FUNCTIONAL ANALYSIS OF *PLASMODIUM FALCIPARUM* MAEBL

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Abstract

by

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Malaria is a disease that affects the tropical areas of the world, killing 1-2 million people every year, particularly in Africa. In order to complete its life cycle, *Plasmodium* has to reproduce asexually in the vertebrate erythrocytes and sexually in an *Anopheles* vector. The invasion of host cells is an essential process in the life cycle and it requires parasite ligands and host cell receptors. Two invasive stages essential for the completion of the life cycle are the merozoites that invade the red blood cells and the sporozoites that invade the mosquito salivary glands and the vertebrate liver cells.

MAEBL is a homologue of the transmembrane Erythrocyte binding proteins (EBP) ligands, first described in erythrocytic stages. It is abundantly expressed in midgut sporozoites and was found to be essential for the invasion of *Anopheles* salivary glands in *Plasmodium berghei*. Alternative splicing generates different MAEBL isoforms and so it is unclear what form is functionally essential.

We examined the function of *Plasmodium falciparum* MAEBL in the erythrocytic stages by creating knockouts in W2mef and NF54 parasites. We found that MAEBL is not essential in erythrocytic stages but it may have a conditional role in *P*. 
*falciparum* blood-stage growth. Therefore, we investigated the function of *P. falciparum* MAEBL in the invasion of salivary glands. Using *P. falciparum* NF54 MAEBL knock outs, we confirmed that MAEBL is essential for the invasion of salivary glands. To identify the MAEBL isoform required for *P. falciparum* (NF54) sporozoite invasion of salivary glands, we created knockout and allelic replacements each carrying CDS of one of the major MAEBL isoforms. Only the transmembrane form of MAEBL is essential and is the first *P. falciparum* ligand validated as essential for invasion of *Anopheles* salivary glands. Understanding what *P. falciparum* sporozoite ligands are critical for mosquito transmission will help validate targets for vector-based transmission-blocking strategies.
To Maria Eugenia and Berenice,

for their unconditional support and forbearance
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1 INTRODUCTION

1.1 Malaria, the disease and its burden

Malaria is a vector-borne infectious disease caused by the parasite of the genus *Plasmodium* that is spread from person to person through the bites of infected *Anopheles* mosquitoes. Approximately, 40\% of the world’s population, mostly those living in the world’s poorest countries, is at risk of malaria. Every year, more than 500 million people become severely ill with malaria. The vast majority of the cases and deaths occur in sub-Saharan Africa (Fig.1-1). However, Asia, Latin America, the Middle East and parts of Europe are also affected. In addition, travelers from malaria-free regions going to areas where there is malaria transmission are highly vulnerable; in fact they have little or no immunity (World Health Organization, 2007).

There are four types of human malaria – *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. *P. falciparum* and *P. vivax* are the most common and *P. falciparum* is by far the most deadly type of malaria infection causing more than a million deaths, particularly in Africa. In fact, one in five of all childhood deaths in Africa is due to malaria. It is estimated that an African child has on average between 1.6 and 5.4 episodes of malaria fever each year and every 30 seconds a child dies from malaria (World Health Organization, 2007).
Figure 1-1: Global distribution of malaria (Sachs and Malaney 2002). The distribution of malaria is currently centered in the tropical areas of the world, affecting largely the poorest countries.

Pregnant women are at high risk not only of dying from the complications of severe malaria, but also spontaneous abortion, premature delivery or stillbirth. Malaria is also a cause of severe maternal anemia and is responsible for about one third of preventable low birth weight babies. It contributes to the deaths of an estimated 10,000 pregnant women and up to 200,000 infants each year in Africa alone (World Health Organization, 2007).

The most important reason for the persistence of malaria in Africa is the presence of the vector Anopheles gambiae. In fact, An. gambiae feeds preferentially on humans and has a relatively long life span, making it particularly effective at transmitting malaria from one person to another. To illustrate this point, the entomological inoculation rate (EIR), that measures the frequency with which an individual is bitten by an infectious mosquito, rarely exceeds 5 per year in Asia or South America but it can reach over 1,000 in several parts of sub-Saharan Africa (Greenwood and Mutabingwa 2002).
Malaria has also a high economic burden, in fact, the global distribution of per-capita gross domestic product shows a remarkable correlation between malaria and poverty, and malaria-endemic countries also have lower rates of economic growth. Indeed, malaria causes an average loss of 1.3% of annual economic growth in countries with intense transmission. Moreover, malaria impedes development in multiple ways, including effects on fertility, population growth, saving and investment, worker productivity, absenteeism, premature mortality and medical costs (Sachs and Malaney 2002), (World Health Organization, 2007).

1.1.1 Symptoms and treatment

Infection with malaria parasites may result in a wide variety of symptoms, ranging from absent or very mild symptoms to severe disease and death. The common first symptoms of malaria are fever, headache, chills, nausea and vomiting, body aches, and general malaise. They usually appear 10 to 15 days after a person is infected. In addition, physical findings may include elevated temperature, perspiration, weakness, and enlarged spleen. In the particular case of P. falciparum malaria, additional findings may include mild jaundice, enlargement of the liver, and increased respiratory rate.

When the disease is not treated promptly with effective medicines, it can cause severe illness that is often fatal. Severe malaria occurs when P. falciparum infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism. The manifestations of severe malaria include cerebral malaria, with abnormal behavior, impaired consciousness, seizures, coma, or other neurological abnormalities besides severe anemia due to hemolysis (destruction of the red blood cells),
hemoglobinuria (hemoglobin in the urine), pulmonary edema (fluid buildup in the lungs), or acute respiratory distress syndrome (ARDS), which may occur even after the parasite counts have decreased in response to treatment, abnormalities in blood coagulation and thrombocytopenia (decrease in blood platelets), cardiovascular collapse and shock (CDC, 2004).

The most common drugs used to treat malaria are active against the parasite forms in the blood (the form that causes disease) and include chloroquine, sulfadoxine-pyrimethamine (Fansidar®), mefloquine (Lariam®), atovaquone-proguanil (Malarone®), quinine, doxycycline and artemisinin derivatives. In addition, primaquine is active against the dormant parasite liver forms (hypnozoites) and prevents relapses (CDC, 2004).

1.1.2 Drug resistance

Drugs that are currently in use as antimalarials have many attributes, but also possess certain liabilities that might be improved by further drug discovery and development. Currently, the main limitation to the use of many drugs is resistance. There is global resistance prevailing against two of the most widely used antimalarial drugs, chloroquine and the antifolate sulphadoxine/pyrimethamine. For many years all the types of human malaria have been treated with chloroquine, a drug from the quinoline group, derived from the quinine, an active ingredient of Cinchona bark. Chloroquine was highly effective and had a low cost, making it a very suitable drug for the treatment of malaria. However, resistance to chloroquine has occurred on a global scale (Ridley 2002).
The antifolate sulphadoxine/pyrimethamine belongs to a class of antimalarial drugs that is not derived from plants. It originates from compounds generated through the knowledge of cell biology and synthetic medicinal chemistry. These components work as inhibitors of dihydrofolate reductase (DHFR) and the dihydroopteroate synthase (DHPS). Resistance of sulphadoxine/pyrimethamine is now widespread.

Other drugs that are being used to treat malaria include other types of quinolines, artemisins, atovaquone/proguanil and antibiotics. Many of these drugs pose problems of resistance and cost. The challenge ahead lies in determining the best alternative therapies and the establishment of mechanisms and projects to ensure that improved drugs are sustainably discovered and developed into the future (Ridley 2002).

1.1.3 The vaccine challenge

The malaria parasite can establish a chronic infection in an immunocompetent host avoiding the immune response of the host. As a result it is very challenging to reproduce sterile immunity that follows natural infection. *Plasmodium* benefits from immune responses if they lead to chronic infection and, as a result, enhance transmission to the mosquito (Richie and Saul 2002). In addition, different stages of the parasite express different antigens, as a result of which, a vaccine effective to one stage of the parasite may not be effective against the others. Moreover, many parasite ligands display polymorphisms, limiting the efficacy of any vaccine not incorporating all the variants of the antigen (Richie and Saul 2002; Matuschewski and Mueller 2007).

Currently, vaccines directed against the different stages of life cycle are being developed (Richie and Saul 2002):
1. Vaccines directed against sporozoites and/or liver stages (collectively termed pre-erythrocytic stages) are designed to prevent blood-stage infection and thereby avoid all manifestations of disease (anti-infection vaccines). This type of vaccine could reduce transmission and morbidity.

2. Vaccines directed against asexual blood stages are designed to reduce clinical severity (anti-morbidity/mortality vaccines). An effective blood-stage vaccine could eliminate blood stages as soon as they emerge from the liver, thereby curtailing both infection and transmission.

3. Vaccines directed against mosquito stages are designed to halt development in the mosquito (transmission-blocking vaccines). This type of vaccine could reduce population-wide malaria infection rates and malaria associated morbidity.

1.2 The life cycle of *Plasmodium*

The life cycle of *Plasmodium* occurs in two different hosts: a vertebrate host, where the asexual part of the cycle takes place and a mosquito of the genus *Anopheles*, where the sexual part of the cycle occurs (Fig. 1-2). The cycle starts when the female *Anopheles* mosquito takes a blood meal. The mosquito releases *Plasmodium* sporozoites under the skin, which traverse the skin and reach a blood vessel. Within 30 to 45 minutes after inoculation, the sporozoites reach the liver parenchyma cells. The sporozoites cross the Kupffer cells of the liver and reach the hepatocytes where they develop into exoerythrocytic schizonts and divide by multiple fission to form thousands of invasive merozoites that upon host cell rupture attach and enter circulating erythrocytes.
In order to complete its life cycle, the malaria parasite has to invade a vertebrate and an invertebrate host. The sporozoites invade the liver where they mature into exo-erythrocytic stages. The asexual stages in the erythrocytes of the vertebrate host are responsible for the morbidity and mortality associated with the disease. The sexual stages of the parasites occur in the invertebrate host.
In *P. vivax*, some of the hepatic forms can remain dormant and can delay their schizogony up to several years, being the cause of relapses (Johnson, Epstein et al. 1980).

The merozoite stage is short-lived and must invade host red blood cells in a rapid manner (Johnson, Epstein et al. 1980). Once the merozoite has invaded the erythrocyte, it undergoes several changes and becomes a ring that changes to a trophozoite. After experiencing multiple divisions, the parasite develops into a schizont that contains 8-32 merozoites. The schizont ruptures the erythrocyte and releases infective merozoites that are able to invade new erythrocytes. The release of merozoites from ruptured schizonts is a synchronized event and is responsible for the cyclic chills and fever classically associated with clinical malaria. A sub-population of the merozoites form gametocytes. Gametocyte maturation is a process that takes 9 to 12 days. The mature gametocytes (male microgametocyte and female macrogametocyte) mature into microgametes and macrogametes in the mosquito midgut. Each microgametocyte can produce up to 8 microgametes. Upon fertilization in the midgut of the female *Anopheles*, a zygote (the only diploid stage in the life cycle of the parasite) is formed, which then develops into a motile oökinete. The oökinete penetrates the mosquito midgut, crosses the epithelium and gets arrested in the basal lamina where it develops into an oocyst. The oocyst development is the longest phase of the life cycle. It undergoes meiosis and 12 mitotic nuclear divisions while it enlarges in size. Then the cytoplasm segregates into regions named sporoblasts. These sporoblasts transform into budding centers for the formation of thousands of sporozoites. When the sporozoites mature, the oocyst ruptures and the sporozoites are released to the hemocoel (Johnson, Epstein et al. 1980).

A small fraction of the sporozoites (19%) make their way to the salivary glands.
(Hillyer, Barreau et al. 2007), and invade mainly the distal lateral and medial lobes. The salivary gland sporozoites are infective to a new vertebrate host and can be transmitted through the bite of a female mosquito.

1.3 Invasion of host cells

An essential process for the completion of the *Plasmodium* life cycle is the invasion of host cells. The merozoite invades the erythrocyte, the oökinete invades the mosquito midgut cells and the sporozoite invades multiple cells, including the salivary gland cells in the mosquito and the liver cells in the vertebrate host. The invasion of host cells in *Plasmodium* occurs in successive steps that can be divided into three distinct phases (Chitnis and Blackman 2000): 1) initial attachment to the host cell, 2) irreversible attachment and reorientation, and 3) parasitophorous vacuole formation (Fig. 1-3).

The first step of the invasion of a host cell is irreversible attachment and reorientation. This is an initial, low-affinity interaction with the host cell and involves adhesive ligands from the surface of the parasite. Reorientation of the bound parasite may be favored by the presence of higher-avidity ligands clustered around the apical prominence. Upon reorientation, irreversible attachment and junction formation may be initiated by exocytosis of micronemal components. The effects of anterior-to-posterior trafficking and/or proteolytic shedding of these and other RBC-binding proteins, linked to the action of a sub-pellicular actomyosin motor, may then aid in driving the parasite into the nascent parasitophorous vacuole (Chitnis and Blackman 2000).
Figure 1-3. Plasmodium invasion of red blood cells (Chitnis and Blackman 2000).
The figure shows the three main steps for the merozoite invasion of red blood:
Initial attachment and reorientation, irreversible attachment and junction formation, and
parasitophorous vacuole and invasion. In addition, the cells and the ligands that could be
involved in these steps are shown.

