PhD Thesis

Title:

Validation of reverse genetic approaches aiming to reveal critical proteolytic steps in early sporogony of *Plasmodium* parasites

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Table of contents

Page

List of figures

List of Tables

SUMMARY

With the rapid emergence of resistance of mosquitoes and *Plasmodium* parasites to the available insecticides and anti-malarial drugs respectively, and the lack of an effective vaccine, malaria remains one of the deadliest diseases claiming more than a million lives annually mostly from developing countries. Therefore, there is an urgent need to identify new targets for developing new antimalarial strategies.

During *Plasmodium* development both in vertebrate host and mosquito vector, central roles in invasion of the host cells/tissues, parasite metabolism, growth and differentiation, as well as, in host immune evasion, invasion/egress of the parasite from the host cell are played by parasitic proteases. As a consequence, in addition to numerous candidates so far identified, *Plasmodium* proteases as potential candidates for malaria interventions have been recently on the rise. However, since the most promising protease targets are the ones that are essential during the parasite life cycle, and therefore resistant to the conventional *Plasmodium* gene knock-out strategies, alternative approaches leading to either conditional silencing, or functional inactivation should develop.

In this study, we explore two such approaches. First we targeted specifically *P. berghei* SUB2 gene, which encodes for a subtilisin-like serine protease expressed both in asexual and mosquito stages. SUB2 is an essential sheddase in merozoites, which makes its disruption unachievable. In an attempt to overcome this obstacle, and identify its functional involvement, as well as, putative substrates in mosquito stages we generated transgenic *P. berghei* parasites in which a ~1kb genomic sequence upstream of the SUB2 open reading frame (ORF), was replaced with the merozoite specific promoter, PbAMA-1. This strategy aimed to restrict expression of SUB2 in merozoites and abolish it in the subsequent stages. Indeed the resultant transgenic parasites developed normally in the vertebrate host. However, contrary to our expectations, the levels of PbSUB-2 in ookinetes were unaffected indicating a more complex regulation of gene expression than initially thought.

In a parallel alternative approach, we expressed in a stage specific manner (under ookinete and sporozoite specific promoters), serine protease inhibitors (serpins) of ovalbumin and Kazal families. The derived transgenic parasites exhibited normal development in the vertebrate. Moreover, *in vitro* and *in vivo* gametocyte to ookinete transition was not affected. However, while the LGI-expressing parasites (under CTRP promoter) showed a mild reduction in oocyst formation, the NcPI-S-expressing parasites showed a severe (97-98%) inability to form oocysts.

Detailed characterization of NcPI-S-expressing ookinetes, involving biochemical studies using *in vitro* ookinete cultures, as well as cell biology studies implicating gametocyte and ookinete feeding experiments, revealed an inability of the transgenic ookinetes, despite their normal motility, to associate tightly with mosquito midgut epithelium, a pre-requisite step for invasion. At the molecular level although expression of two surface associated proteins (P25, CTRP), critical for the mosquito midgut invasion, was found unaffected in NcPI-S expressing ookinetes, protein levels of the secreted essential micronemal proteins WARP and SOAP, were severely reduced.

In a remarkable contrast to the NcPI-S expression in the ookinete, NcPI-S expression in sporozoites under CS promoter did not affect their development or their ability to invade mosquito salivary glands, and subsequently to establish a successful infection in the vertebrate host with similar pre-patent periods to the wt control.

In summary, this study has established *P. berghei* ookinetes as a test tube system to screen *in vivo* macromolecular protease inhibitors. Such molecules could be subsequently used to generate attenuated *Plasmodium* parasites as part of the efforts towards live attenuated vaccine. Alternatively, MPIs can be used for the development of innovative transmission blocking interventions.

CHAPTER 1: INTRODUCTION

1.0 Malaria

In addition to AIDS and tuberculosis, malaria is one of the major infectious disease killers in the world, accounting for 1-3 million deaths per year (Breman *et al*., 2001; Greenwood and Mutabingwa, 2002). As it is estimated 300-660 million malaria cases occur annually with the vast majority of the deaths being those of children under the age of 5 years (WHO-UNICEF 2005, snow *et al.,* 2005). Most of these cases are registered in the endemic regions comprising mainly developing countries of the globe. In contrast to the other two diseases (AIDS and TB), *Plasmodium*, the causative agent of malaria is mainly transmitted between mammalian hosts by female *Anopheline* mosquitoes, in which the parasite accomplishes its complex sexual developmental program.

Major challenges in the efforts to combat the disease are the rapid emergence of resistance of the parasites and mosquitoes to the available anti-malarial drugs and insecticides respectively. This urgently necessitates identification of new targets for establishment of reliable anti-malarial interventions. Tireless efforts have discovered critical molecules, as drug targets or vaccine candidates. Some are already under clinical trial, while some other potential candidate molecules are still under investigation.

Antimalarial strategies targeting blood stages of the parasites intend to prevent the parasite development in the host and cure the infected individual, while those targeting parasite development in the mosquito do not directly protect an individual from malaria but limit the number of infectious vectors thus reduce the parasite transmission from one person to another. Therefore are collectively

called, Transmission Blocking Strategies (TBSs). Until recently, TBSs were underestimated, despite the significant evidence indicating that the parasite suffers its greatest population losses in the mosquito.

A great collection of data point lately beyond any doubt to a vital role of *Plasmodium* proteases across the parasite development both in the vertebrate host and the mosquito vector, therefore parasite encoded proteases are among the most attractive anti-malarial targets currently under study. Elucidation of the functional role of these proteases during parasite development in the mosquito will boost substantially the efforts to develop innovative TBSs. This can be achieved through delivery of specific protease inhibitors as drugs or by expressing macromolecular protease inhibitors in transgenic mosquitoes.

In the present thesis, we investigate the importance of proteolytic processes during the early parasite development in the mosquito and the subsequent mosquito midgut invasion by applying transgenesis to express protease inhibitors (serpins) in the malaria model parasite, *Plasmodium berghei.* We also used this malaria model to target, by conditional stage specific disruption, a *Plasmodium* Subtilisin-2, one of the essential proteases in the blood stages, with a potential role during ookinete-oocyst transition.

HUMAN **MOSQUITO**

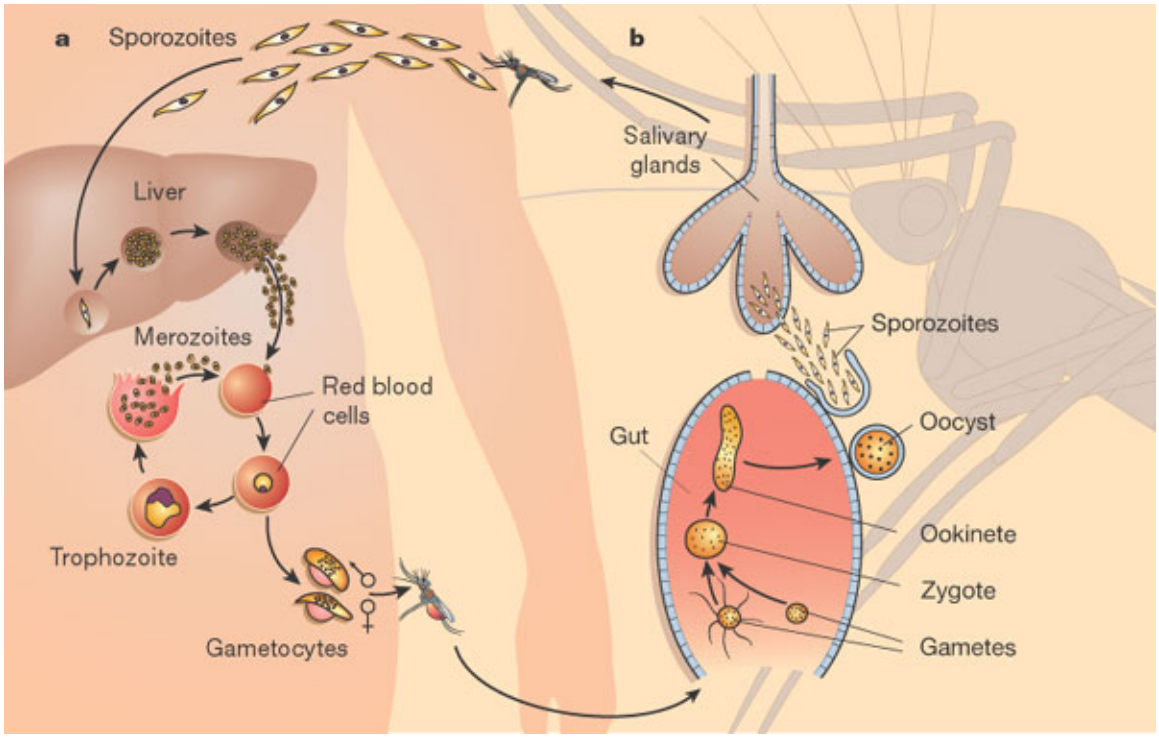

Figure 1: Schematic representation of malaria parasite life cycle (Wirth 2002).

