampton High School in Hampton, Virginia. Then she attended Delaware State University (DSU), where she graduated Summa Cum Laude with a Bachelor of Science degree in Biology and an emphasis in Chemistry. She also met her lifelong love and best friend, Dr. Denzil Roberts at DSU.

After graduation, Renee began pursuing her Doctor of Philosophy in the Molecular Microbiology & Immunology / Veterinary Pathobiology graduate program at the University of Missouri-Columbia in August 2005 and performed her research studies in the lab of Dr. Brenda T. Beerntsen. Her research focuses on characterizing parasite proteins to gain a better understanding of malaria parasite biology. She has presented her research at national conferences and university seminars.

During her graduate training, Renee was involved with many organizations. She was the public relations chairperson for the MU Nexus Graduate Association (NGA), CALEB science club mentor, Graduate Student Association (GSA) representative, Mizzourec fitness instructor, T.E. Aikins Healthy For Life program representative and MU recruiter at national research conferences for minority students.