1.3.1 The Plasmodium merozoite

The Plasmodium form that invades the erythrocytes is the merozoite. Upon
release from an infected hepatocyte or erythrocyte, Plasmodium merozoites enter the
blood circulation of the host and invade new erythrocytes. The merozoite has a set of
secretory organelles that contain proteins involved in the invasion of the red blood cells.
The three main apical organelles of the merozoite are the micronemes, the rhoptries and
the dense granules. Micronemes are small vesicles of varying electron density that
frequently show a neck-like extension. Rhoptries are large, usually paired, pear-shaped
organelles filled with proteins and phospholipids. These organelles discharge at the
anterior tip of the parasite in the process of invasion of the red blood cells (Bannister, Hopkins et al. 2000). Dense granules are spherical organelles with a size intermediate between the micronemes and the rhoptries (Culvenor, Day et al. 1991).

1.3.2 The invasion of red blood cells

After the initial contact, the merozoite reorients itself such that the apical end of the merozoite faces the erythrocyte membrane. Once the apical end is apposed toward the erythrocyte membrane, a membrane-to-membrane adhesion occurs and a tight junction is formed between the two cells (Bannister, Butcher et al. 1975; Aikawa, Miller et al. 1978). While the initial contacts between the merozoite and erythrocyte are reversible, the formation of a tight junction is irreversible. At this point, the merozoite is committed to invade this particular erythrocyte.

After the formation of a tight junction, there is movement of this junction around the merozoite toward its posterior pole with the entry of the merozoite into the red blood cell in an active process and requires energy and involves an actin-based parasite molecular motor. Simultaneously to this process, the contents of the rhoptries are released and finally, there is an alteration of the erythrocyte membrane architecture followed by an invagination of the erythrocyte membrane forming the parasitophorous vacuole membrane (PVM) and a parasitophorous vacuole (PV). Once the merozoite is completely inside its new host erythrocyte, the junction fuses and closes (Aikawa, Miller et al. 1978; Aikawa and Miller 1983). The merozoite that is completely inside the erythrocyte then transforms into an intracellular-ring stage trophozoite that begins to grow by feeding on the RBC hemoglobin.
1.3.3 Molecules in the *Plasmodium* invasion of erythrocytes

1.3.3.1 Merozoite surface proteins

Nine merozoite surface proteins MSPs (1-9) have been identified in either *P. falciparum* or in *P. vivax* and a number of them have been described for other *Plasmodium* species. These proteins are thought to be involved in the initial recognition and attachment with the erythrocyte surface (Epstein, Miller et al. 1981; David, Hadley et al. 1984; McKean, O'Dea et al. 1993; Toebe, Clements et al. 1997; Holder, Guevara Patino et al. 1999).

*P. falciparum* MSP-1 is the best characterized of the MSPs. It is a leading vaccine candidate (Holder, Guevara Patino et al. 1999; Uthaipibull, Aufiero et al. 2001) present on the surface of merozoites as a noncovalent complex of four fragments 83, 30, 38 and 42 kDa (Holder and Freeman 1984; Perkins 1984). The C-terminal fragment binds the complex to the surface of the merozoite via a glycosylphosphatidylinositol (GPI) anchor. Further processing of this fragment yields two fragments of 33 and 19 kDa. Monoclonal antibodies against the C-terminal end of MSP1 have been shown to inhibit merozoite invasion (Chappel and Holder 1993).

1.3.3.2 Microneme proteins

One of the vastly studied micronemal proteins is the apical membrane antigen 1 (AMA-1), which plays a role in binding erythrocytes. AMA-1 is found in different *Plasmodium* species and in *Toxoplasma* (Donahue, Carruthers et al. 2000). AMA-1 is a Type I integral membrane protein that is synthesized as an 83 kDa (*P. falciparum*) or a 66
kDa protein (other species). AMA-1 is then redistributed to the surface of the merozoite near the time of invasion and hence thought to play a role in the initial attachment of the merozoite to the erythrocyte (Narum and Thomas 1994). It has been shown that anti-AMA-1 MAb antibodies inhibited \textit{P. knowlesi} invasion in vitro (Deans, Alderson et al. 1982) and it was recently shown that anti-AMA-1 antibodies block sporozoite invasion of hepatocytes, validating the efficacy of AMA-1 as a leading antimalarial vaccine candidate (Bannister, Hopkins et al. 2003).

An important group of micronemal proteins that have functions in the invasion of red blood cells are the DBL-EBPs (Duffy binding like erythrocyte binding proteins) (Adams, Sim et al. 1992). Some of the members of this group bind to glycoproteins on red blood cells. Previous studies have confirmed that the erythrocyte membrane glycoprotein carrying the Duffy blood group antigen epitope is a key receptor for \textit{P. vivax} and \textit{P. knowlesi} merozoites (Miller, Mason et al. 1975; Barnwell, Nichols et al. 1989). \textit{P. vivax} and \textit{P. knowlesi} merozoites do not invade human erythrocytes lacking the Duffy glycoprotein and antibodies raised against the Duffy-specific epitopes inhibit \textit{P. knowlesi} and \textit{P. vivax} invasion (Miller, Mason et al. 1975; Barnwell, Nichols et al. 1989). Experiments using \textit{P. knowlesi} have shown that interaction between the merozoites and the erythrocyte Duffy glycoprotein is a key process in the formation of the apical tight junction (Miller, Aikawa et al. 1979). The merozoite proteins that bind the Duffy were identified to be a 135 kDa protein in \textit{P. knowlesi} and a 140 kDa protein in \textit{P. vivax} (Haynes, Dalton et al. 1988; Wertheimer and Barnwell 1989). In order to invade the red blood cells \textit{P. falciparum} merozoites use the sialic acid residues on erythrocyte membrane glycophorins (Miller, Haynes et al. 1977; Pasvol, Wainscoat et al. 1982).
specifically binds to sialic acid residues of glycophorin A on the erythrocyte (Camus and Hadley 1985; Sim, Orlandi et al. 1990; Orlandi, Klotz et al. 1992). Other members of this family are BAEBL, a 140 kDa microneme protein, that has been shown to bind to glycophorin C (Lobo, Rodriguez et al. 2003; Maier, Duraisingh et al. 2003), JESEBL, a 180 kDa microneme protein, and EBL-1 (Peterson and Wellems 2000).

The ebl gene family in Plasmodium encodes the Duffy binding-like-erythrocyte binding proteins (DBL-EBPs). Members of this family include the \textit{P. vivax dbp} and \textit{P. knowlesi dbps}, \textit{P. yoelii} and \textit{P. berghei maeb}, \textit{P. reichenowi} and \textit{P. cynomolgi ebp}, and \textit{P. falciparum baebl, eba-175, ebl-1, jesebl, maeb} and \textit{pebl} (Adams, Sim et al. 1992; Okenu, Malhotra et al. 1997; Adams, Blair et al. 2001) (Fig. 1-4). The features shared by the members of this family of genes include: similar multi-exon structures, conserved exon/intron boundaries, single copy genes, and amino and carboxyl cysteine-rich domains. The exonic structure of the \textit{ebls} includes a putative signal sequence followed by the amino cysteine-rich Duffy binding-like (DBL) domain. The DBL domain acts as the extracellular ligand domain for the parasite to bind erythrocytes. \textit{P. vivax} and \textit{P. knowlesi} DBPs have a single DBL domain, but there is a tandem duplication of the DBL domains of the DBL-EBPs in \textit{P. falciparum} and \textit{P. reichenowi}. The carboxyl cysteine-rich domain is highly conserved among the DBL-EBPs with approximately 35\% amino acid identity. Cysteine residues are perfectly conserved in position and number in all the DBL-EBPs except for EBL-1 which has only four cysteine residues instead of the conventional eight (Adams, Sim et al. 1992; Blair, Witney et al. 2002). The 3D structure of this region was recently elucidated showing that it is a homodimer, containing in each
subunit a compact five-alpha-helix core that is stabilized by four conserved disulfide bridges. It shows no similarity to the Duffy-binding-like domains of EBA-175 involved in erythrocyte binding, indicating a distinct role (Withers-Martinez, Haire et al. 2008). Following the carboxyl cysteine-rich regions are a transmembrane domain and two intracellular domains. The cytoplasmic tail of EBA-175 appears to play a role in the invasion process by being a part of the parasite machinery for the invasion of erythrocytes (Gilberger, Thompson et al. 2003).

Figure 1-4 Schematic representations of *P. falciparum ebl* gene structure. There are six *ebl* genes in *Plasmodium falciparum* while there is only one in *P. vivax*. Ligand domains of *maebl* are different than the other *ebls* and are similar to those of *ama-1*. *ebl-1* lacks four of the eight cysteine residues in the c-cys domain that are conserved in the other members of the family (Adams et al 2001).
1.3.3.3  *maebl*: a unique member of the *ebl* gene family

MAEBL is found in all the different species of *Plasmodium* and is a paralogue of the DBL-EBP family. MAEBL was first characterized in *P. berghei* and *P. yoelii* (Kappe, Curley et al. 1997; Kappe, Noe et al. 1998) and later in *P. falciparum* (Blair, Kappe et al. 2002). *maebl* is a chimeric molecule, it shares its exonic structure with the *ebl* and it has similarities with this gene family in the transmembrane and cytoplasmic domains. On the other hand, the amino cysteine-rich region of *maebl* has a tandem copy of domains (M1 and M2) that share similarity to the cysteine-rich domain 1 and 2 of AMA-1 and not the DBL domains (Kappe, Noe et al. 1998; Noe and Adams 1998; Michon, Stevens et al. 2002). *maebl* transcripts are found in late rings / early trophozoites of the blood stage development, whereas, the rest of the *ebls* are found in the late stages (Blair, Witney et al. 2002; Bozdech, Llinas et al. 2003). Finally, MAEBL seems to be localized to the rhoptry organelles in blood-stage merozoites whereas BAEBL, EBA-175 and JESEBL have all been shown to localize to the micronemes (Mayer, Kaneko et al. 2001; Blair, Kappe et al. 2002; Gilberger, Thompson et al. 2003).

1.3.3.4  Rhoptry proteins

A number of *Plasmodium* rhoptry proteins are involved in the invasion process. One of these is the high molecular weight complex, RhopH with three different proteins of 140, 130 and 110/105 kDa (Holder, Freeman et al. 1985). The other rhoptry protein complex is composed of two lower-molecular mass proteins of RAP-1 (80 kDa), RAP-2 (42 kDa) and RAP-3 (37 kDa). The exact functions of these protein complexes is not
very well known, but proteins of both complexes have been found associated with the erythrocyte membrane shortly after invasion (Lustigman, Anders et al. 1988).

Another rhoptry family of proteins that play a role in the attachment of merozoites to the erythrocyte is the *P. yoelii* 235 kDa and its homologues in other species (Holder, Freeman et al. 1985). A monoclonal antibody against the Py235 protein protected mice from infection with *P. yoelii* YM strain by restricting the parasites to invade only reticulocytes (young RBCs), which suggests that this protein is involved in the invasion of mature erythrocytes (Freeman, Trejdosiewicz et al. 1980; Holder and Freeman 1981). Homologues of Py235 are found in different *Plasmodium* species. In *P. vivax*, these proteins were termed reticulocyte binding protein 1 and 2 (PvRBP-1 and PvRBP-2). In fact, the ability of *P. vivax* to invade only reticulocytes has been attributed to these two proteins. In *P. falciparum*, the PfRh family of proteins comprises five different proteins, PfRh1, PfR2ha, PfR2hb, PfRh3 and PRh4 (Triglia, Thompson et al. 2001). PfRh1 is an orthologue of PvRBP1 and binds to a receptor on RBC that is trypsin resistant and sialic acid independent (Triglia, Duraisingh et al. 2005). PfRh1 also binds erythrocytes lacking glycophorin B thereby suggesting a novel pathway of erythrocyte invasion. PfRh1 is localized to the apical end of the merozoite. The protein PfRh4 was found to be involved in a sialic acid independent invasion pathway and is essential for switching invasion pathways (Stubbs, Simpson et al. 2005).

1.3.3.5 Dense granules proteins

Only a few proteins have been found in the dense granules of malaria merozoites. After the closure of the parasitophorous vacuole membrane, the dense granules migrate to
the periphery and fuse to the plasma membrane of the merozoite, releasing its contents into the parasitophorous vacuole. RESA (ring-infected erythrocyte surface antigen) is a dense granule protein which after release is found under the membrane of the erythrocyte and is thought to be associated with the cytoskeleton (Aikawa, Torii et al. 1990; Culvenor, Day et al. 1991). RIMA (ring membrane antigen) is a 14 kDa protein that remains localized to the early trophozoite plasma membrane (Trager, Rozario et al. 1992). Also, two subtilisin-like proteases, PfSUB-1 and PfSUB-2, are present in the dense granules. Both these proteases are thought to play a role in the proteolytic processing of invasion molecules (Hackett, Sajid et al. 1999).