a. When a parasite-infected mosquito feeds on a human, it injects the parasite in the form of sporozoite. Sporozoites travel to the liver, where they develop through several stages, finally producing merozoites that bud off from the infected hepatocytes and infect blood cells. Inside the blood cells parasite multiplies multiply, via the trophozoite stage, into huge numbers of merozoites. Eventually, up to 10% of all red cells become finally infected. Parasites in a subset of infected red blood cells develop into gametocytes. **b,** When a mosquito bites an infected individual, it takes up blood containing gametocytes, which inside the mosquito midgut develop further into male and female reproductive

cells (gametes). Gametes are fused to form a zygote, which in turn develops into a motile ookinete that crosses the mosquito midgut wall and forms a sporozoite-filled oocyst. When the oocyst bursts, the sporozoites move to the mosquito's salivary glands, and the lethal cycle continues.

1.1. *Plasmodium* Life cycle

1.1.1. *Plasmodium* development in the vertebrate (human) host

The life cycle of malarial parasites is extremely complex, with multiple intracellular and extracellular developmental stages in both, the vertebrate host and the *Anopheline* mosquito vector (Figure 1). In the vertebrate host, an infection is initiated when haploid *Plasmodium* sporozoites, present in the salivary glands of an infected female *Anopheles* mosquito, are introduced into the bloodstream of the host with saliva as the mosquito probes for blood. The sporozoites travel through the bloodstream until they reach the liver, where they invade and infect hepatocytes. In addition to the two well described major surface proteins of sporozoites, the circumsporozoite protein (CS) and thrombospondin-related anonymous protein (TRAP) (Frevert 2004, Sultan *et al*., 1997), two more recently identified proteins the thrombospondin-related sporozoite protein (TRSP) and S6 (Labaied *et al*., 2007, Steinbuechel and Matuschewski 2009) have been linked to the journey of sporozoites and the invasion of hepatocytes. In the hepatocyte, sporozoites undergo schizogony and form pre-erythrocytic schizonts containing thousands of merozoites. Upon rupture of the pre-erythrocytic schizont, the merozoites are released into the blood stream, where they invade erythrocytes.

Like in other apicomplexan parasites, the erythrocyte invasion by merozoites is an active and rapid process mediated by adhesive receptor–ligand interactions and driven by an actinomyosin motor (Carruthers and Blackman 2005, Soldati *et al*., 2004). Light and electron microscopic studies have shown that initial attachment to the host erythrocyte is followed by re-orientation of the merozoites such that its apical end contacts the cell surface. This results in the formation of an irreversible zone of contact, or tight junction, between the apical end of the parasite and the host cell surface. The host cell membrane then invaginates, and forms a parasitophorous vacuole (PV) into which the parasite is propelled; during the later process the junction sweeps around the periphery of the parasite with a concomitant ‘‘shaving’’ of the parasite surface, ultimately sealing behind the intracellular parasite (Figure 2). The entire process is thought to be completed within 30 seconds (O’Donnell and Blackman 2005, Dvorak *et al*., 1975). Merozoite surface protein-1 (MSP-1), Apical membrane antigen 1 (AMA-1), *Plasmodium* subtilisin like protease 2 (PfSUB-2) have been linked with this invasion process (Harris *et al*., 2005, O’Donnell and Blackman 2005, Chitnis and Blackman 2000).

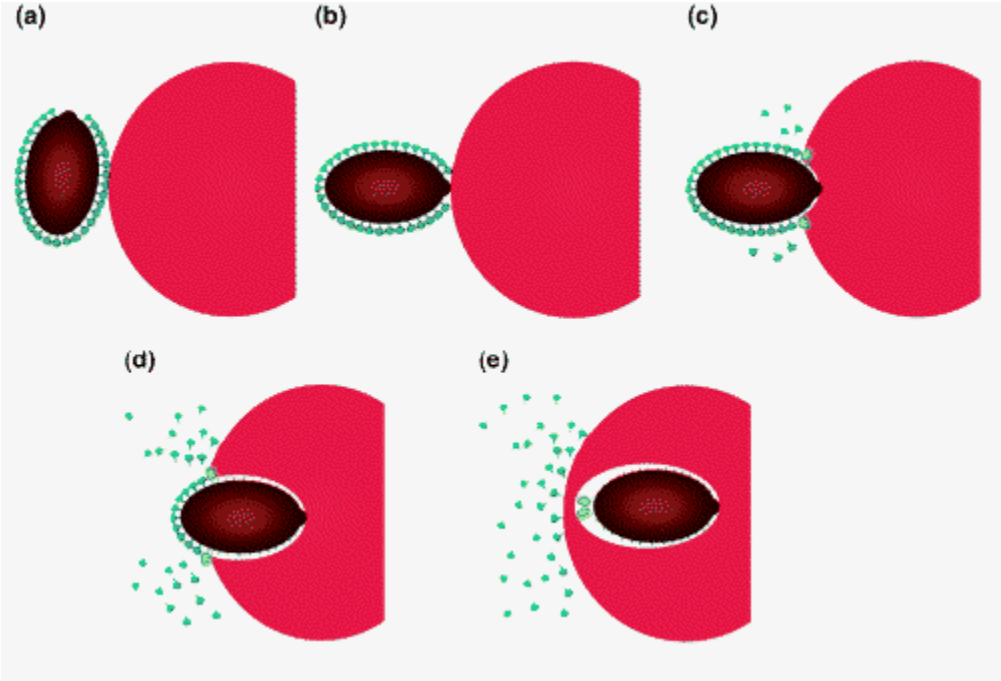

Figure 2: Erythrocyte invasion by *Plasmodium* merozoites as proposed by (O'Donnell and Blackman 2005) Initial binding of the malaria merozoite (brown) (a) is followed by reorientation and junction formation (b), which is mediated by apically-restricted adhesins that are released from micronemes, merozoite surface sheddase (MESH) (green) is then also released from micronemes onto the merozoite surface (c) and remains fixed at the moving junction, which is translocated over the parasite surface as the latter enters the parasitophorous vacuole (d,e). During the process, MSP1 (blue) is cleaved at a juxtamembrane site releasing the bulk of the complex extracellularly. AMA1 is similarly processed by MESH.

Within an erythrocyte, the merozoite develops into a ring form, which undergoes mitotic replication within the parasitophorous vacuole (PV) to become

a mature schizont packed with approximately 8-24 merozoites. The mature schizont then ruptures releasing invasive merozoites, which invade new erythrocytes to repeat the cycle. The repeated steps of invasion, replication and schizont rupture cause the clinical manifestations of the disease, including anemia, fever, and in severe cases, coma and death (Miller *et al*., 2002). A subset of the erythrocytic parasites ceases asexual multiplication and commits into a sexual development (Smith *et al*., 2000, Silvestrini *et al*., 2000, Alano 2007) to become gametocytes, the precursor forms of the sporogonic development in the female *Anopheles* mosquito.

1.1.2. *Plasmodium* development in the invertebrate (mosquito) vector

Parasite development inside the mosquito vector begins when a mosquito takes up a blood meal from an infected vertebrate host containing arrested sexual stages (male and female gametocytes) (Ghosh *et al*., 2000). The gametocyte transformation is activated by a shift in pH from 7.2-8.0, a 5°C drop in temperature and the mosquito derived factor xanthurenic acid (Billker *et al*., 1997, 1998, and 2000). Within 10-20 minutes, both male and female gametocytes escape from the enveloping erythrocytes. The female gametocyte differentiates into a single, spherical female gamete whereas the male gametocyte undergoes rapid three rounds of DNA replication (Janse *et al*., 1986), nuclear division, resulting in the production of eight, motile gametes in a process termed exflagellation (Figure 3).