1.3.4 The *Plasmodium* sporozoite

1.3.4.1 Structure of the sporozoite

The sporozoite of the *Plasmodium* species is the only invasive stage that can invade more than one different type of cells in two different hosts: the salivary glands in the mosquito, the Kupffer cells and hepatocytes in the vertebrate host. The sporozoite, like the other invasive stages, has a unique set of secretory vesicles, termed micronemes and rhoptries, defining the apical complex. They have proteins with functions in apicomplexan motility, host cell invasion, and the generation of the nonphagosomal parasitophorous vacuole (PV). The apicomplexan invasive forms have a triple membrane pellicle, consisting of the outer plasma membrane and a closely juxtaposed inner membrane complex (IMC) (Aikawa 1967; Dubremetz and Torpier 1978; Morrissette and Sibley 2002). Underlying and supporting the triple membrane are rows of microtubules.
(MTs) (Sinden and Strong 1978; Adams and Todd 1983; Krettli and Miller 2001; Mota, Pradel et al. 2001; Mota and Rodriguez 2002; Kappe, Buscaglia et al. 2004) (Fig. 1-5). An actin-myosin motor essential for parasite motility and invasion is located in the narrow space between the plasma membrane and the outer membrane of the IMC (Kappe, Buscaglia et al. 2004).

![Diagram of Plasmodium sporozoite](image)

**Figure 1-5.** The *Plasmodium* sporozoite (Kappe, Buscaglia et al. 2004).

The apical complex of the *Plasmodium* sporozoite is characterized by micronemes, rhoptries and the polar ring. An inner membrane complex (having two membranes) (IMC) is underlying the plasma membrane and interacting with the actomyosin motor.
1.3.4.2 The glideosome

The *Plasmodium* sporozoites are capable of invading host cells by gliding motility. Indeed, most apicomplexan species are able to glide on solid substrates, including host cell surfaces. Gliding motility, specific to Apicomplexa, is defined by the absence of any obvious modification in the shape of the moving cell (Menard 2001).

The glideosome is the molecular machine that promotes gliding motility in Apicomplexa and comprises the motor complex myosin A (MyoA), GAP48 (gliding associated protein 48) and GAP50 (gliding associated protein 50), anchored in the outer membrane of the IMC and connected via actin (F-actin) and aldolase to a microneme – protein or protein complex (MIC2 in *Toxoplasma* and TRAP in the case of *Plasmodium*). These microneme proteins interact with host-cell receptors (Buscaglia, Coppens et al. 2003; Keeley and Soldati 2004) (Fig. 1-6). These sets of molecules are coordinately involved in the process of gliding motility and invasion of the host cells.

1.3.5 The sporozoite invasion of *Anopheles* salivary glands

Mosquito salivary glands are paired organs localized in the thorax. In females, each gland is formed by two similarly constructed lateral lobes and a shorter and wider medial lobe, while large secretory cells separated by a narrow region formed by non-secretory cells form the medial lobe. Two regions can be identified in the lateral lobes, proximal and distal, separated by a narrow transitional region. Cells in the distal regions are similar to those forming the medial lobe and their secretions participate in blood feeding. The proximal regions are narrow and are similar to those forming the salivary glands of males and their secretions participate in sugar feeding (Fig. 1-7)
Figure 1-6. Gliding machinery in Apicomplexa (Soldati and Meissner 2004). The figure shows the current understanding of the molecular machinery responsible for gliding motility and host cell invasion in apicomplexan parasites. The three membranes (IMC bilayer and plasma membrane). Myosin (MyoA) and its light chain (MLC) is anchored to the parasite cytoskeleton (comprising the inner membrane particles (IMP), the subpellicular filaments, IMC network, and the microtubules) by gliding-associated proteins (GAP) 45 and GAP50. On the other hand, actin (F-actin) is anchored to the host cell by aldolase and the microneme protein MIC2 (Toxoplasma) or TRAP (Plasmodium). The interaction between the two parts of the motor complex (actin and myosin) results in moving the parasite relative to its extracellular anchor point. Finally, in order to complete the movement and force for invasion, the microneme proteine must be cleaved by a protease like the microneme protein protease 1 (MPP1) in the case of Toxoplasma.

Figure 1-7. Salivary gland of a female of *Anopheles stephensi*. The different lobes of the gland are shown (Jariyapan, Choochote et al. 2007). PL: Proximal lobe, DL: Distal lobe, ML: Medial lobe.
Midgut sporozoites are transported by the circulation of the hemolymph to then invade the salivary glands. Only 19-20% of the sporozoites that are released from the oocysts are able to reach the salivary glands (Hillyer, Barreau et al. 2007). The invasion of salivary glands is an important step for the maturation of the sporozoites. Several studies have shown that the salivary glands sporozoites have higher infectivity than the midgut sporozoites (Vanderberg 1975; Daher and Krettli 1980). In addition, salivary gland sporozoites are not able to invade salivary glands from a different mosquito (Touray, Warburg et al. 1992). These data imply a requirement of the sporozoites to invade the salivary glands to be infective to a new host.

The fact that the parasites invade only specific regions of the salivary glands implies the presence of specific sporozoite receptors in these areas. In addition, it has been suggested that the accumulation of parasites in the vicinity of the preferred invasion regions of the glands would involve the participation of a taxic mechanism in the homing (Golenda, Starkweather et al. 1990). This could be mediated by soluble molecules released by salivary glands or by a topological distribution of ligand carbohydrate residues; however, this hypothesis has not been proved.

The mechanism of invasion of the female mosquito salivary glands has been described in detail for the *Plasmodium gallinaceum* invasion of the *Aedes aegypti* glands using electromicrocopy. Eight steps of invasion were defined for this process (Pimenta, Touray et al. 1994) (Fig. 1-8). First the sporozoite attaches to the basal lamina of the salivary gland followed by the invasion of the basal lamina. Then, the sporozoites invade
the plasma membrane of the salivary cell; as a result, a vacuole is formed surrounding it. The sporozoites are released to the salivary cavity, where they accumulate until they are able to pass through the salivary duct to be transmitted to another host (Pimenta, Touray et al. 1994).

The sporozoite invasion of the salivary cell plasma membrane requires that the sporozoites attach to the membrane, re-orientate and form an irreversible junction with the membrane of the cell. The molecular interactions of this process are just starting to be elucidated. In fact, it is known that gliding motility is playing an essential role for the completion of the salivary gland invasion (Kappe, Bruderer et al. 1999) but all the molecules involved in this process are not known.

It is probable that specific carbohydrate molecules on the surface of salivary glands function as parasite receptors; however, specific molecules participating have not been identified. Glycosaminoglycan chains (GAG) are receptors for the sporozoites in the liver (Frevert, Sinnis et al. 1996); however, these have not been identified in salivary glands. Some possible receptors for the sporozoites in the salivary glands have been identified but not characterized. Using a phage display strategy, a 12-amino acid peptide (PCQRAIFQSICN) that binds to the midgut luminal side and salivary glands (named SM1) of An. stephensi was identified. SM1 blocks midgut and salivary gland invasion by P. berghei, indicating the presence of common binding ligands for the two invasive forms of the parasite on the surface of these organs (Ghosh, Ribolla et al. 2001). Recently, SGS1 a protein present in the basal lamina of distal lateral lobes of the salivary glands has been described. Anti-aaSGS1 antibodies inhibit sporozoite invasion into the salivary glands in vivo, confirming aaSGS1 as a candidate sporozoite receptor. Two An. gambiae
Figure 1-8. *Plasmodium* sporozoite invasion of *Anopheles* salivary glands (Pimenta et al., 1994).

In order to invade the salivary glands and be transmitted to another host, the sporozoites go through a number of successive steps: 1. Sporozoite attachment to the basal lamina. 2. Sporozoite invasion of the basal lamina. 3. Sporozoite invasion of salivary cell plasma membrane. 4. Vacuole formation surrounding the sporozoite and mitochondria. 5. Sporozoite is released to the salivary cavity. 6. Vacuole formation in the salivary cavity. 7. Accumulation of the sporozoites in the salivary cavity. 8. Passage of the sporozoites though the salivary duct.
SGS genes display salivary gland specific expression like aaSGS1. SGS proteins possess heparin-binding domains, and have among the highest density of tyrosine sulphation sites of all *An. gambiae* proteins (Korochkina, Barreau et al. 2006). Previously, a molecule of 100 kDa in *An. gambiae* salivary glands was identified as a putative receptor for *P. yoelii* sporozoites (Brennan, Kent et al. 2000). Recently, this protein, designated Saglin was partially characterized and suggested to exist as a disulphide-bonded homodimer of 50 kDa subunits. In addition, it is suggested that Saglin is a secreted protein and is involved in the process of invasion of *A. gambiae* salivary glands by sporozoites (Okulate, Kalume et al. 2007).

1.3.6 *Plasmodium* sporozoite proteins involved in the invasion of salivary glands

Three proteins have been shown to be involved in the process of invasion of *Anopheles* salivary glands: CSP (circumsporozoite surface protein)(Menard, Sultan et al. 1997; Sinnis and Nardin 2002), TRAP (trombospondin related anonymous protein)(Sultan, Thathy et al. 1997) and MAEBL (Kariu, Yuda et al. 2002). These three proteins are found in the micronemes and localize to the surface of the sporozoite before or during invasion.

1.3.6.1 Circumsporozoite surface protein (CSP)

CSP is a protein unique to *Plasmodium* species and is the main sporozoite surface protein of the sporozoite. CSP is a single copy gene, and its C-terminal region encodes a GPI attachment signal (Nussenzweig and Nussenzweig 1989; Kappe, Buscaglia et al. 2004). It is synthesized as a 50- to 70-kDa precursor that is later processed into a 40- to
60-kDa mature surface protein (Nussenzweig and Nussenzweig 1985; Coppi, Pinzon-Ortiz et al. 2005). This molecule generally presents common structural characteristics, with a variable central region composed of tandem repeat amino acid sequences and two highly conserved portions (regions I and II). Region I, localized in the N terminus, contains the conserved KMKQP motif in different malarial parasites in mammals. A peptide from the region I (82DEKLRKPKHKKLQADG100), called I-plus, binds saturably to heparin and inhibits CSP recombinant protein binding to heparin (Ancsin and Kisilevsky 2004). The central tandem repeat region is formed in the *P. falciparum* CSP by 37 repeat units with the NANP sequence and 4 repeat units with the NVDP sequence (Dame, Williams et al. 1984). It has been suggested that the central CSP repeat region is involved in initial stages of host cell invasion or that it could perform a structural role in the CS protein (Godson, Ellis et al. 1983; Hoffman, Nussenzweig et al. 1991). The II-plus region, localized in the CSP C-terminal portion, has significant homology with thrombospondin cell (TSR) and properdin domain adhesion (Lawler and Hynes 1986; Robson, Hall et al. 1988) as well as with some other sporozoite surface proteins such as TRAP and TRAP-related protein (CTR) (Trottein, Triglia et al. 1995). This region consists of 18 amino acids and is involved in binding to HSPGs (Fig. 1-9) (Cerami, Kwakyte-Berko et al. 1992).

CSP has long been the primary focus of pre-erythrocytic vaccine development and several CSP based vaccines have been produced; some are undergoing human trials. In fact, immunization with irradiated sporozoites leads to robust protection against malaria infection in rodents, monkeys and humans by eliciting antibodies to CSP that inhibit sporozoite infectivity (Nussenzweig, Vanderberg et al. 1967; Clyde, McCarthy et
al. 1973; Gwadz, Cochrane et al. 1979; Romero, Maryanski et al. 1989; Kumar, Sano et al. 2006).

Figure 1-9. Schematic representation of the genes coding for CSP, TRAP and MAEBL (Kappe et al., 2003).

CSP is a GPI anchored molecule with two main regions RI and RII. trap and maebl code for type 1 transmembrane proteins. TRAP has two adhesive domains (A domain and TSR) a transmembrane domain and an acidic cytoplasmic domain. MAEBL has two ligand domains M1 and M2, a transmembrane domain and an acidic cytoplasmic domain.

CSP is an important multifunctional molecule for the parasite. It is an essential protein in both, the formation of the sporozoites (Menard, Sultan et al. 1997; Sinnis and Nardin 2002), and in the sporozoite binding to liver cells and inactivating host cell protein synthesis machinery (Cerami, Kwakye-Berko et al. 1992; Frevert, Sinnis et al. 1993). Recently, it was reported that CSP promotes the development of the liver stages of the parasite by influencing the expression of over one thousand host genes involved in diverse metabolic processes (Singh, Buscaglia et al. 2007).
It is suggested that CSP would be participating in the first steps of *Anopheles* salivary glands invasion, and binds preferentially to salivary glands when compared to other organs exposed to the circulating hemolymph. In addition, a peptide from region I of CSP, which is highly conserved in rodent and primate parasites inhibits binding of CS to mosquito salivary glands (Sidjanski, Vanderberg et al. 1997).

1.3.6.2 Thrombospondin anonymous protein (TRAP)

TRAP (also known as Sporozoite surface protein 2, SSP2) is a type I transmembrane protein that is expressed in the micronemes of the midgut sporozoites and is relocated to the surface prior to invasion of host cells (Rogers, Malik et al. 1992; Kappe, Kaiser et al. 2003). TRAP just like CSP is an important vaccine candidate. In fact, mice immunized with a mixture of TRAP and CSP transfectants were completely protected against malaria (Bodescot, Silvie et al. 2004).

TRAP has two adhesion domains in the extracellular portion: a type A domain and a second domain with a motif similar to that of the thrombospondin type I repeat (TSR) (Robson, Hall et al. 1988), that shares the region II-plus with CSP. A putative TRAP parologue protein (CTRP) in the *Plasmodium* ookinete stage has been described, and other putative protein orthologues have been identified in other Apicomplexa such as *Toxoplasma* (MIC2), *Eimeria* (Etp100), and *Cryptosporidium* (TRAPC1) species (Trottein, Triglia et al. 1995; Carruthers, Sherman et al. 2000; Kappe, Kaiser et al. 2003). The members of this family have in common two extracellular domains (A and TSR), in addition to an acidic cytoplasmic domain.
TRAP is involved in the process of gliding motility of the sporozoite, the invasion of salivary glands and the invasion of hepatocytes in the vertebrate host (Sultan, Thathy et al. 1997; Kappe, Bruderer et al. 1999; Matuschewski, Nunes et al. 2002). Both the extra cellular adhesive domains and the cytoplasmic domain of TRAP are important in invasion of salivary glands and hepatocytes. It is suggested that both domains are necessary for the recognition of specific ligands in the salivary glands and liver cells by recognizing specific receptors (Matuschewski, Nunes et al. 2002). In particular, mutations in TRAP adhesive (A) domain do not affect motility but decrease sporozoite invasion of host cells (Matuschewski, Nunes et al. 2002) and sporozoites that have mutations in both extracellular domains have motility but do not invade salivary glands. Ligands for specific domains of TRAP in the hepatic cells have been identified. Indeed, heparin binds the A domain of TRAP in vitro and the TSR domain binds heparan sulfate (McCormick, Tuckwell et al. 1999).

The disruption of the *P. berghei* TRAP showed that it is a critical protein in invasion of salivary glands and in gliding motility of the sporozoites on solid substrates in vitro but not initial attachment to the glands (Sultan, Thathy et al. 1997; Kappe, Bruderer et al. 1999; Wengelnik, Spaccapelo et al. 1999; Kappe, Kaiser et al. 2003). In addition, mutant parasites lacking the cytoplasmic domain of TRAP do not associate with salivary glands, are not infective to the liver cells, and do not show gliding motility (Kappe, Bruderer et al. 1999). By allelic replacement of the cytoplasmic domain, they showed that it is functionally homologous to the cytoplasmic domain of the *Toxoplasma* MIC2. Furthermore, by amino acid substitutions it was determined that the conserved residues of the cytoplasmic domain were essential for invasion and gliding motility. In particular,
a terminal tryptophan residue was found to be essential in the process of gliding motility and invasion (Kappe, Bruderer et al. 1999).

The way of action of TRAP in the invasion of host cells has been determined. The adhesive domains of TRAP bind to a receptor in the host cell while essential residues of the cytoplasmic domain bind to aldolase, a protein that directly interacts with the actomyosin motor of the parasite.

Recently, many ligands previously thought to be present only in blood stages were identified in sporozoites (Florens, Washburn et al. 2002; Le Roch, Zhou et al. 2002). In particular, EBA-175, and AMA1 (apical membrane antigen), two important vaccine candidates involved in invasion of erythrocytes are also expressed in sporozoites. Their exact function in salivary gland invasion is, nevertheless, unknown (Gruner, Brahimi et al. 2001; Silvie, Franetich et al. 2004).

1.3.6.3 MAEBL in Anopheles stages

MAEBL is a sporozoite microneme protein, paralogue of erythrocyte binding proteins (EBP), implicated in the merozoite invasion of erythrocytes (Kappe, Noe et al. 1998; Fu, Saenz et al. 2005). Importantly, EBP family ligands share structural homology with MAEBL in the extracellular carboxyl cysteine-rich domain and in the presence of transmembrane and cytoplasmic domains, but do not appear to interact with the glideosome in the same way as TRAP or its homologues. Amino terminal cysteine-rich domains of MAEBL have similarity to AMA-1 ectodomain and exhibit cytoadhesive properties in vitro (Kappe, Noe et al. 1998). MAEBL is highly expressed in midgut sporozoites and on the surface of salivary glands sporozoites (Le Roch, Zhou et al. 2003;
Preiser, Renia et al. 2004; Singh, Preiser et al. 2004; Srinivasan, Abraham et al. 2004). It is strongly induced during late stages of oocyst maturation and indirect immunofluorescence revealed that the localization of MAEBL in the cell changes as the sporozoite matures. In *P. berghei* immature sporozoites, the protein is restricted to the apical end, but in mature midgut sporozoites and in salivary gland sporozoites, MAEBL is uniformly distributed on the surface (Srinivasan, Abraham et al. 2004). In addition, the molecular form of the protein in the midgut is different that in the salivary glands. Indeed, the full length 240 KDa MAEBL is expressed on the surface of salivary gland sporozoites suggesting an importance of MAEBL in the liver stages of the parasite. This hypothesis was reinforced by the fact that antisera developed against the cysteine-rich regions of the extracellular portion of MAEBL inhibited sporozoite development to exoerythrocytic forms in vitro (Preiser, Renia et al. 2004).

A targeted disruption of *P. berghei* MAEBL showed that it is essential for attachment and invasion of salivary glands (Kariu, Yuda et al. 2002). No sporozoites were found in salivary glands infected with MAEBL knockout parasites, while the number of sporozoites in the midgut and hemolymph were comparable to the wild type (Kariu, Yuda et al. 2002).

### 1.3.6.4 Alternative splicing of *maebl*

MAEBL has alternative splicing of its carboxi terminal end and two dominant isoforms are produced in all the *Plasmodium* stages (Singh, Preiser et al. 2004) (Fig. 1-10. These two isoforms have been named ORF1 and ORF2. ORF 1 is a transmembrane isoform, product of the canonical transcript and ORF2 is a potential soluble isoform, in
which exon 2 binds exon 3, 16 nucleotides before the canonical site. Inclusion of this extra sequence creates a shift in the ORF causing an early stop in exon 4 and switching deduced transmembrane amino acid residues from hydrophobic to hydrophilic. In addition, a third transcript named ORF3, in which exon 2 binds exon 5 has been found only in *P. yoelii*. The expression of ORF 1 and ORF2 transcripts is differentially regulated in midgut and salivary gland sporozoites. The canonical transcript, ORF1 starts to be expressed on day 6 post feeding in *P. yoelii* and reaches a peak of expression in mature midgut sporozoites. On the other hand, ORF2 has some expression on day 3 post feeding and is expressed again on day 6 post feeding reaching a peak of expression in mature midgut sporozoites. These results show surprisingly complex post transcriptional mRNA processing in Apicomplexa (Singh, Preiser et al. 2004).

Figure 1-10. Alternative splicing of MAEBL (modified from Singh et al., 2004). Two main transcripts occur in all different stages of *Plasmodium*. ORF1 that is the canonical form and ORF2, in which exon 2 binds exon 3, sixteen nucleotides before the canonical site. ORF2 codes for a potential soluble protein.
Because of this complexity in the expression of MAEBL isoforms, the functional significance of the transmembrane (ORF1) and soluble forms (ORF2) of MAEBL for the invasion of salivary glands is not known.

1.4 Aims of the research

The main goal of my research was to understand the function of MAEBL in the life cycle of *P. falciparum*. In particular, I wanted to understand the function of the two major isoforms of MAEBL in the sporozoite invasion of salivary glands.

The aims of my research were:

1. Understand the role of MAEBL in the erythrocytic stages of *Plasmodium falciparum*.

2. Determine the role of MAEBL isoforms in sporozoite invasion of mosquito salivary glands.

In order to accomplish these aims, the main technique used was the genetic modification of *P. falciparum* parasites and the phenotypic characterization of the parasites in the invasion of host cells in the vertebrate and the mosquito hosts.

I hypothesized that the transmembrane domain of MAEBL is essential in the process of invasion of salivary glands because it is the main form expressed in the mature sporozoites and the expression of MAEBL appears to be dependent on the transmembrane form (Singh, Preiser et al. 2004). By studying the role of the different forms of MAEBL, a highly conserved protein between the different *Plasmodium* species (Michon, Stevens et al. 2002) and an essential ligand for the transmission of the parasite life cycle (Kariu, Yuda et al. 2002), it will be possible to understand the biology of the
Plasmodium sporozoite and, as a result, generate information useful for the development of tools such as a transmission blocking vaccine to stop the parasite passage into the vector.
2 MATERIALS AND METHODS

2.1 Parasite culture and maintenance

Clones of *Plasmodium falciparum* NF54, 3D7 and W2mef were obtained from the Biomedical Research Institute, Rockville, Naval Medical Research Center, Walter Eliza Hall Institute (WEHI), respectively and maintained in culture according to standard methods at 37°C and gassing (5% O2, 5% CO2, Nitrogen balanced) with 5% hematocrit in RPMI 1640 (Invitrogen) supplemented with 0.5% Albumax I (Invitrogen) or 10% human AB sera (Interstate Blood Bank), 0.25% sodium bicarbonate and 0.01mg/ml gentamicin. Human red blood cells were obtained from Indiana Blood Bank or local donors and washed three times with RPMI 1640 (Invitrogen), resuspended to 50% hematocrit and stored at 4°C. Only fresh blood, less than 2 weeks old, was used to culture the parasites in vitro.

2.2 Synchronization of blood stage parasites

*Plasmodium falciparum* blood stage parasites were synchronized at either the ring stages or the late-schizont stages using the following methods.
2.2.1 Sorbitol synchronization of ring stage parasites

Parasite cultures were centrifuged at 1200 g for 5 min at room temperature. The supernatant was removed and equal volume of 5% sorbitol (Sigma) in RPMI 1640 was added to the pellet and mixed. The mixture was then incubated at room temperature for 10 min with intermittent mixing. The above mixture was centrifuged at 2000g for 5 min at room temperature and the supernatant was removed. The parasite pellet was washed twice with complete media and resuspended in complete media to the initial culture volume and returned to culture. The procedure was repeated after 42 hrs to obtain highly synchronized ring stage parasites.

2.2.2 Purification of mature schizonts by use of a magnetic column

Late-stage parasites were purified from culture by passage through a MACS magnetic column (Miltenyi Biotech). In brief, 20 ml of parasite culture at 5-10% late stage parasitemia was passed through the MACS column with a magnet attached. The column was then washed with 30 ml of incomplete RPMI 1640 to remove uninfected RBCs and early stage parasites. The column was then removed from the magnet and the late-stage parasites still bound to the column were eluted in 20 ml of incomplete RPMI 1640 and pelleted by centrifugation at 2500 rpm for 5 minutes without brakes. The pellet of purified parasites was washed once with complete media, resuspended in complete media and reintroduced into culture with 5 ml of complete media at 5% hematocrit.
2.3 Genomic DNA and RNA extraction

*Plasmodium falciparum* genomic DNA was purified from blood-stage parasites using a standard phenol/chloroform method. Briefly, parasite cultures were lysed in 0.15% saponin in TSE (50 mM tris, pH 8.0, 50 mM EDTA and 100 mM NaCl), incubated (30 minutes at 37°C), and washed twice in TSE. The DNA containing pellet was resuspended in TSE containing 2% SDS and proteinase K (100 ug/ml), and incubated (37°C) overnight. Extraction was performed twice in phenol/chloroform (1:1) and once in chloroform. DNA was precipitated from the aqueous phase with 3 M sodium acetate (1/10 volumes) and ice cold 100% ethanol (2 volumes), allowed to incubate 5 minutes at -20°C, centrifuged (12,000 x g without brake), and washed twice with 70% ethanol. The pellet was partially air-dried and resuspended in purified water or TE (10mM Tris-HCl pH 8.0, 1 mM EDTA).

Total RNA was isolated using TRI REAGENT (Molecular Research Center, Inc) RNA isolation protocol. Parasite-infected erythrocytes were lysed in TRI REAGENT (approximately 1 ml per 5 x10^6 cells) and the homogenate was incubated for 5 minutes at room temperature before the addition of chloroform (0.2 ml/ 1 ml TRI REAGENT added). The mixture was vigorously mixed, allowed to incubate (10 minutes at room temperature) and centrifuged (12,000 x g for 15 minutes at 4°C). The aqueous phase containing the RNA was isolated and precipitated with isopropanol (0.5 ml/ 1 ml TRI REAGENT added), incubated (5 minutes at room temperature), and centrifuged (12,000 x g for 8 minutes at 4°C) to pellet RNA precipitate. The pellet was washed with 75% ethanol (1 ml/ 1 ml TRI REAGENT added) by vortexing and centrifuged (12,000 x g for 8 minutes at 4°C). The ethanol wash was then removed and the RNA pellet was air-dried.
for 5 minutes at RT. RNA was resuspended in DEPC-treated water. The concentration and purity of DNA and RNA was determined by measuring the absorbance at 260/280 nm wavelength with a GeneQuant RNA/DNA calculator (Pharmacia) or with ND-1000 spectrophotometer (Nanodrop Technologies).