Fertilization occurs within 1hr upon gametocyte activation to form zygotes. The zygote differentiates over the next 18-24 hrs into an elongated, motile, invasive form called the ookinete. The ookinetes escape from the blood bolus and penetrate the acellular, chitin-rich peritrophic matrix (PM) surrounding the blood meal (Sieber *et al*., 1991). They then invade and traverse the midgut epithelium (Han *et al*., 2000, Zieler *et al*., 2000a), emerging at the basal side of the midgut, where they differentiate beneath the basal lamina into oocysts. The parasite in the oocyst undergoes rounds of endomitosis generating thousands of sporozoites, a process which takes about 10-14 days to be completed. Upon oocyst rupture, thousands of sporozoites are released into the hemocoel (Figure 3). Only a fraction of sporozoites ultimately invades the salivary glands, from where they can be transmitted into the next vertebrate host.

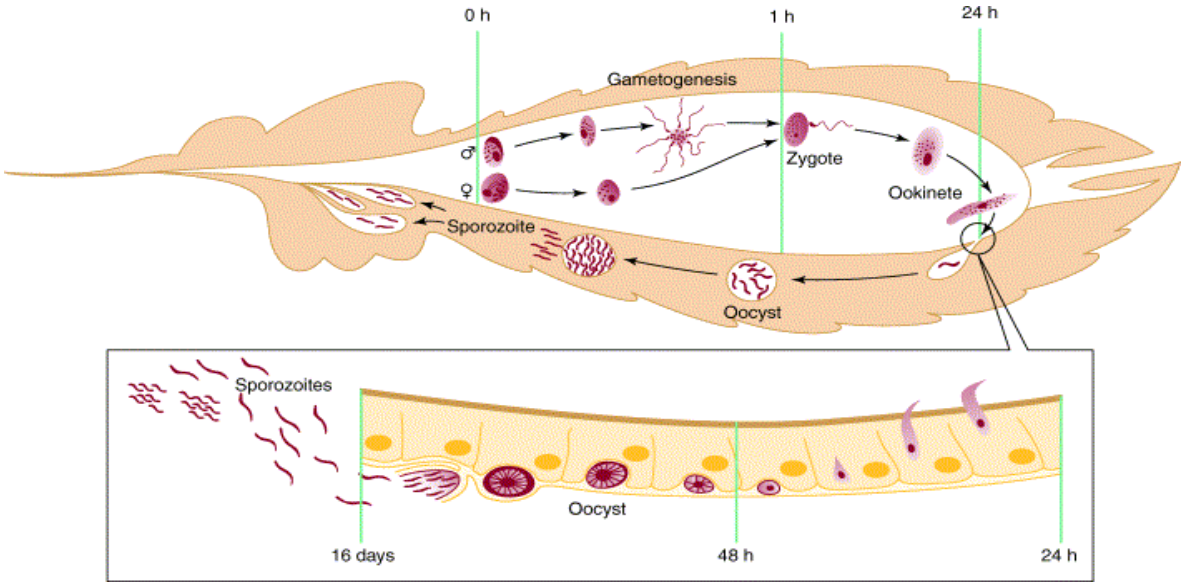

Figure 3: Life cycle model of *Plasmodium* in the mosquito (Ghosh *et al.*, 2003).The approximate developmental time at which each stage occurs in *Plasmodium berghei* (maintained at 20°C) is indicated. Transmission starts when a mosquito ingests an infected blood meal (at 0 h). Within minutes, gametocytes develop into gametes (the star-shaped figure illustrates exflagellation, which is the formation of male gametes) that fuse to form the zygote. At 24 h, the motile ookinete invades the midgut epithelium and differentiates into an oocyst. About two weeks later, the oocyst ruptures, releasing thousands of sporozoites into the mosquito body cavity. The sporozoites then invade only the salivary gland and transmission is completed by release of sporozoites from the salivary glands into when the mosquito bites another vertebrate host.

1.1.2.1. Ookinete specific genes as targets for the development of transmission blocking strategies

The *Plasmodium* parasite suffers severe population losses during the ookinete to oocyst transition. Observations in the field indicate that out of ten thousands of gametocytes ingested by the mosquito, just 50-100 ookinetes are formed and fewer than 5 produce mature oocysts on the midgut wall (Sinden and Billingsley, 2001). Furthermore, depending on parasite/vector combination, it is estimated that up to 100,000 fold parasite losses occur during the sexual development of the parasite in the mosquito (Alavi *et al*., 2003, Vaughan *et al*., 1992, 1994). Destruction of ookinetes by an internal cell death program (that leads to over 50% apoptosis; Al-Olayan *et al*., 2002a), digestive enzymes secreted into the mosquito midgut, components of the vertebrate immune system that may remain active in the blood meal, and immune factors secreted by the mosquito, may account for these losses (Sinden, 2002; Blandin and Levashina 2004; Osta *et al*., 2004; Dimopoulos *et al*., 1998). Therefore, the ookinete-to-oocyst transition is crucial for successful parasite establishment in the mosquito required for malaria transmission, representing the most vulnerable target for transmission interventions.

Molecular players involved into these processes may provide excellent transmission blocking targets. Parasite-derived antigens for the development of transmission blocking vaccines have been identified and are currently under clinical trial phase (Wu *et al*., 2006, Kubler-Kielb *et al*., 2006). At the top of the list are surface proteins P25 and P28, abundantly expressed and evenly distributed

on the ookinete surface (Tsuboi *et al*., 1997, Winger *et al*., 1988, Tomas *et al*., 2001, Duffy *et al*., 1993). Concomitant disruption of p25 and p28 results in dramatic reduction in oocyst numbers (Tomas *et al*., 2001). The same is observed when antibodies against the two proteins mixed with parasites are fed to mosquitoes through laboratory membrane feeders (Hisaeda, *et al*., 2000). In addition to the P25 and P28, other ookinete-expressed proteins that are important for ookinete-to-oocyst transition have been suggested as potential transmission vaccine targets. An ookinete-specific chitinase (Shahabuddin *et al*., 1993) is among them. Chitinase-deficient *P.falciparum* and *P.berghei* parasites are significantly impaired in their ability to form oocysts in the mosquito midgut (Tsai *et al*., 2001, Dessens *et al*., 2001). Depletion of other ookinete micronemal proteins, (associated with micronemes; the only secretion specialized organelle present in ookinetes), such as: circumsporozoite and thrombospondin-related adhesive protein (CTRP) (Dessens *et al*., 1999, Yuda *et al*., 1999, Templeton *et al*., 2000), membrane attack ookinete protein (MAOP) (Kadota *et al.,* 2006), secreted ookinete adhesive protein (SOAP), (Dessens *et al*., 2003), cell-traversal protein for ookinetes and sporozoites (CelTOS) (Kariu *et al*., 2007) significantly interferes with midgut invasion and the subsequent oocyst formation in the mosquito. More recently, disruption of the gene encoding for one of the LCCL/lectin adhesive-like protein (LAP) family members expressed in ookinetes (Reine *et al*., 2007) severely impaired the parasites sporogonic cycle. Finally, antibodies against an ookinete specific von Willebrand factor A domain-related micronemal protein (WARP) (Yuda *et al*., 2001), CTRP and the *Plasmodium*

chitinase when fed to mosquitoes, together with infected blood; block malaria transmission (Li *et al*., 2004).