2.4 Construction of transfection plasmids

For MAEBL-KO clones, a plasmid construct for single homologous recombination was created by using the plasmid pHD22Y. A 750 bp region of maebl was amplified from the *P. falciparum* NF54 genomic DNA by using primers JA-1410 (5’ AGATCTCCAACATGTACTGAAAAAGG 3’) and JA-1411 (5’ CTCGAGCCTAGAAGATTGTACAATAATAGC 3’). The conditions for PCR were 94°C for 1 min followed by 35 cycles of 15 s at 94°C; 30 s at 49°C; 1 min at 65°C. The PCR product was cloned into pGEM-T easy vector (Promega) and excised as an EcoR I fragment and cloned into the vector pHD22Y. For MAEBLΔORF1 and MAEBLΔORF2 the plasmids constructs for single homologous recombination were created by using the plasmid pHH1 (Reed et al., 2000). A 1100 bp Region of the 3’ end of MAEBL was amplified from the *P. falciparum* NF54 cDNA using the primers JA-1441 (5’ AGATCTGAAGATGAAAAAAGAATGGAAGTA 3’) and JA-1442 (5’ CTCGAGCAAAAAAAAAATAACCCCACAAAAAGTAC 3’). The conditions for RT-PCR were 45°C for 30 min 94°C for 2 min followed by 35 cycles of 15 s at 94°C; 30 s at 48°C; 1 min at 65°C. The PCR products were cloned into a pGEM-T easy vector (Promega), sequenced to identify ORF1 and ORF2 DNA and excised as a Bgl II XhoI
fragment. The fragments were cloned into the vector pHH1 to make the pHH1-ORF1 and pHH1-ORF2 plasmids.

The plasmid screening for inserts was done by lysing the transformant bacteria in 10mM Tris HCl pH8, 1mM EDTA, 15% sucrose, 2mg/ml lysozyme, 0.2 mg/ml RNase, 0.2 mg/ml BSA and boiling for 1 minute. A restriction digest screening was used in every case to confirm the insertion. The plasmid preps were performed using a Wizard miniprep kit (Promega) and ligase 4 (New England Biolabs) was used to ligate the purified pieces (GeneClean II, MP Biomedicals). The bacterial transformations were done by temperature shock electroporation (45 seconds at 42 °C in XL10 gold Chemical Competent Cells (Stratagene) or by electric shock electroporation (1.2 volts) using XL10 gold Electro Competent Cells (Stratagene).

2.5 Stable transfection of *P. falciparum* and selection of transformed parasites

Transfection of *P. falciparum* NF54 was achieved by parasite invasion of plasmid DNA-loaded RBCs. For plasmid DNA loading, 250 µl of processed RBCs were washed once with 250 µl of incomplete cytomix and combined with the desired amount of plasmid DNA and incomplete cytomix to a final volume of 400 µl. The mixture was then transferred to a 0.2 cm cuvette (Bio-Rad), chilled on ice and electroporated using a Bio-Rad Gene Pulser II and standard conditions of 0.31 KV and 950 µF of capacitance. The electroporated blood was then washed two times with incomplete RPMI 1640 and resuspended in incomplete RPMI 1640 to 50 % hematocrit. To ensure parasite invasion of only DNA-loaded RBCs, mature blood stage parasites were purified by using a magnetic column (Miltenyi Biotech). The purified parasites were then reintroduced into
culture with 5 ml of complete media at 5% hematocrit of loaded RBCs to obtain a parasitemia of 0.1%.

After 48 hrs post-transfection, 2.5 nM of WR99210 was added to the culture and the parasites were maintained in drug until the appearance of parasites in Giemsa-stained smears. The concentration of WR99210 was increased to 5 nM at this point. Parasites were cultured with and without drug three to six times for two weeks each to get rid of episomal DNA.

2.6 Limiting dilution of parasite clones and parasite lactate dehydrogenase (pLDH) assay

Drug-resistant parasites were cloned by limiting dilution at 0.5 and 0.25 parasite per well, in a 96 well plate. Parasites were counted by Giemsa-stained smears and the parasites were diluted in RPMI such that there were 10 parasites/µl. For each plate, 500 parasites were mixed with 19.2 ml RPMI media and 0.8 ml 50% hematocrit. 200 µl of this mixture was added to each well in a 96-well plate to obtain a final dilution of 0.5 parasite/well at 2% hematocrit. Similarly, 250 parasites were used as above to obtain a dilution of 0.25 parasite/well. The culture media was changed and 0.4% hematocrit added on day 7 and day 14. On day 17, the presence of parasites was detected by direct microscopical observation or by pLDH assay. Briefly, 20 µl of parasite cultures were lysed by freeze-thawing three times and 100 µl of MALSTAT reagent (Flow inc.) was added. Ten µl of 1 mg/ml NBT (Sigma) and 10 µl of 2 mg/ml diaphorase (Sigma) were then added to the mixture. The mixture was incubated at room temperature for 20 minutes and wells positive for parasites were identified by colorimetric analysis.
2.7 Characterization of *P. falciparum maebl* transformant clones in blood stages

2.7.1 Southern blot hybridization

Two µg of genomic DNA extracted from transformed parasites was digested with 10 units of EcoR V, EcoRI or Nsi I overnight and separated on a 0.8% agarose gel. The DNA was depurinated in 0.25 M HCl, denatured in 0.5 M NaOH/1.5 M NaCl, neutralized in 1M Ammonium acetate/0.02N NaOH and blotted overnight to a nylon membrane. The blot was then hybridized in 5X SSC (1X SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), 5X Denhard’s reagent, 1 % SDS to a *maebl* M2 region probe that was amplified from *P. falciparum* 3D7 genomic DNA using primers JA-302 and JA-310. Alternatively, the probe used was a human *dhfr*. The probe was labeled with 32P using the RadPrime labeling system (Invitrogen). The blot was washed 3 times in 0.2X SSC/0.1% SDS for 15 min and exposed to a Kodak photographic film at -80°C to visualize the hybridized fragments.

2.7.2 Indirect immunoflorescence assays (IFA)

Mature schizont-stage parasites were purified from cultures of W2mef, 3D7, NF54 and *maebl* disruptant clones w2mef B7, D8, and NF54 F7, G3 and G7, by passage through a MACS magnetic column (Miltenyi Biotech). The schizont-enriched *P. falciparum* infected erythrocytes were smeared on glass slides and stored at -80°C until use. Thawed slides were fixed in 1% formaldehyde, preincubated with 3% BSA 1%Triton in PBS at room temperature for 1 hour, and then reacted with primary antibody (MAB 2C11) diluted 1:100. Secondary antibody was FITC-conjugated goat anti-mouse
IgG antibody (Alexa fluor®488 goat anti-mouse IgG; Molecular probes) at 1:70 dilution. The slides were mounted in Fluoromount-G (Southern Biotechnology Associates, Inc., USA) and viewed by fluorescent microscopy (Nikon ECLIPSE E400, Japan). Digital images (Spot insight QE, USA) were obtained and were prepared for presentation using PhotoShop CS2 for PC or PhotoShop 7.0 for Mac OSX (Adobe).

2.7.3 Enzymatic treatment of erythrocytes

Washed erythrocytes were treated with either 1 mg/ml trypsin (TPCK treated) (Sigma), 1 mg/ml chymotrypsin type-VII (Sigma), or 0.5 Unit/ml neuraminidase type II (Sigma) for 1 hr at 37°C with gentle shaking, followed by treatment with the appropriate protease inhibitor for 10 min. Erythrocytes were then washed with RPMI supplemented with 0.2% NaHCO3 and stored at 4°C until needed.

2.7.4 Determination of parasite invasion phenotype

*P. falciparum* W2mef and NF54 wild-type and *maebl* disruptant clones were synchronized by sorbitol treatment. A culture mainly composed by schizonts was allowed to invade normal and enzymatically treated erythrocytes. The cultures were initiated in a 96-well plate at 1% parasitemia with 1% hematocrit in low hypoxanthine media. After 20 hrs of growth, 0.5 μCi 3H was added to the cultures. Cultures were incubated overnight (20 hr), and the culture plate was frozen at –80°C for 4hr. The cells were harvested on filter (filtermat), and were counted by Microbate 1450. Each treatment was done in triplicates and the % invasion in each treatment was calculated by using the formula:
Average cpm in Treated RBCs \( \times 100 \)

Average cpm in Normal RBCs

To further confirm the results, the parasitemia was determined microscopically after 60 hours of incubation with the treated erythrocytes.

An analysis of variance (ANOVA) was used to determine the significance of the difference in percentage of invasion between the wild type parasites 3D7 and w2mef and the mutant parasites.

2.8 \textit{Plasmodium falciparum} passage through mosquitoes

In order to investigate the importance of MAEBL in the mosquito stages of \textit{Plasmodium falciparum}, we produced gametocytes and fed \textit{Anopheles} female mosquitoes to verify the presence or absence of oocysts in midguts and sporozoites in salivary glands (Fig. 2-1).

2.8.1 Production of \textit{Plasmodium falciparum} gametocytes

Mature gametocytes were produced in NF54 isolate and NF54 transformants were cultivated in vitro as previously described (Ifediba and Vanderberg 1981). Briefly, 10 ml cultures were started at 0.1\% parasitemia in 6\% blood. After 4 days the volume of media was doubled and the media was changed every day until the majority of gametocytes were mature. The gametocytes were checked for exflagellation in an optical microscope in 60\% AB human serum.
Figure 2-1. Transmission of *Plasmodium falciparum* to *Anopheles*

The parasites were transmitted to mosquitoes by membrane feeding and the infection in midgut and salivary glands was determined by microscopical observation. The total time of each experiment from the start of the gametocyte production to the characterization of the salivary gland invasion takes 32-36 days.
2.8.2 *Anopheles* membrane infections with *P. falciparum* gametocytes

After confirming exflagellation, the gametocyte cultures were fed to *Anopheles freeborni* and *An. stephensi* mosquitoes. For that purpose, a feeding apparatus was assembled (water bath at 37.5°C, mosquito cages, feeders and hog casing membranes) and human sera was warmed at 37 °C for at least 10 min.

The gametocyte cultures were spun down for 2 minutes at 2500 rpm and the pellet mixed with 100% of red blood cells maintained at 37 °C (to have a final gametocytemia of 0.5%) and mixed with human serum at 37 °C to make it 60% of the final volume. The mix was carefully placed in the warmed feeders and the mosquitoes were allowed to feed for 10-20 minutes. 50-200 female *Anopheles* mosquitoes were fed from each parasite culture.

2.9 Characterization of transformant parasites in mosquito stages

2.9.1 Mosquito dissections

The midguts of female mosquitoes were dissected on days 10-14 and the salivary glands were dissected on days 15-20. In order to dissect the infected mosquitoes, the infected cages (having 25 to 30 females each) were placed in CO2 containers for 5-10 minutes and then the cage was placed on ice. The mosquitoes were removed one by one and dissected in PBS. The midguts were removed by inserting a dissection needle in the thorax and pulling the second to last segment of the abdomen (using another needle).
The midguts were cleaned from all the hepatic secca and other tissues and briefly washed in PBS prior to staining or processing.

The salivary glands of each female mosquito were removed by separating the head and thorax of each mosquito in order to expose them. Then, each salivary gland was pulled out and cleaned from remnants of any other tissue previous to staining or processing.

2.9.2 Collection of midgut and salivary gland sporozoites

Midgut and salivary gland sporozoites were collected by pulling together 10-20 midguts or 30-40 salivary glands and smashing the collected tissue in PBS. The resulting mix was centrifuged (40g for 5min at 4°C). The supernatant containing the midgut and salivary gland sporozoites were then concentrated by centrifugation or directly observed under the microscope.

2.9.3 Collection of hemolymph

The hemolymph of the mosquitoes was collected by first extracting salivary glands from at least 20 mosquito thoraxes and then smashing the thoraxes prior to centrifuging in a 0.5 ml tube with glass wool adapted to a 1.5 ml tube at 2000 rpm for 5 min. Alternatively, hemolymph was collected from a pinhole in the abdomen by the injection of 10 µl Medium 199 (Sultan, Thathy et al. 1997).

2.9.4 Staining of parasites in the Anopheles vector

In order to observe the presence of oocysts in midguts and sporozoites in salivary glands of Anopheles mosquitoes, the DNA stain dihydroethidium was used. The midguts
and salivary glands were stained for 10 minutes in 0.5 mg/ml of dihydroethidium (invitrogen). Alternatively, the presence of oocysts was confirmed by staining the midguts with mercurochrome.

2.9.5 Infected mosquitoes and oocyst quantification

The number of infected midguts per infection (out of 8 counted midguts), the total number of oocysts in each midgut, the number of sporozoites in the hemolymph and in the salivary glands were counted to assess the effect of the knockouts and allelic replacements in these stages. A t-student test was used to determine if there was statistical difference between the transformant parasites and the parental clone NF54.

2.9.6 RNA extraction of Anopheles tissues

The midguts and salivary glands collected in PBS, perhaps stained in dihydroethidium were washed in sterile PBS and 8 midguts or 20 salivary glands were homogenized in 50 µl of Trizol Reagent (Invitrogen) and frozen (-80 °C). The RNA was extracted using 10 µl of chloroform, spinning for 15 min at 15000 g, followed by a precipitation of aqueous phase using 25 µl of isopropanol and a final 70% ethanol wash. RNA was resuspended in DEPC-treated water. The concentration and purity of DNA and RNA was determined by measuring the absorbance at 260/280 nm wavelength with ND1000 spectrophotometer (Nanodrop Technologies).

2.9.7 RT-PCR analyses of midgut and salivary gland sporozoites

The total RNA from Anopheles midguts and salivary glands was used for RT-PCR. The RNA from midguts and salivary glands of the parental NF54 and the
transformant clones was treated with DNase I (Invitrogen) and the reaction stopped by heating at 65°C for 10 minutes. The RT-PCR reaction was done using Superscript III (Invitrogen). The set of primers used were: \textit{maebl} m2sd1 :

5’AACCCGCAACAAAAATATATG3’ and

5’TAAAGGGTATCTAGGTGGACATTT3’

Region upstream of \textit{maebl}:

5’GAACAAGAAGAATATTTTTTAGTACTGC3’ and

5’GGCATGCATGGAGAACTA3’

Carboxi-terminal end of \textit{maebl}:

5’GTGATTATATGAAGGATAATATTTCATCACG3’ and

5’CCGAATAACTTAAAGGATTGCTTAC3’

\textit{csp}:

5’-GCTAATGCCAACAGTGCTGTA-3’ and

5’-GGAACAAGAAGGATAATACC-3’

\textit{Anopheles ITS2}:

5’-TGTGAACTGCAGGACACAT-3’ and

5’-TATGCTTAAATTGAGGGGT-3’

The conditions for the One-step PCR were: One cycle of 45°C for 30 minutes and 94°C for 2 minutes, followed by 35 cycles of 94°C for 15 minutes, 45°C for 30 minutes and 65°C for 30 sec-1 minute.
Alternatively Superscript II (Invitrogen) and First Strand cDNA Synthesis (Fermentas) were used for the RT-PCR reaction followed by a PCR using the previous conditions.

2.9.8 Immunolocalization of MAEBL and CSP in sporozoites

Sporozoites isolated from midguts and salivary glands were purified by centrifugation and spotted in glass slides, dried and stored at -80°C until use. Thawed slides were fixed in PBS, 1% formaldehyde for 5 min. The slides were then preincubated 3 times for 10 minutes with blocking buffer (PBS, 1 % Triton X-100, 0.3 ng/ml goat serum) and then reacted with primary antibody (M2SD2 sera or MAB 2C11 diluted 1:100 or CSP sera diluted 1:500) for 1 hour at 37°C in a humidified container. Secondary antibody was FITC-conjugated goat anti-rat (for M2SD2 sera), FITC-conjugated goat anti-mouse (for MAB 2C11), or Rhodamine-conjugated goat anti-rabbit (for CSP sera) at 1:60 dilution. The slides were mounted in Fluoromount-G (Southern Biotechnology Associates, Inc., USA) or in anti-fade Prolong Gold mounting media (Invitrogen) and allowed to dry overnight before sealing. The slides were observed by fluorescent microscopy (Zeiss Axioskop, Olympus IX71 Delta Vision System (Applied Precision)).

2.9.9 Image capturing and analysis

The images were captured using the Axiovision software: AxioVs40V4.6.3.0 (Carl Zeiss Imaging Solutions) in addition to the Worksuites software for Delta Vision system (Applied Precision). The images were edited and processed using 2D lateral and
3D z-stack deconvolution. Digital images (Spot insight QE, USA) were prepared for presentation using PhotoShop CS2 for Windows or PhotoShop 7.0 for Mac OSX (Adobe) and images were organized and labeled in Canvas X (ACD systems inc).
3 RESULTS

3.1 MAEBL in the erythrocytic stages of *Plasmodium falciparum*

3.1.1 Disruption of *Plasmodium falciparum maebl*

*maebl* disrupted parasite lines were created to examine the possible importance of MAEBL in erythrocytic invasion pathways. For this purpose, homologous integration into the *maebl* locus was carried out by a strategy similar to disruption of the homologous *eba-175* (Reed et al., 2000), using the plasmid pH1/Δ*maebl* containing the selection marker hDHFR and targeting sequence that disrupted *maebl* coding sequence (CDS) after the second ligand domain (M2).

A cloned parasite line of the *P. falciparum* W2mef isolate was transfected with this construct and two independent clones, B7 and D8, were obtained through intermittent selection with WR99210. These parasites were generated in the laboratory of Alan Cowman at the Walter and Eliza Hall Institute of Medical Research and provided to the Adams lab for phenotype analysis.
3.1.2 Characterization of the MAEBL-KO parasites

In order to confirm that pHH1/hDHFRΔmaebl had integrated through single crossover homologous recombination into the maebl locus, genomic DNA from parental wild type W2mef and W2mef Δmaebl clones were analyzed by Southern blot hybridization probed with hDHFR and maebl M2 CDS. Integration of multiple copies of the whole plasmid into the maebl locus was observed in B7 and D8 clones (Fig. 3-1).

RT-PCR analysis in parental and Δmaebl clones indicated that parts of maebl continued to be actively transcribed (Fig. 3-2). Primer pairs were designed to amplify mRNA segments before and after the disruption and similarly spliced transcripts were detected in both wild type (3D7, W2mef) and Δmaebl clones (B7, D8) of P. falciparum. Standard PCR was performed as a reference for the size of the unspliced primary transcript or genomic DNA. Similar RT-PCR analysis of eba-175 gene was included as a control. The region amplified at the 3’ end of the maebl locus spanned where introns 2-4 occur in the primary transcript in order to determine if normal splicing occurred independent of the promoter used to express the mRNA. We detected both the canonical ORF1 mRNA and alternative ORF2 mRNA of splicing patterns of the maebl 3’ exons (Fig 3-2), which were verified by sequence analysis.

3.1.3 Disruption results in loss of MAEBL expression in erythrocytic stages of P. falciparum

Monoclonal antibodies (MAB) against recombinant M2 domain subdomain 1 (rM2sd1) were produced by Jun Fu in our laboratory. These antibodies were used to confirm MAEBL expression in P. falciparum erythrocytic stages and to confirm whether
Figure 3-1 schematic representation of the transfection plasmid used to disrupt the *P. falciparum* *maebl* locus and analysis of the transformed parasites.

(A) The region of *maebl* encoding the M2 domains was cloned into the plasmid pHH1, which contains the hDHFR selection cassette. This resultant plasmid pHH1/hDHFR/Δ*maebl* was used to disrupt *maebl* in *P. falciparum* W2mef to create independent *maebl* knockout clones (W2mefΔ*maebl*), which were designated B7 and D8. The bracket indicates the approximate boundaries between which two copies of pHH1/Δ*maebl* have integrated. *EcoRI*(E) and *HaeIII* (Ha) restriction sites used in mapping the plasmid integration events are shown. The probe of *maebl* M2 region and *hdhfr* used for Southern blot analysis in B is indicated by a solid bar. (B) Integration of the whole plasmid into the *maebl* locus was confirmed by Southern blot hybridizations using probes to *hdhfr* and the *maebl* M2 region. Southern blot analysis of *EcoR*
1, *HaeIII* and *HinfI* restricted genomic DNA isolated from 3D7 (reference clone), W2mef and knockout clones B7 and D8 indicating complete disruption of the endogenous *maebl* locus.
Figure 3-2. RT-PCR analysis of *maebl* mRNA expressed in intact *P. falciparum* clones (3D7, W2mef) and W2mefΔ*maebl* clones (B7, D8).

Approximate locations of each primer pair are shown as arrows on the schematic diagrams on the right-side of the figure. RT-PCR and PCR products separated by agarose gel electrophoresis are: A. 5’ end of *maebl* before the disruption; B. 3’ end of *maebl* after the disruption and bracketing introns 2-4; C. 3’ end of *eba-175* included as a control. Standard PCR of the genomic DNA (gDNA) were performed as references for the sizes of the unspliced primary sequences. Similar RT-PCR analysis of *eba-175* gene was included as a control.
MAEBL was functionally knocked out in the Δmaebl clones. Three MABs (1H2, 2C11, 5D2), that were developed with rM2sd1, were screened for reactivity against metabolically-labeled parasite-expressed proteins of P. falciparum 3D7 extracted with non ionic or ionic detergent. MAB 2C11 immunoprecipitated a MAEBL protein migrating at approximately 140-155 kDa from the SDC-extracted parasite fraction, but not the TX-100-extracted parasite fraction. PfMAEBL was not expressed in erythrocyte stages of Δmaebl parasites lines B7 and D8 (Fu, Saenz et al. 2005).

Indirect immunofluorescence assays using MAB 2C11 against MAEBL in wild type parasites showed punctate apical fluorescence in free merozoites and in late stage schizonts of 3D7 and W2mef, but not B7 and D8 clones (Fig. 3-3). No reactivity was observed in either ring stages or trophozoite-infected erythrocytes.

3.1.4 Erythrocyte invasion phenotypes of W2mef Δmaebl clones

The invasion phenotype of the W2mef Δmaebl clones was determined by an in vitro assay that measured their short-term growth rates in intact and enzyme-treated human erythrocytes. Neuraminidase (N), trypsin (T), and chymotrypsin (C) were used to treat A+ human erythrocytes. W2mef, the parent clone of the Δmaebl clones, and clone 3D7 of P. falciparum were included for reference. Invasion rates were inferred from the growth rates, which were determined relative to growth rates in intact untreated erythrocytes and all treatment groups were done in triplicate. Assay data are the averaged result from at least three experiments and standard error bars are shown (Fig. 3-4). Although growth rates of B7 and D8 in neuraminidase-treated erythrocytes (14.8% and 10.0%, respectively) were consistently higher than W2mef (5.1%), these differences
were not significant. Surprisingly, the growth of Δmaebl clones in trypsin-treated erythrocytes was much higher (131%, 126%, respectively) compared to W2mef. These differences in B7 and D8 growth rates for trypsin-treated erythrocytes were significant compared to W2mef and 3D7 (p<0.05 ANOVA). Similar results were found when using the 3H-hypoxanthine incorporation method (Su, Kirkman et al. 1997) to measure the growth of parasites (data not shown). There was no significant difference in any of these four clones when grown in chymotrypsin-treated erythrocytes (Fig. 3-4).

Figure 3-3. Immunolocalization analysis with anti-MAEBL MAB 2C11 to wild type (3D7, W2mef) and Δmaebl clones (B7, D8).

The top row shows the immunofluorescence images and the bottom row the corresponding brightfield images. MAB 2C11 reacted with late stage schizonts in wide-type 3D7 and W2mef, demonstrating a punctate apical localization pattern. The punctate fluorescent pattern is characteristic of antigen localized within the apical complex of merozoites. Consistent with the immunoprecipitation results the MAB 2C11 did not react with the Δmaebl clones (B7, D8). Multiple formed merozoites and centrally located hemozoin are apparent in the brightfield images of the mature segmented schizonts (Fu, Saenz et al. 2005).
Figure 3-4. Comparison of parasite growth rates in untreated and enzyme-treated erythrocytes between wild type and Δmaebi clones.

The invasion phenotype was determined by an in vitro assay that measured their short-term growth rates in intact and neuraminidase, trypsin, and chymotrypsin treated A+ human erythrocytes. The percentage of invasion represents the average of three independent assays, each performed in triplicate. Asterisk (*) indicates the treatment groups, which include Δmaebi clones B7 and D8, had a significant difference (p<0.05 ANOVA) in invasion into trypsin-treated erythrocytes compared to W2mef and 3D7.

3.2 Staining techniques for the characterization of Plasmodium stages in Anopheles

The detection of Plasmodium oocysts and sporozoites in midguts and sporozoites in hemolymph and salivary glands is usually done by direct microscopic observation without any stain or using commercially available stains. The identification of Plasmodium in mosquito tissues is often a challenge because the parasites are usually not evident in non stained tissues. The most commonly used stain to evidence oocysts in mosquito midguts is mercurochrome (0.1-2%) a topical antiseptic used for minor cuts and scrapes (Gouagna, Bonnet et al. 1999). Mercurochrome is based on merbromin that is an organomercuric disodium salt compound and a fluorochrome. The oocysts stained with mercurochrome appear as bright orange or pink. The main handicap of using mercurochrome is that it is not possible to obtain DNA or RNA from the tissues after
staining, which is a challenge given the shortage of infected material in *P. falciparum* infected mosquitoes. In order to facilitate the detection of *Plasmodium* in the midguts and salivary glands of *Anopheles*, we tested a different staining technique in addition to the widely used mercurochrome. In particular, the life staining dihydroethidium that binds to DNA, was used to stain live *Anopheles* tissues. Dihydroethidium was used at a concentration of 0.5 mg/ml and we show that it is particularly useful in recognizing oocysts in midguts and sporozoites in hemolymph and salivary glands. The high concentration of nuclei in oocysts of the newly forming sporozoites, appears as brighter circles. Once the sporozoites were released to the hemolymph, these were easily identified in purified suspensions or in solutions contaminated with other mosquito tissues. Finally, the sporozoites could be identified inside the salivary glands with dihydroethidium for their small nuclei as compared to the salivary cell nuclei. The results for the different staining methods and tissues are shown in Fig. 3-5.

3.3 *maebl* knockout and allelic replacements

3.3.1 Plasmids constructs

The plasmid pH22YΔmaebl was obtained by introducing a 720 bp region of *maebl* into the EcoRI site of the plasmid (done by Bharath Balu).

The plasmids pH1ΔMAEBL ORF1 and pHH1ΔMAEBL ORF2 were obtained by introducing a 1000 bp region of the 3’ end of *maebl* ORF1 and ORF2 transcripts
Figure 3-5. Dihydroethidium highlights *Plasmodium* in different mosquito stages.

The figure shows bright field (BF) and dihydroethidium (DH) stained *Anopheles* midguts (MG), oocysts (OO), non-purified hemolymph (HE), and salivary glands (SG). The nuclei of the sporozoites appear smaller than the nuclei of the surrounding cells. The arrows point to the position of the parasite.
(obtained by RT-PCR) in the Xho I and Bgl II sites of the pHH1 plasmid (Reed, Saliba et al. 2000) (Fig. 3-6, Fig. 3-7).