1.1.2.2. Peptides and exogenous proteins as transmission blocking agents

A number of exogenous molecules and peptides have also been shown to interfere with *Plasmodium* transmission in the mosquito vector. For instance, bee venom derived phospholipase 2 (PLA2) expressed in transgenic mosquitoes strongly inhibits *P. berghei* oocyst formation (Moreira *et al*., 2002). A similar inhibition on *P. gallinaceum* oocyst development was observed with PLA2 purified from snake venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*) when fed to mosquitoes together with infected chicken blood (Zieler *et al*., 2001). A similar inhibitory effect on oocyst development was achieved with SM1 peptide (salivary gland and midgut binding peptide 1). SM1 was initially isolated from a bacteriophage-expressed peptide library due to its strong affinity for midgut and salivary gland epithelia. When SM1 was mixed with infectious blood meal and fed to mosquito, or alternatively when it was expressed in transgenic mosquitoes, significantly inhibited the formation of oocysts (Ghosh *et al*., 2001, Ito *et al*., 2002). The SM1 peptide has been recently shown to bind to saglin, a mosquito salivary gland surface protein thought to interact with thrombospondin-related anonymous protein (TRAP) (Ghosh *et al*., 2009), and to a second sporozoite surface protein required for the salivary gland invasion by the sporozoites (Sultan *et al*., 1997). Thus SM1 peptide may disrupt critical receptor-ligand interactions taking place at the sporozoite/salivary gland

interface. Finally, a cell penetrating peptide (CPP) designated TP10 significantly blocks zygote to oocyst transition in mosquitoes (Arrighi *et al*., 2008).

Most of the Transmission Blocking vaccines (TBv) and Transmission Blocking peptides (TBp), however, intend to target surface molecules putatively implicated in parasite's developmental signaling and differentiation, or in "parasite/mosquito tissue" interactions. Polymorphic variations among different parasite species, though, and/or clonal variation may hamper the efforts of choosing the right target molecule and the design of a wide-ranging strategy.

It is reasonable to speculate that enzymatic activities with an essential role in the sporogonic development and/or mosquito tissue invasion may constitute better targets of a transmission blocking strategy. For example, a high degree of conservation is expected in invasion related proteases among different parasite/vector combinations; therefore, the concept of functionally disrupting proteolytic steps vital for parasite survival and tissue invasion in the mosquito should be systematically explored.

1.2. Proteases

Proteases or proteolytic enzymes catalyze the degradation of peptide bonds in proteins and peptides and are widespread in the plant and animal kingdoms. Several mechanistic classes of proteases have evolved to handle the peptide bond hydrolysis workload of the cell (Rawlings and Barrett 1993). Serine, the largest group, threonine, and cysteine proteases rely upon the nucleophilic character of the serine, threonine, and cysteine side chains, respectively, to

directly attack the peptide bond, forming a transient, covalent enzyme-substrate intermediate. Aspartic proteases utilize two aspartic acid side chains in catalysis, while metalloproteases contain a metal ion in the active site that plays a key role in substrate binding. Both aspartic and metalloproteases employ a water molecule for nucleophilic attack and do not form a covalent intermediate. Proteases can be further categorized based on their ability to cleave at the N or C terminus of the substrate (exopeptidases) or internally (endoproteases).

In higher organisms, activation of enzymes (including other proteases), maturation of structural and signaling proteins, hormone biosynthesis and receptor activation, apoptosis, inflammation, blood clotting, complement activation, fibrinolysis, and other signaling pathways involve proteolytic events mediated by proteases.

In the parasitic organisms, proteases mediate a wide range of biologically relevant processes such as invasion of the host cells/tissues, metabolism within the host, host immune evasion, parasite remodeling as the parasites transform from one developmental stage to another, degradation of host cytoskeleton during invasion or egress from the host cell (McKerrow *et al*., 1993). Because of their central role in pathogenesis, proteases of many pathogens have been generally targeted for therapeutics, as well as, vaccine development. One of the best examples is HIV/AIDS where many antiretroviral drugs currently in use were developed based on their inhibitory potency against HIV protease (Wlodawer and Vondrasek 1998). For instance, saquinavir is a potent inhibitor against the HIV aspartic protease currently used to treat advanced HIV infection (Lin 1997).

1.2.1. The role of proteolysis in *Plasmodium* and other apicomplexan parasites

Plasmodium and other protozoan proteases are considered potential targets for chemotherapy due to their crucial roles in the parasite life cycle, and to the feasibility in designing specific inhibitors (McKerrow *et al*., 1993, Rosenthal 1998; Blackman 2000; Rosenthal 2002). Genome annotation has predicted 92 proteases in *Plasmodium falciparum,* the majority of them conserved across *Plasmodium* species (Wu *et al*., 2003), and in some other members of apicomplexan family. Parasite proteases are essential not only during the asexual development, where they have been extensively studied, but also for several key events during the sexual development of the *Plasmodium* parasites in the mosquito, as demonstrated by the use of broad range of chemical inhibitors.

For instance, during gametogenesis and fertilization, proteolysis has been implicated in the parasite egress from the erythrocyte, exflagellation of the microgametocyte and cell contact between male and female gamete prior to their fusion. Recent studies showed that exflagellation in both *P. berghei* and *P. falciparum* is blocked by the cysteine/serine protease inhibitors TPCK/TLCK as well as by the metalloprotease inhibitor 1,10-phenanthroline (1mM), indicating that related proteases are involved in exflagellation (Torres *et al*., 2005, Rupp *et al*., 2008). Furthermore, a membrane-permeant cysteine protease inhibitor E64d significantly inhibited oocyst production (80 to 100%) (Eksi *et al*., 2007).

During the erythrocytic development of *Plasmodium* parasites, proteases have been implicated in a wide range of processes. Two *Plasmodium falciparum* subtilisin-like proteases, PfSUB-1 and PfSUB-2), play a critical role. PfSUB-1 processes serine repeat antigen (SERA) proteins (also predicted to be proteases), secreted into the parasitophorous vacuole, in order to promote erythrocyte rupture and facilitate merozoite release (Yeoh *et al*., 2007, Arastu-Kapur *et al*., 2008, Blackman 2008). PfSUB1 also mediates the primary proteolytic processing of essential merozoite surface proteins, MSP1, MSP6 and MSP7 required for erythrocyte invasion (Koussis *et al*., 2009). PfSUB-2 is responsible for the processing of merozoite surface proteins that is vitally linked to erythrocyte invasion (Harris *et al*., 2005). Interestingly, PbSUB2 is shed from the invading ookinete in the cytoplasm of mosquito midgut epithelial cells (Han *et al*., 2000). Their orthologues in other *Plasmodium* species and in some members of apicomplexan family have been also identified. The homologues of PfSUB-1 and PfSUB-2 in *Toxoplasma gondii*, TgSUB1 and TgSUB2, are localized in the secretory organelles; micronemes and rhoptries respectively, whereas it is hypothesized they are involved in the processing of several micronemal and rhoptry proteins (Miller *et al.,* 2001, 2003).

A cascade of proteolytic events implicating proteases of cysteine, aspartic and metalloprotease families facilitates the degradation of hemoglobin to supply amino acids for the intraerythrocytic development of the parasite (Rosenthal and Meshnick 1996, Ersmark *et al*., 2006). It has been reported that proteolysis is also involved in the cleavage of cytoskeletal proteins of infected erythrocytes, a

process postulated to cause membrane instability, which, in turn, facilitates parasite release *in vivo* (Hanspal *et al.,* 2002).

On the other hand, some proteases are dispensable for intra-erythrocytic development of the parasite but are essential during sporogonic development. Disruption of the cysteine protease falcipain-1 results in reduced oocysts (Eksi *et al*., 2004); while a papain-like cysteine protease of *Plasmodium* SERA family, named egress cysteine protease 1 (ecp1), is involved in sporozoite egress from fully matured oocysts (Aly and Matuschewski 2005). Some protease genes have been found specifically expressed in mosquito stages e.g., a putative third *Plasmodium* subtilisin PfSub-3, the cysteine protease metacaspace II, the aspartic protease plasmepsin 6 (Wu *et al*., 2003) and ECP1 (Aly and Matuschewski 2005), while others, such as falcipain-1 (Eksi *et al.,* 2004) and SUB-2 (Barale *et al.,* 1999, Hackett *et al.,* 1999, Uzureau *et al*., 2004, Han *et al*., 2000) are expressed in both asexual and sexual stages.

The mechanisms by which parasite encoded proteases contribute to the invasion process in the mosquito are not clear and the putative targets are unknown. Generally, ookinete secreted or surface associated proteases could act as propellers interacting with surface moieties on host cells and/or extracellular matrix components pushing the ookinete deeper into the midgut epithelium. For instance, in the pathogenic bacteria *Streptococcus pneumoniae,* a surface-associated serine protease named choline-binding protein G has been implicated in host cell adherence (Mann *et al.,* 2006).