Figure 3-6. Schematic representation of the transfection plasmids for the allelic replacement of *maebl*.

The cDNA for the the two isoforms of MAEBL, ORF1 and ORF2 were cloned in the pHH1 plasmid. ORF2 has 16 extra nucleotides after exon 2.

3.3.2 Targeted integration for allelic replacement of MAEBL

Targeted integrations at the *maebl* locus were achieved in *P. falciparum* NF54 by single homologous recombination events. Two different allelic replacements of the 3’ end of *maebl* were created in NF54 parasites to express either the ORF1 or ORF2 isoform. Multiple independent clones were obtained as ORF1-only (ORF1) or ORF2-only (ORF2) MAEBL expression mutants. Three independent *maebl* knockout clones
Figure 3-7. Allelic replacement of the carboxyl terminal end of *maebl*.

A. Schematic representation of the disruption of the 5’ end of *P. falciparum maebl* using the vector pHHD22YΔMAEBL that has a human *dhfr* selectable marker (made by Bharath Balu). B. Schematic representation of the allelic replacement of the 3’ end of *P. falciparum maebl* using the vector pHH1ΔMAEBL that has a human *dhfr* selectable marker. The 3’ end of *maebl* with the cDNA sequence corresponding to ORF1 or ORF2 was cloned into the pHH1 vector. The alignment shows the nucleotide and amino acid sequences of ORF1 and ORF2. ORF2 has 16 extra nucleotides that cause a shift in the ORF switching from hydrophobic to hydrophilic amino acids and an early stop in exon 4. C. Screening for transformed clones by Southern blot hybridization analyses of genomic DNA. Transformed drug resistant parasites identified plasmid integration into the *maebl* locus in drug resistant populations. ORF1 mutant clones C4, C9 and E9 show the allelic replacement at the 3’ end. ORF2 clones A10, B3, C8, F11, G10, H3, H11 show the allelic replacement at the 3’ end. C. MAEBLKO mutant clones F7, G3 and G7 show plasmid integration between the M1 and M2 domains.
were also created as negative controls and NF54 parental parasites were used as positive controls for invasion of the mosquito salivary glands. The expected integration into the maebl locus was confirmed for each mutant (Fig. 3-7). There were no differences in blood-stage growth or the erythrocyte invasion phenotype for any of the genetically mutated parasites. Growth assays in neuraminidase, trypsin and chemotrypsin treated red blood cells using MAEBL mutant parasites did not show any significant difference with the parental clone NF54 (transformed parasites were generated by Dr. Bharath Balu). In addition, all the parasites were able to produce gametocytes that matured and formed male and female gametes similar to the parental clone NF54. Indeed, the microgametes of all the mutant parasites showed exflagellation in vitro comparable to NF54 (Fig. 3-8).

3.3.3 Mosquito infections: oocyst and midgut sporozoites

Anopheles freeborni and An. stephensi mosquitoes were fed mature P. falciparum NF54 gametocytes (days 15 and 16) using a biological membrane feeding system. Infections in the midgut, hemolymph and salivary glands at days 10, 18, and 20 post blood meal feeding, respectively, were identified by microscopic observation in Nomarski bright field microscopy and by fluorescence microscopy using dihydroethidium as a nuclear stain. In addition, transcript analysis for csp was used to confirm the presence or absence of sporozoite infections in each of the mosquito organs. An average of 50% of the midguts were infected and we obtained an average of 9 oocyst per mosquito midgut. In some cases 100% of midguts were infected and some midguts had up to 120 oocysts. Fig. 3-9 shows a heavily infected midgut.
Figure 3-8. Microgametes of *P. falciparum* NF54 and mutant parasites.

NF54, MAEBL KO, ORF1, and ORF2 male gametes are shown in the process of exflagellating. 1-8 flagella can be seen around the main body. All the clones expressing isoforms of MAEBL and the *maebl* mutants show micro and macrogametes presenting similar phenotypes to the parental clone NF54.
The presence of oocysts in the anopheline midguts was confirmed on day 10 post blood meal feeding. The MAEBL mutant parasites produced morphologically and developmentally normal, or wild type (WT), oocysts in the midguts of *Anopheles* (Fig. 3-10). The oocysts of the genetically modified parasites typically required 10-14 days to mature and sporozoites were observed budding from sporoblasts similar to the parental clone NF54 (Fig. 3-10). Quantitative analysis indicated there were no significant differences in the number of infected midguts among the mutant clones relative to WT NF54 parasites (P value NF54 vs MAEBL KO: P(0.2962): 0.30, NF54 vs ORF1: P(0.6018): 0.6, NF54 vs ORF2, P(0.0902) or in the number of oocysts per midgut (P value NF54 vs MAEBL KO: P (0.1428): 0.30, NF54 vs ORF1: P(0.1508): 0.6, NF54 vs ORF2, P(0.5622) (Table 1, Table 2). Microscopic observation of the hemolymph of infected mosquitoes confirmed the presence of sporozoites for all the mutant parasites, including the MAEBL knockout lines (MAEBL KO, ORF1, ORF2). Indeed, sporozoites
Figure 3-10. Oocysts (day10) of wild type and transformed parasites. NF54, MAEBL KO, ORF1, and ORF2 Anopheles midgut oocysts are shown. Sporozoites can be seen forming around sporoblasts in all the transformed parasites. All the clones expressing isoforms of MAEBL and the maebl mutants show oocysts presenting similar phenotypes to the parental clone NF54. In addition, all parasite lines required the same time (12-14 days) to develop and rupture.
<table>
<thead>
<tr>
<th>PARASITE</th>
<th>NF54</th>
<th>ORF1</th>
<th>ORF2</th>
<th>MAEBL KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUMBER</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OF MIDGUTS</td>
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</tr>
<tr>
<td>INFECTED</td>
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<td>(OUT OF 8)</td>
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<td>1</td>
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* SIGNIFICANCE
TABLE 2.

AVERAGE NUMBER OF OOCYSTS PER MIDGUT IN NF54 AND MUTANT PARASITES ON DAY 10 POST FEEDING

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<th>PARASITE</th>
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<td>2.6</td>
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* SIGNIFICANCE
released into the hemolymph from the oocysts of mutant parasites appeared similar in abundance, and morphology (Fig. 3-11).

Figure 3-11. Midgut sporozoites of NF54 wild-type parasites and mutants of MAEBL KO, ORF1 and ORF2 observed microscopically in the hemolymph. The pictures were taken in 1000X in Nomarski bright field (left) and by fluorescent nuclear stain dihydroethidium (right). All the transformed parasites produced sporozoites that were released to the hemolymph in the same time as the parental parasite NF54.

Normal expression patterns of MAEBL were confirmed in mutant parasites by RT-PCR (Fig. 3-12) and by IFA (Fig. 3-13, fig. 3-14). Immunofluorescence localization patterns in the ORF1 and ORF2 allele-specific parasites, using antibodies against the M2 ligand domain of MAEBL, were similar to NF54. For example, midgut sporozoites at day 13 of NF54, ORF1-specific mutants and ORF2-specific mutants showed distribution of MAEBL on the surface of the sporozoite (Fig 3-13, fig. 3-14). As expected, MAEBL KO sporozoites did not express MAEBL whereas CSP immunofluorescence patterns
were similar in NF54 and all the mutant parasites, including the MAEBL knockout. These results suggest that the localization of MAEBL in ORF2, ORF1 and NF54 parasites is the same in spite of the structural differences in MAEBL proteins.

Figure 3-12. *maebl* transcripts expression using primers for M2sd1. There is no expression in MAEBL KO parasites. On the other hand, there is expression of MAEBL transcript in NF54, ORF1 and ORF2.
Figure 3-13. IFA localization using anti MAEBL serum (M2sd2 Ab).

Midgut sporozoites from NF54 parasites, MAEBL KO, ORF1 and ORF2 were purified as previously described (Sultan, Thathy et al. 1997) and incubated with M2sd2 antibody (against the M2 ligand domain of MAEBL) and DAPI (nuclear stain). ORF1 and ORF2 sporozoites show a similar expression of MAEBL as the parental clone NF54. MAEBL appears to be distributed throughout the body and on the surface of ORF1 and ORF2 sporozoites. As expected, MAEBL KO sporozoites do not express MAEBL. All the genetically modified parasites express CSP on the surface of the sporozoite similar to the parental clone NF54.
Figure 3-14. IFA localization using anti-MAEBl (MAb 2C11).

Midgut sporozoites from NF54 parasites, MAEBl KO, ORF1 and ORF2 were purified as previously described (Sultan, Thathy et al. 1997) and incubated with monoclonal antibody 2C11 (Fu, Saenz et al. 2005). ORF1 and ORF2 sporozoites show a similar expression of MAEBl as the parental clone NF54. MAEBl appears to be distributed in all ORF1 and ORF2 mutant sporozoites, but MAEBl KO sporozoites do not express MAEBl. All the mutant parasites express CSP on the surface of the sporozoite similar to the parental clone NF54.
3.3.4 Sporozoites in the salivary glands

*Plasmodium falciparum* sporozoites usually begin to infect the salivary glands of *Anopheles* mosquitoes beginning day 14 post blood meal. This was confirmed in NF54 parasites, where thousands of sporozoites were observed inside the salivary glands (Fig. 3-15). Similar to the wild type NF54, ORF1 mutant parasites had abundant sporozoites in the salivary glands (Table 3 and 4). In contrast, no sporozoites were observed or detected in the salivary glands of mosquitoes fed the MAEBL KO parasites or ORF2 mutant parasites (Fig. 3-16), even though midgut sporozoites were readily observed beginning day 10 in the hemolymph of these mosquitoes infected with the MAEBL KO or ORF2 mutant. The absence of sporozoites in the salivary glands determined by microscopic analysis was confirmed with RT-PCR using primers for *csp*, the most highly expressed protein in sporozoites (Fig. 3-17).
Figure 3-15. Salivary gland lobe of *An. freeborni* infected with *P. falciparum* NF54.

Some of the infected *Anopheles* females had thousands of sporozoites in the salivary glands distal lateral lobes.
Figure 3-16. ORF1 parasites invade salivary glands cells.
A. Sections of salivary glands of *Anopheles* mosquitoes infected with NF54, ORF1, ORF2 and MAEBL KO on day 19 post feeding are shown in Nomarski bright field images (top lane) and by nuclear fluorescence using dihydroethidium (bottom lane). The sporozoites can be seen as small nuclei inside the salivary cells. B. The Brightness and contrast of an area showing sporozoites has been changed (rectangle) in order to evidence the sporozoites. NF54 and ORF1 sporozoites are visible inside salivary glands cells whereas no sporozoites of ORF2 or MAEBL KO clones were found in salivary glands.
Figure 3-17. ORF1 parasites invade salivary glands cells.
RT-PCR of NF54, ORF1, ORF2 and MAEBL KO in *Anopheles* midgut (mg) day 10 and *Anopheles* salivary glands (sg) day 20. Primers for *P. falciparum* csp were used to detect the parasites and primers for *Anopheles* its2 were used as controls. A band is observed in the midguts and salivary glands of the NF54 and ORF1 infected *Anopheles*. While the midguts of ORF2 or MAEBL KO infected mosquitoes are positive, the salivary glands do not show any band. The controls show that there was *Anopheles* material present in all the samples.
### TABLE 3.

**P. FALCIPARUM INFECTIONS IN ANOPHELES**

<table>
<thead>
<tr>
<th>Parasite (clone)</th>
<th>Midgut oocysts n</th>
<th>Av (SE)</th>
<th>%</th>
<th>Midgut oocysts</th>
<th>Hemol Spz Av (SE)*</th>
<th>%⁺</th>
<th>Saliv. gland Spz Av (SE)</th>
<th>%⁺</th>
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<tbody>
<tr>
<td>NF54</td>
<td>289</td>
<td>9 (2.1)</td>
<td>72</td>
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<td>20.2 (16.1)</td>
<td>67</td>
<td>701 (130)</td>
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<td>ORF1 (C9, E9)</td>
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<td>14.5 (3.3)</td>
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<td>565 (151)</td>
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<td>ORF2 (A10, H11)</td>
<td>144</td>
<td>7.5 (1.3)</td>
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<td>20 (17.7)</td>
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<td>MAEBL KO (G3, G7)</td>
<td>154</td>
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<td>22.6 (24.0)</td>
<td>60</td>
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</table>

* Pulled from 8 mosquitoes

+ Percentage of infected mosquitoes
### TABLE 4.

**SUMMARY OF *P. FALCIPARUM* INFECTIONS OF *AN. FREEBORNII* AND *AN. STEPHENSI***

<table>
<thead>
<tr>
<th>Parasite (clone)</th>
<th>Midgut oocysts</th>
<th>Hemolymph sporozoites</th>
<th>Salivary gland sporezoites</th>
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<tr>
<td>NF54</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>ORF1 (C9, E9)</td>
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<td>ORF2 (A10, H11)</td>
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<td>MAEBL KO (G3,G7)</td>
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4 DISCUSSION

4.1 *Plasmodium falciparum* MAEBL in the invasion of red blood cells

MAEBL is a chimerical molecule; it has similarity with two different groups of malarial ligands: the DBL-EBP family and AMA-1. These two groups are known to be important in the merozoite invasion of host erythrocytes. Consistent with a possible role in erythrocytic stage development, the cysteine-rich ligand domains of MAEBL were experimentally shown to have erythrocyte binding activity (Kappe, Noe et al. 1998; Ghai, Dutta et al. 2002). In order to investigate the role of MAEBL in the merozoite invasion of erythrocytes, the erythrocyte invasion process in parasites having a disruption of MAEBL was characterized. We found that MAEBL is not essential in the erythrocytic cycle, but it may have a role in the *P. falciparum* blood-stage growth.

The expression of MAEBL in *P. falciparum* erythrocytic stages was confirmed using an antibody against the M2 domain. The principal MAEBL product in schizonts appears to be a 140–155 kDa protein soluble in ionic detergent, but not in non ionic detergent (Ghai, Dutta et al. 2002; Fu, Saenz et al. 2005). Targeted disruption of *maebl* in *P. falciparum* caused a total loss of this 140–155 kDa protein expressed in erythrocytic stages as determined by immunoprecipitation and IFA using MAB 2C11. The total loss of MAEBL in the disruptants was confirmed by a independent set of *P.falciparum* MAEBL disruptants in NF54.
The ability to disrupt the *P. falciparum* maebl demonstrates that MAEBL is not required for parasite survival in blood-stage development under ideal culture conditions. Using standard in vitro culture methods in untreated human erythrocytes, growth curves for Δmaebl clones were comparable to the parent clone W2mef. Yet erythrocyte invasion by *P. falciparum* is a very complex process that requires multiple ligand-receptor interactions, with apparent redundancies at different steps and with different pathways, so phenotypes may not be evident except under certain conditions. There are at least three invasion pathways identified by sensitivity to enzyme treatment: sialic acid-dependent/trypsin-sensitive (associated with glycophorin A); sialic acid-dependent/trypsin-insensitive (glycophorin B); and sialic acid-independent/trypsin-sensitive (receptor X) (Dolan, Proctor et al. 1994; Stubbs, Simpson et al. 2005). Using these defined invasion pathways, we compared the erythrocyte invasion phenotypes of the Δmaebl clones and the parental clone W2mef. Just the same as the parent clone W2mef, the invasion of Δmaebl clones was almost eliminated by treatment with neuraminidase, indicating that invasion is dependent on sialic acid residues on the erythrocyte surface. However, invasion into erythrocytes treated with trypsin was significantly enhanced in the parasites with the disrupted *maebl* gene (Fig. 3-4). This invasion pathway was different from the glycophorin B pathway, because treatment by chymotrypsin (which removes glycophorin B) had no effect on invasion in these parasites (Fig. 3-4). This result showed that loss of MAEBL expression in merozoites alters the W2mef invasion pathway to a novel alternative pathway, which is sialic acid dependent and trypsin insensitive. This pathway may be similar to several isolates of *P. falciparum* (e.g. Indochina I) that invade erythrocytes through a sialic acid dependent/trypsin-
resistant pathway independent of glycophorin B (Gaur, Storry et al. 2003). However, these results contrast with the findings that the disruptants of *P. falciparum* NF54 *maebl* did not show a difference in growing in any of the enzymes (work done by Bharath Balu). This apparent contradiction likely results from inherent differences in the repertoire of invasion pathways used by NF54 parasites, which can invade neuraminidase, trypsin, and chymotrypsin treated erythrocytes, while W2mef does not. Indeed, differences in invasion pathways between isolates add complexity to understanding results from the crude analysis of available invasion assays. The enhanced invasion of W2mef MAEBL disruptant parasites by this alternative pathway presents the possibility that some ligands such as MAEBL have a function to control or restrict invasion mechanisms or processes of other invasion-related proteins. It is unknown whether switching invasion phenotype of the malaria parasite may have arisen as a mechanism to adapt to evolving polymorphisms of human erythrocyte receptors or to evade naturally occurring inhibitory immune responses to a parasite ligand.

One of the discoveries in our analysis was the finding that the canonical and alternative splicing of the 3’ exons was not altered in the ∆*maebl* clones. Despite the disruption of the *maebl* locus with multiple copies of the targeting plasmid, the 3’ end of the gene was still actively transcribed and the mRNA processed in a manner indistinguishable from that in the intact parasites. Orientation of the hDHFR selection cassette within the plasmid is inverse to the ORF of the *maebl* targeting sequence (Reed, Caruana et al. 2000), so the 5’ CAM regulatory region lies adjacent to the 3’ CDS of the disrupted *maebl* locus. This strong bidirectional promoter is likely driving mRNA synthesis of the 3’ end of *maebl* after the disruption instead of the resident *maebl*. 

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promoter, which would be over 20,000 nt upstream (Wickham, Thompson et al. 2003). In higher eukaryotes, transcription and pre-mRNA processing are functionally linked, so similar experimental changes in promoter structure can alter splicing patterns by presumably changing associated co-factors responsible for splicing (Cramer, Pesce et al. 1997; Cramer, Caceres et al. 1999; Kadener, Fededa et al. 2002). Our results show that definition of exon structure, including recognition of suboptimal alternative splicing junctions, occurs independently of promoter usage. This suggests that pre-mRNA processing is likely to be an inherent structural property of the RNA.

MAEBL is not essential for *P. falciparum* blood-stage development under normal culture conditions, but may have a direct or indirect role in an alternative trypsin-insensitive invasion pathway. The importance of MAEBL in the invasion of erythrocytes may involve its interactions with other molecules that facilitate invasion. As with other merozoite adhesion molecules, the role or importance MAEBL may vary depending on the genetic background of the parasite and the phenotype of the target host erythrocyte to be invaded.

MAEBL is not essential in the erythrocytic stages of *P. falciparum*; nevertheless, it was demonstrated that *P. berghei* MAEBL has an essential role in the sporozoite invasion of salivary glands. The importance of MAEBL in this process needed to be confirmed in *P. falciparum* in addition to the elucidation of the isoform essential for the process of invasion.
4.2 *Plasmodium falciparum* MAEBL in the invasion of *Anopheles* salivary glands

Sporozoite invasion of the anopheline salivary gland is critical for malaria parasite transmission and yet it is one of the most poorly studied stages of this deadly pathogen. One of the ligands that has been shown to be essential in the invasion of salivary glands in the rodent parasite *Plasmodium berghei* is MAEBL (Kariu, Yuda et al. 2002). We found that *P. falciparum* MAEBL is not essential for the erythrocytic stages; yet, we confirm that it is essential in the sporozoite invasion of *Anopheles* salivary glands. This is the first characterization of a *P. falciparum* ligand essential for the invasion of the mosquito salivary glands. The only other *Plasmodium* ligand confirmed to be essential for the invasion of salivary glands is TRAP, which was studied in *P. berghei*; however, the importance of this protein in the human parasite has not been confirmed.

This study shows that only the transmembrane isoform of MAEBL is essential for the invasion of salivary glands. Since alternative splicing of *maebl* is conserved among all *Plasmodium* species examined (Singh, Preiser et al. 2004), it was not possible to know from previous studies what MAEBL isoform is essential for invasion (Kariu, Yuda et al. 2002). These results suggest an involvement of the cytoplasmic domain of MAEBL in the invasion process but by a mechanism distinct to the TRAP interactions with the parasite’s glideosome (Kappe, Bruderer et al. 1999; Gilberger, Thompson et al. 2003). No MAEBL knockout and ORF2 mutant sporozoites were present inside the salivary gland cells and sporozoites were not arrested in the invasion process on the surface of the glands.
This observation that the MAEBL deficient mutants cannot attach to salivary glands coincides with previous studies (Kariu, Yuda et al. 2002) and contrasts with the data for TRAP in which TRAP deficient sporozoites were found on the surface of the salivary glands, but not inside the salivary cells (Sultan, Thathy et al. 1997). Importantly, MAEBL appears lacking in the critical residues at the C-terminus of its cytoplasmic domain to mediate interactions with aldolase and the glideosome complex. Although there is an acidic motif at the C-terminus of \textit{P. falciparum} MAEBL, these terminal residues are not conserved among MAEBL products of other \textit{Plasmodium} species and the MAEBL C-terminus is lacking the critical penultimate tyrosine residue for binding to aldolase (Fig. 4-1). These observations for MAEBL imply it has a distinct role before TRAP establishes the moving junction that enables the parasite to strongly attach to and then invade the salivary cell.

![Figure 4-1](image_url)

**Figure 4-1.** Comparative alignment of the cytoplasmic tails of invasion proteins. AMA-1, TRAP, \textit{P. berghei} and \textit{P. falciparum} MAEBL, and EBA175. The acidic carboxy terminal ends of TRAP and MAEBL lack similarity.
Based on its homology to the EBPs, which are critical for junction formation during merozoite invasion of erythrocytes, MAEBL is anticipated to be important in a similar step after initial attachment of the sporozoites to the salivary gland cell. AMA-1 plays a major role in *P. falciparum* merozoite attachment and reorientation to erythrocytes and its high level of expression midgut sporozoites suggests that it may have a similar role in sporozoite invasion processes. This would place the function of MAEBL after AMA-1 and before TRAP involvement during the sporozoite invasion process into salivary glands. Therefore, we conclude that we do not see sporozoites attached to the salivary glands for the MAEBL deficient mutants, because similar to merozoite’s initial attachment the sporozoite attachment to salivary glands is an unstable and reversible step when invasion does not proceed to junction formation. Normal activity and movement of the mosquitoes or the process of dissection are likely sufficient to dislodge attached sporozoites from the surface of the salivary gland. These results demonstrate an importance for *P. falciparum* MAEBL as an essential ligand in the early stages of the invasion process.

The fact that a transmembrane form of MAEBL is essential in the process of invasion of salivary glands validates the importance of alternative splicing in *Plasmodium*. As in higher eukaryotes, alternative splicing may be a little appreciated mechanism for the expression of different malaria parasite products that could be generating product diversity (Mironov, Fickett et al. 1999; Muhia, Swales et al. 2003; Meister, Kanzok et al. 2005; Fonager, Cunningham et al. 2007). At this point we can only speculate about the function of ORF2, which may be participating in a different phase of the interactions with the host cell or alternative splicing may be participating in
the regulation of gene expression through a nonsense mediated decay mechanism (Lewis, Green et al. 2003; Wollerton, Gooding et al. 2004).

4.3 Conclusions and future directions

In this work, I intended to provide experimental results to understand the importance of MAEBL in two invasion steps of the life cycle of *P. falciparum*. For this purpose, homologous recombination was used to disrupt MAEBL expression and to complete allelic replacement of the 3’ end of *maebl* to force the production of the different alternatively spliced isoforms. I found that MAEBL is not essential in the red blood cell invasion but it may be participating in an alternative invasion pathway. Moreover, *P. falciparum* NF54 *maebl* disruptant parasites and ORF1 and ORF2 parasites were used to produce gametocytes and feed *Anopheles* in order to identify the isoform essential for the invasion of salivary glands. I found that ORF1, the trasmembrane form of MAEBL is the essential form for this process. These findings imply a role of MAEBL in the invasion of salivary glands, distinct from the role of TRAP.

The fact that the transmembrane isoform of MAEBL is essential in the sporozoite invasion of salivary glands implies a role of the transmembrane and cytoplasmic domains in this process. The cytoplasmic domain of MAEBL could be interacting with a hypothetical protein of the parasite to mediate invasion prior to the formation of the glideosome complex (Fig. 4-2). Consequently, the invasion role of the cytoplasmic domain, and the specific regions participating should be further investigated.

Furthermore, the transmembrane domain of MAEBL could be necessary in the process of invasion because it may contain a target sequence for the cleavage by rhomboid
proteases. Indeed, co-transfection of MAEBL with TgROM5 and PfROM4 resulted in strong cleavage of the protein (Baker et al., 2006). Therefore, the importance of proteases in the sporozoite invasion of salivary glands and the mechanism of action with their targets need further investigation.

![Diagram](image)

Figure 4-2. Hypothetical role of MAEBL in the sporozoite invasion of salivary glands.

In order for the parasite to form a junction, MAEBL ligand domains have to bind a receptor in the salivary gland cell surface. Simultaneously, the cytoplasmic domain would interact with a hypothetical sporozoite cytoplasmic protein. This would occur before the formation of the glideosome.

In order to participate in the process of invasion, the AMA-1-like ligand domains of MAEBL would interact with a receptor in the salivary glands. The nature of this interaction and the determination of the identity of the *Anopheles* salivary gland receptor
for MAEBL need to be elucidated for a better understanding of the process of the sporozoite invasion of the salivary glands. Finally, antibodies against MAEBL inhibit sporozoite development in vitro. This strongly suggests a role for MAEBL in the pre-erythrocytic stages of *Plasmodium*. As a result, the determination of the function of MAEBL in the liver stages should be a priority.

Transmission of malaria is dependent upon successful completion of parasite development in the mosquito. In this work, I show that the *P. falciparum* MAEBL transmembrane protein is essential for the infective stages to enter the salivary glands. In addition, my study demonstrates the feasibility of using genetically modified *P. falciparum* to study host-parasite interactions in the vector stages of development. The essential role of MAEBL for the sporozoite invasion of the mosquito salivary gland is a weakness in the parasite’s biology that provides a potential opportunity for vector-based intervention strategies to disrupt malaria transmission.


Center for disease control and prevention. 2004 <http://www.cdc.gov/malaria/disease.htm>


Orlandi, P. A., F. W. Klotz, et al. (1992). "A malaria invasion receptor, the 175-kilodalton erythrocyte binding antigen of Plasmodium falciparum recognizes the