Alternatively, such proteases may activate ligands on parasite surface in a way similar to that observed in merozoites (Harris *et al.,* 2005). This process could lead to conformational changes, facilitating adherence of the parasite to the mosquito midgut cell, a requirement for the subsequent invasion steps (e.g. reorientation, motility etc). In *Trypanosome cruzi*, a serine protease, prolyl oligopeptidase (POP) which specifically hydrolyzes human collagen (types I and IV) and fibronectin, has been implicated in the parasite's adhesion to host cells and cell entry (Grellier *et al*., 2001).

Recent studies identified mosquito expressed proteases e.g. alanyl aminopeptidase (APN) as essential for the parasite to establish infection in the vector. Antibodies against these molecules or their depletion significantly blocked the parasite development in the mosquito (Lavazec *et al*., 2007, Dinglasan *et al.,* 2007a, 2007b, 2003, Zieler *et al.,* 1999, 2000b). These findings indicate that not only parasitic but mosquito proteases as well, may have a critical role in the mosquito midgut invasion process.

1.2.2. *Plasmodium* Subtilisins

Serine proteases of the subtilisin superfamily appear to be critical and conserved in apicomplexa. In addition to *Plasmodium*, they have been reported in *Toxoplasma gondii, Babesia divergens,* and *Neospora caninum, Cryptosporidium parvum* and *Cryptosporidium hominis* (Miller *et al.,* 2003, 2001, Louie *et al.,* 2002, and Montero *et al.,* 2006, Feng *et al*., 2007).

Of particular interest in the current study is *Plasmodium* SUB-2. The protease exhibits primary structural characteristics of a type 1 integral membrane protein (Barale *et al.,* 1999, Hackett *et al*., 1999, Uzureau *et al*., 2004). Conservation in the activation process and the subcellular localization of the protease in the micronemes of merozoites (Harris *et al*., 2005) mirrors a functional conservation of the protease in the two species, *P. falciparum* and *P. berghei*, in which it has been extensively studied. It has been demonstrated that in merozoites the protease is involved in the processing of multiple proteins among them the two essential proteins for invasion, AMA-1 and MSP-1 (Harris *et al*., 2005). The subcellular localization of SUB2 in ookinetes and sporozoites is still unknown; however, SUB2 seems to be expressed in all three invasive stages merozoites, (Hackett *et al*., 1999, Barale *et al.,* 1999, Uzureau *et al.,* 2004), ookinete (Han *et al*., 2000) and sporozoite (Barale, personal communication). Efforts to disrupt *SUB-2* in all three species where it was attempted (*P. falciparum, P. berghei and T. gondii*) have been proven fruitless (Withers-Martinez *et al*., 2004, Uzureau *et al.,* 2004) supporting its vital role and its choice as a drug target.

1.3. Serine proteinase inhibitors (Serpins)

In any living organism excess proteolysis or its ill-timed activation can cause substantial pathological problems therefore it is controlled in many different ways among which by serpins (Potempa *et al*., 1994). Serpins are found in multiple forms in numerous tissues of animals and plants as well as in

microorganisms (Gettins 2002). Especially serpins of the ovalbumin family share a suicide-substrate inhibitory potency which results into complexes of serpins and their irreversibly inactivated target proteases.

Generally, the primary physiological function of a serpin is the regulation of proteolytic processes mediated by serine proteinases. In mammals, they play roles in a diverse range of processes, including blood clotting, fibrinolysis, inflammation, complement activation, and turnover of extracellular matrix (Potempa *et al*., 1994). In some parasites and other pathogenic microbes, serpins have been hypothesized to not only regulate endogenous parasitic proteases but also to protect the parasites from the protease-rich environment they encounter in the course of their establishment. For instance, the expression of three serpins in *T. gondii* has been linked to the parasite's resistance to trypsin and hence its survival in a protease-rich environment such as the one encountered by the parasite in the small intestine (Lindh *et al*., 2001 Pszenny *et al*., 2000, 2002; Morris *et al*., 2002; Morris and Carruthers, 2003). In addition to the parasite/microbe survival in their harsh environments, host innate immune evasion and immuno-subversive roles have been also attributed to serpins. The latter is well illustrated in the case of Myxoma virus that encodes a serpin, SERP-1, which directly interacts with effector elements evading the host immune system (McFadden *et al*., 1995). From the other side, host derived serpins have been also suggested to play roles in the defense mechanisms against pathogens by simply inhibiting the proteases secreted by pathogens and abolishing the host tissue/cell colonization (Abraham *et al*., 2005, Kanost 1999). The reverse has

been also described. For example, in *Anopheles gambiae* mosquitoes, a mosquito-derived serpin, SRPN2, expressed in mosquito midgut epithelium facilitates midgut invasion by the malaria parasite *Plasmodium berghei* (Michel *et al*., 2005).

Serpins are primary inhibitors of serine proteases. However, a few cases of cross-class inhibition have been reported, where the serpins inhibit other classes of proteases mainly *in vitro*. The viral serpin CrmA and, to a lesser extent, PI9 (SERPINB9) inhibit the cysteine proteinase, caspase 1 (Komiyama *et al*., 1994, Simonovic *et al*., 2000), and the serpin SCCA11 (SERPINB3) neutralizes papain-like cysteine proteinases, cathepsins L, K, and S (Schick *et al*., 1998).

Extensive biochemical and biophysical studies have provided a comprehensive picture of the mechanism by which an ovalbumin type serpin recognizes and inhibits its target protease. The majority of ovalbumin type serpins are thought to act as “suicide inhibitors” that react only once with their target protease, forming SDS-stable complexes. Most ovalbumin serpins possess at least one C-terminal domain referred to as the reactive site (or centre) loop (RSL) (Figure 4). RSL is an exposed flexible stretch of residues that acts as bait for target serine proteases, mimicking their preferred cleavage site. Cleavage of serpins is associated with an increase in their stability that is required for the irreversible inhibitory mechanism. Upon cleavage of the scissile bond, the RSL transforms into an additional strand that is inserted into β-sheet A followed by conformational changes of the whole molecule. The protease first

forms a non-covalent Michaelis-like complex with the residues flanking the scissile bond. Attack of the scissile bond leads to the formation of an acyl-intermediate between the active serine residue of the protease and the P1 residue of the serpin and subsequent cleavage of the scissile bond (Ye and Goldsmith 2001, Silvermann *et al*., 2001, Zhou *et al*., 2001, Gettins 2002). At this point, the RSL is inserted into the β-sheet A, dragging the bound protease with it. Upon complete loop insertion, the catalytic center of the protease is distorted. *In vivo* the entire complex is marked for clearance (Huntington *et al*., 2000, Ye and Goldsmith 2001, Lawrence *et al*., 2000, Zhou *et al*., 2001).

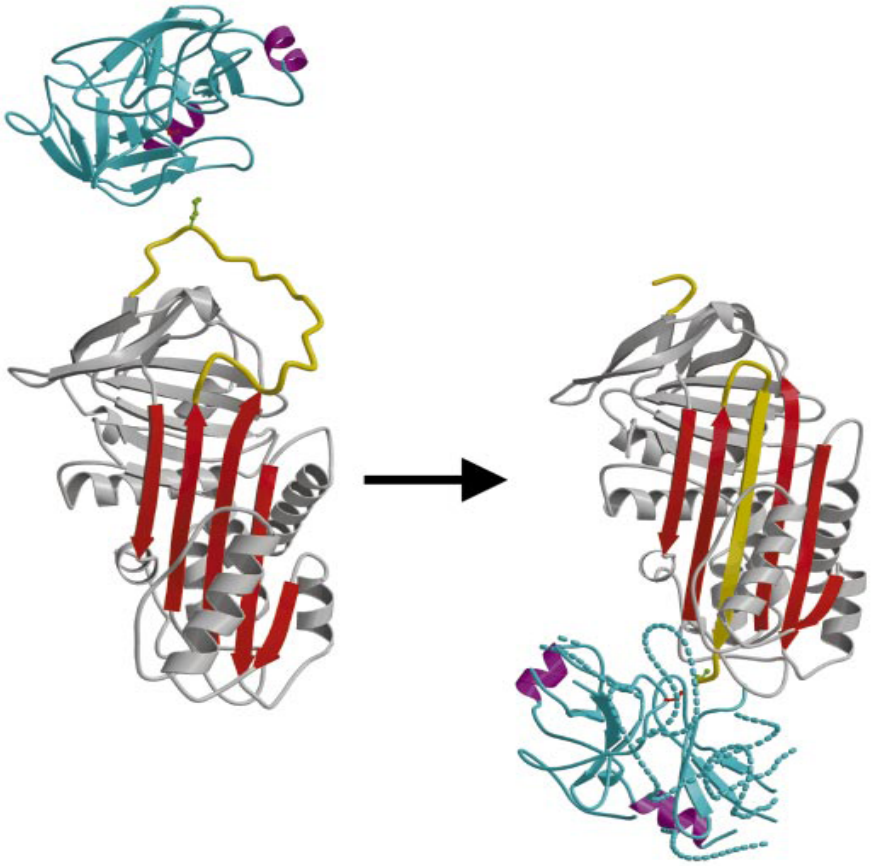

Figure 4: A model of a canonical serpin-protease complex formation. Ribbon depictions of native α1-antitrypsin8 with trypsin aligned above it in the docking orientation (left), and of the complex showing the shift of the P1 methionine of α1-antitrypsin, with full insertion of the cleaved reactive centre loop into the A-sheet (right). Regions of disordered structure in the complexed trypsin are shown as interrupted coils projected from the native structure of trypsin. Red, α1-antitrypsin β-sheet A; yellow, reactive-centre loop (RCL); green ball-and-stick, P1 Met (is left exposed to interact with its cognate protease); cyan, trypsin (with helices in magenta for orientation); red ball-and-stick, active serine 195 (Huntington *et al*., 2000).

In other types of serpin for instance, Kazal, Bowman-Birk and others (Krowarsch *et al*., 2003), a single domain of approximately 50-60 residues can be repeated 2-15 times to generate a multidomain (Figure 5), single chain inhibitor capable of interacting with several protease molecules.

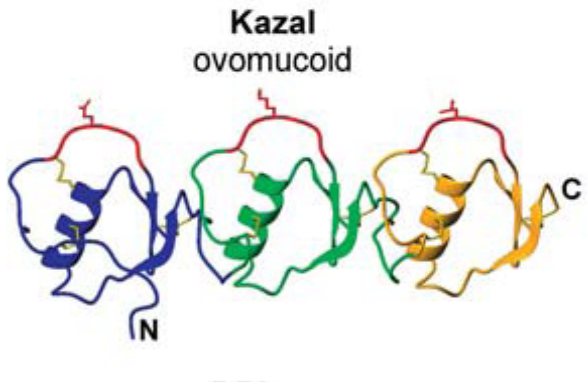

Figure 5: Example of a multidomain serine protease inhibitor: A three domain structure model of ovomucoid structure based on OMSVP3 crystal structure (2ovo); Binding loop and P1 side chain residue are marked in red under each domain, P1 is left exposed to interact with its cognate protease, N and C denote amino and carboxy terminals of the molecule respectively (Krowarsch *et al*., 2003).

Kazal inhibitors are among the most understood family. They are found in mammals (pancreatic secretory trypsin inhibitors, mammalian seminal acrosin inhibitors), birds (avian ovomucoids, chicken ovoinhibitor) (Laskowski and Kato 1980), leeches (leech-derived tryptase inhibitor) (Stubbs *et al*., 1997), insects (rhodniin) (van de Locht *et al*., 1995) and protozoan parasites (Pszenny *et al*., 2000, 2002; Morris *et al*., 2002, 2004; Morris and Carruthers, 2003 and Bruno *et*

al., 2004). Most Kazal serpins are characterized by their conserved stretch of six cysteine residues linked in three disulfide bridges making these molecules more stable. They can further be categorized as classical and non classical. The classical Kazal serpins have their last cysteine residue un-bridged, whereas in the non-classical the last cysteine residue participates in disulfide bridging (Laskowski and Kato, 1980). Each individual segment of a Kazal serpin contains an exposed RSL that binds to the target protease as a normal substrate. The P1-P1' residues remain close together upon cleavage of the scissile bond due to the rigid disulfide-bridged structure holding the RSL in place. The inhibitory effect is believed to be achieved through a very slow dissociation rate of the inhibitor from the protease's active site (Lu *et al*., 2001).

A rare member of Kazal serpin called NcPI-S was characterized in *Neospora caninum*, a member of Apicomplexan family. The serpin is a unique single domain molecule expressed specifically in tachyzoites of *N. caninum*. *N. caninum* is a protozoan parasite responsible for clinical disease in dogs, its definitive host and also a major cause of abortion in cattle with infection of horses and sheep also reported (Dubey 1999). NcPI-S is a proven strong inhibitor of subtilisin *in-vitro* (Morris *et al*., 2004, Bruno *et al*., 2004). Amino acid at P1 position of Kazal serpins has been described as the most important residue for dictating the specificity of inhibitor in this family (Laskowski and Kato, 1980). In contrast to other serpins, however, substitution of methionine with alanine at P1 position in the NcPI-S neither alters its potency against subtilisin nor inhibits elastase type of enzymes (Morris *et al*., 2004) for which most inhibitors with

alanine at P1 position have high affinity (Laskowski and Kato, 1980) suggesting that its inhibitory mechanism may be independent of the P1 residue. This phenomenon has been previously also observed in non-Kazal serpin, ecotin expressed in *E. coli* which also has methionine at P1 position whose mutation does not significantly affect its inhibitory functionality (Eggers *et al*., 2001).

1.3.1. Engineered serpins as tools for novel therapeutic strategies

In addition to natural protease inhibitors, molecularly engineered inhibitors emerge as promising therapeutic agents. Especially for ovalbumin serpins mutatagenesis within the RSL has produced novel serpins with altered specificity. Insertion of the minimal consensus sequence required for efficient processing by furin (Molloy *et al*., 1992) in the RSL of α1-antitrypsin generated a highly potent and selective inhibitor of furin called α1-PDX. In two separate studies, intracellular expression of the α1-PDX cDNA showed that α1-PDX blocks the furin-dependent processing of proteins such as HIV-1 gp160 and measles virus F0 and the production of infectious progeny (Anderson *et al*., 1993, Watanabe *et al*., 1995). In addition, when this bioengineered serpin was provided exogenously, it dramatically reduced the production of infectious human cytomegalovirus (HCMV) by blocking the furin dependent processing of the essential envelope proprotein, HCMV gB (Jean *et al*., 2000).

However, since many serpins have similar P1 residues but inhibit different proteinases, serpin's specificity may not be dictated solely by the RSL (Djie *et al*., 1997). The overall serpin scaffold has been shown to play also an important role

in the inhibitory activity and therefore the choice of an appropriate scaffold is important in the design of novel variants (Bottomley and Stone 1998). For example, limited amino acid modifications of serpin α1-proteinase inhibitor (α1-PI) outside RSL generated a specific and more potent inhibitor of factor Xa and factor IXa compared to their cognate inhibitor, antithrombin (Izaguirre *et al*., 2009).

Objectives of the study

In this study we validate two alternative methods in order to approach the function of essential proteases and/or protease groups. Both methods aim to disrupt proteolytic events during early sporogony without affecting asexual parasite development in the vertebrate host.

The first method targets a particular and essential protease, *Plasmodium* SUB2. We validate a method to accomplish stage specific disruption of Pbsub-2 by replacing its native promoter with Pbama-1 promoter. Pbama-1 is a protein exclusively expressed in late schizonts/merozoites and salivary gland sporozoites.

In the second approach we use serine protease inhibitors (serpins); an engineered variant of an ovalbumin serpin (LGI), and a single domain non-classical Kazal, NcPI-S, derived from the apicomplexan parasite *Neospora caninum*. Both serpins are expressed in a stage specific manner under CTRP (ookinete specific) and CSP (sporozoite specific) promoters respectively. Although those serpins were selected on the basis of their inhibitory activity against bacterial subtilisins *in vitro* (thus again SUB2 consisted our prime target), this second approach, in a more general way, validates the usefulness of *P. berghei* ookinetes, as a test tube system to screen for macromolecular protease inhibitors (MPIs), natural or engineered, putatively active against essential parasitic proteases.

CHAPTER 2:

MATERIAL AND METHODS

2.1 PLASMID CONSTRUCTIONS

2.1.1. Construction of CTRP-serpins (NcPI-S or LGI) targeting vector

For serpin expression in ookinete, targeting vectors were constructed by placing serpins (NcPI-S or LGI),N-terminally fused to signal peptide (sp) of *Pbsub-2* and *V5* epitope (SP-V5-NcPI-S/LGI) between zygote-ookinete specific 2.6kb CTRP promoter and the 0.7 kb *Pbs21* 3' UTR (Vlachou *et al*., 2004).

The signal sequence of the Pbsub-2 (SPSUB2) was amplified from *P. berghei* genomic DNA using primer pairs; SPsub-2F 5'-GGATCCATGTTGAGAACA-3' (BamHI site underlined) and SPsub-2R 5'-AAGCTTAGATCTCTGGCCATTGTG- 3' (HindIII and BglII sites underlined). The V5 epitope was amplified from a pIZ/V5-His vector (Invitrogen) using primers; V5for: 5'-AGATCTGGTAAGCCTATCCCTAAC-3' (BglII site underlined) and V5Rev: 5'-AAGCTTGGCCGGCCCGTAGAATCG-3' (HindIII and FseI sites underlined). SpSUB2 and V5 epitope tag fragments were separately cloned into pGEM-T (Promega). After sequence verification, V5 tag fragment was fused to spSUB2 as BglII – HindIII generating the pGEM-T/Sp-V5 plasmid. The two serpin ORFs (LGI and NcPI-S) were subcloned, in frame, as FseI – NotI fragments into the pGEM-T/Sp-V5 to generate plasmids pGEM-T/Sp-V5 (-LGI) or (-NcPI-S).

LGI was generated by molecular engineering of CAM. Briefly, the Reactive Site Loop (RSL) of CAM serpin was amplified by PCR using as template pQE30/CAM (Danielli et al., 2003), and as a forward primer, LGI_5' (5'GACCGGTATGTCGATGGATCTGCTCGGAATTGATCCGAAACATATGTTTA-3'), which includes the introduced nucleotide modifications and reverse primer

CAMB-H (5'GAAGCTTAGATCTTTAAACTTTAAGTATTTTA-3'). The 0.1-kb PCR fragment was digested with AgeI/HindIII and replaced the RSL of pQE30/CAM (an AgeI site exists just before the P1 residue), resulting in pQE30/LGI.

NcPI-S ORF (excluding the signal sequence) was amplified from a pQE30-NcPI-S plasmid (Morris *et al*., 2004) kindly provided by Dr. Carruthers (USA) using the primer set; NcPI-SF: 5'-GGCCGGCCCAAGGAAAATGAAG-3' (FseI site underlined) and NcPI-SR: 5'- GCGGCCGCTTAATTAATGTTTTTG-3' (NotI site underlined). The SP-V5-LGI or NcPI-S fusion minigenes were subcloned in the vector pCp~S (kindly provided by Dr. D. Vlachou) as BamHI – NotI fragments between the CTRP promoter and Pbs21 3' UTR. The resulting expression cassettes CTRP-SP-V5-(NcPI-S or LGI)-3'UTR were subsequently inserted into the pDEF-TgDHFR/DSSU transformation vector (kindly provided by the Leiden parasitology group)) as EcoRV fragments to generate the knock in targeting plasmids, [CTRP-SP-V5-(NcPI-S OR LGI)] pDEFSSUToxo. These final targeting plasmids contain a *dssu-rrna* fragment that serves as a target for integration and a pyrimethamine resistant gene (*Toxoplasma gondii dhfr/ts* under the control of the *pbeef-1αa* promoter). Prior to their use for transfection, the targeting plasmids were linearized at unique ApaI ([CTRP-SP-V5-(NcPI-S)]pDEFSSUToxo) or SacII ([CTRP-SP-V5-(LGI)]pDEFSSUToxo) restriction sites within the *d-ssu-rrna* sequence.

2.1.2. Construction of the CSPpromoter-NcPI-S knock in targeting vector

The *SP-V5-NcPI-S* sequence was amplified from the genomic DNA of NcPI-S cl3 transgenic line and inserted into pSEO3 knock in transfection vector (kindly provided by Dr. K. Matuschewski, Berlin) as BamHI fragment, in frame with circumsporozoite protein (*CSP)* promoter generating the knock in targeting vector. In addition to the *CSP* promoter, the vector contains also pyrimethamine selectable marker *tgdhfr/ts*. Prior to its use for transfection, targeting vector [*csp-SP-V5-NcPI-S-3'utr*]*Pbdhfr* was linearized at a unique KpnI restriction site within the *CSP* promoter sequence.

2.1.3. Construction of pAKM-14-*sub2* promoter knock in vector (SUB2 promoter exchange vector via single cross over recombination)

A 2kb sequence starting with the initiator ATG of the *Pbsub-2* ORF was PCR amplified from *P. berghei* gDNA using the primer set; Pbsub-2fwd: 5'-GGATCCATGTTGAGAACATTTTATG-3' and Pbsub-2rev 5'-ACTAGTGCGCTCAAAATTTTTTCTTCTATTTCAG-3' (BamHI and SpeI sites are underlined). The resulting fragment was subcloned into pGEM T-easy vector (Promega) and sequenced. Upon sequence confirmation, it was subcloned as BamHI and SpeI, in frame with PbAMA-1 promoter, in the *P.berghei* transfection vector pAKM-14 (kindly provided by Dr. K. Matuschweski, Berlin), generating pAKM-14-*sub2* vector. The vector contains in addition *Toxoplasma gondii dhfr/ts* as a selectable marker for resistance to pyrimethamine. For parasite transfection

pAKM-14-*sub2* vector was linearized at a unique BlpI restriction site within the 2 kb Pbsub-2 sequence.

2.1.4. Construction of promoter knock out vector pAKM-14-*sub-2p* (SUB2 promoter exchange vector via double cross over recombination)

For the construction of the vector used to disrupt and replace the native putative *Pbsub-2* promoter via double cross over recombination, a second targeting region of 1.16kb upstream of the putative native *Pbsub-2* promoter was PCR amplified from gDNA of *P.berghei* using the primer set; sub-2utrF: 5'-CCTGCAGGTACCCATCGCTTACATTCCCTTTTGCTTATCC-3', and sub-2utrR: 5'-CCTGCAGGCTGAAACATGTAATAGTTACTGCC-3' (SbfI, and Kpn1 sites are underlined). The fragment was cloned as SbfI into pAKM-14-sub2 vector upstream of the 5' UTR of *Pbdhfr-ts* (Figure 6A) generating the final promoter knock out vector. pAKM-14-*sub-2p* was sequentially digested at unique restriction sites first with BlpI within the 2 kb Pbsub-2 sequence and then with KpnI upstream of the dhfr 5' UTR releasing the targeting cassette for transfection.

2.2. Parasite techniques

2.2.1. Parasite maintenance

All parasites, the original *P. berghei* ANKA strain and its derivative GFP expressing strain 507cl1 (Janse *et al*., 2006a) used as recipient strains, and the derived transgenic parasites, were maintained in 6-10 week-old Theiler's Original

(OlaTO) mice by serial mechanical passages of infected blood intraperitoneally. Parasitemia of infected mice was examined by light microscopy at 1000X in blood-smears counterstained with Giemsa solution (Sigma). One to two hundred ml of blood of an infected mouse (parasitaemia 5–15%) was always injected into a naive mouse. A maximum of 10 passages were performed before a fresh aliquot of blood stage parasites was defrozen. To increase parasite loads, mice were pretreated with phenylhydrazine (6 mg ml-1, 200 ml intraperitoneal injection), 2-3 days before the passage. Only mice with erythrocyte infection rates between 10%-20% and >10 exflagellation centers per field (400X magnification) in 3–4 days post infection were used for mosquito infections and ookinete cultures.

2.2.2. *P. berghei* transfection and genotyping

Two strains have been used as recipients of transfections; the native *P. berghei* ANKA and its genetically modified derivative strain 507cl1 which constitutively expresses GFP (Janse *et al*., 2006a). Transfection procedure was performed as previously described (Janse *et al*., 2006b). Briefly, *in vitro* culture mature schizonts containing fully developed merozoites were purified on a Nycodenz gradient. Approximately 0.5-1x10^7 of the purified schizonts were transfected with 5-10μg DNA of the desired linearized transfection construct, using program U-33 of the Nucleofector® device (Amaxa GmbH).

Transfected schizonts were immediately intravenously injected into a mouse. Positive selection of transgenic parasites was performed by treating the

mice with pyrimethamine, provided in the drinking water 24-30hrs after transfection, for a period of 5-7 days. Selected parasite populations were checked for the plasmid integration into the expected locus either by Southern analysis of parasites' chromosomal DNA separated on 1% agarose gel under pulsed-Field Inversion Gel Electrophoresis (PFIGE) system or by genomic PCR. For PFIGE –Southern, DNA was transferred onto Hybond-N+ nylon transfer membrane (Amersham) and then hybridized with *P. berghei* radio-labeled *dhfr-ts* 3'UTR probe as described (Janse *et al*., 2006b).

Genomic PCR genotyping was performed in all cases in the resulting transgenic clones. Briefly, gDNA derived from the clones and wt control was isolated using QIAamp DNA kit and used as template in the PCR reactions. The PCR products were run on 1% agarose gel and visualized and photographed under UV. In the PCR genotyping of CTRP-LGI and CTRP-NcPI-S clones the following integration specific primer sets were used : L665: 5'-GTTGAAAAATTAAAAAAAAAC-3'; L740: 5'-CTAAGGTACGCATATCATGG-3' (Franke-fayard *et al*., 2004). Clone purity as well as the position of integration (into *c* or *dssu* locus), were checked by using forward primers; *cssu* specific L270: 5'- GTGTAGTAACATCAGTTATTGTGTG-3' (van Spaendonk *et al*., 2001) and *dssu* specific L260: 5'- ATACTGTATAACAGGTAAGCTGTTATTGTG -3' in combination with the reverse L740 primer common to both loci.

In the case of CS-NcPI-S transgenic parasites, clones were genotyped by diagnostic PCR using two integration specific primer combinations: A) Tgfwd: 5'-CCCGCACGGACGAATCCAGATGG3'/CStest:5'GCAAGTAATCTGTTGACTGTA

TTTCG-3' and B) CS_{wt}: 5'-CCAACCTTAGGAACTAATATTTG-3'/NRev: 5'-GGATCCTTAATGTTTTTGCTTGGATTTATCC-3' each combination specific for the right and the left part of the recombinant site. The purity of the clones was verified using the wt specific primer combination; CS_{wt}/ CStest.

Clones derived from Pbsub2 promoter exchange experiments, either via single or via double cross over, were genotyped using integration specific primer sets F1:5'-TTTATAACTCATGAAAGTTG-3 and 3R: 5'-TGTGTCTATATTACCAACTC-3'; F4: 5'-GGAATATAATTCAAAATGATTTGACAC-3' and 5R: 5'-CATCTTCATTCTCTCCTGTTTCTG-3'. Purity of the clones was verified by using the WT specific primer combination: F1 and 2R: 5'-GGCGTCTTTCATTTTCTTCAGC-3'.

2.2.3. *In-vitro* ookinete cultures

For ookinete culture and purification, we adopted a previously described protocol (Winger *et al*., 1988, Paskewitz and Shi 2005) with minor modifications. Briefly, blood from infected mice was collected by cardiac puncture into a heparinized syringe 3-4 days post-infection. Blood was diluted 1:10 in ookinete culture medium and the mixture was incubated for 18-24 hrs at 19°C (Sinden 1997, Al-Olayan *et. al*., 2002b). To purify the ookinetes from the 18-24hr ookinete culture, the culture was spun at 2000rpm, room temperature for 10 minutes and the supernatant carefully removed without disturbing the pellet. The pellet was then resuspended in 0.9% (w/v) cold Ammonium chloride (50ml per

0.8-1ml of infected blood added in the culture), followed by incubation on ice for 20-30 minutes in order to lyse red blood cells. The culture was then centrifuged at 4°C, 1500rpm for 5 minutes, followed by 2-3 successive washes of the ookinete pellet in phosphate buffer saline (PBS). The resulting ookinete pellet was resuspended in appropriate volume of PBS and counted on a hemocytometer. For higher enrichment of ookinetes, (with minimum asexual stage contamination), resuspended ookinetes were further purified by magnetic beads (Dynabead®) coated with the 13.1 monoclonal antibody to P28 (Siden-Kiamos *et al*., 2000). However, macrogametes and zygotes, that also express P28, are present to a varying degree in this purified ookinete preparation.

Ookinete culture medium: Dissolve16.4g RPMI-1640 with Hepes (25mM) and glutamine (2mM, Sigma), 2g sodium bicarbonate ($NaHCO_3$), 10ml penicillin (5000U/ml)/Streptomycin (5mg/ml), 50mg hypoxanthine, adjust pH to 8.0 with NaOH and bring the total volume to 1 liter with sterile water, filter sterilize through 0.4µm filter and add 10% foetal calf serum just before use.

2.2.4 Immunofluorescence staining (IFA) of *in vitro* cultured ookinetes

Immunofluorescent staining of ookinetes was carried out as described (Dessens *et al*., 1999) with slight modifications. Briefly, *in vitro* cultured ookinetes (24hrs) were spotted on microslide chambers, air dried, and fixed in 4% paraformaldehyde for 10 min at room temperature. The fixative was washed out and ookinetes were blocked with PBT solution (1% BSA/1% Triton-100/PBS) for 30min-1 hour at room temperature. CTRP-LGI ookinetes were stained with a

purified rabbit anti-SRPN10 antibody (dilution: 1: 333; Danielli *et al*., 2003), while CTRP-NcPI-S ookinetes either with a monoclonal anti-V5 (dil: 1:100; Invitrogen) or with an anti-NcPI-S mouse serum (dil: 1:100; gift of Dr. V. Carruthers) diluted in PBT for 1 hour at RT or overnight at 4°C. After three washes of 10 minutes in PBT, samples were incubated with Alexa 555-conjugated goat anti-rabbit or anti-mouse IgG (Invitrogen) for 1 h at room temperature, washed three times in PBT, and finally mounted in vectorshield mounting medium (Molecular Probes) and examined under a fluorescence microscope. Parental wild type ookinetes treated under similar conditions were used as controls.

2.2.5 *In vitro* ookinete motility assay

Ookinetes were cultured *in vitro* as described above. After 24 hours, 1ml was taken from the culture and spun down for 5 minutes at 500rpm. Most of the supernatant (~800 µl) was removed and the pellet was resuspended in the remaining medium and put on ice for a few minutes. Matrigel was defrost overnight at 4°C, gently inverted and kept on ice. Using a cut tip, 20 µl of the resuspended culture and an equal volume of matrigel were gently but thoroughly mixed. Six microliters of this mixture were spotted on a glass slide, covered with a cover slip, sealed and left to set at room temperature for 30 minutes to 1 hour. Samples were monitored at 630X magnification in a microscope fitted with a CCD camera by taking a series of pictures, at an interval of 10 seconds for ~5-10 minutes. Movies were reconstructed from isolated pictures and ookinete motility of NcPI-S expressing strain was compared to that of the parental strain.

2.2.6. Sporozoite gliding motility assay

Approximately ten thousand salivary gland sporozoites were seeded into BSA coated labtek chamber slide (10,000/well) and incubated at 37°C for 15-30 minutes. The supernatant was gently aspirated. As they glide on a solid support *Plasmodium* sporozoites shed circumsporozoite protein trails (Stewart and Vanderberg 1988). The adherent sporozoites and the shed CSP trails were fixed in 4% paraformaldehyde for 10 minutes at room temperature. The samples were then washed with washing solution (1% FBS, 0.01% saponin in PBS) and sporozoites were permeabilized with 0.05% saponin in PBS for 5 minutes on ice. After a quick wash with washing solution, the samples were blocked overnight with 10% FBS in PBS at 4°C. After the blocking, samples were washed once again and incubated with a mAb against *P. berghei* circumsporozoite protein (α-PbCSP, 1:1000) (Potocnjak *et al*., 1980). Staining was revealed using Alexa Fluor 555–conjugated anti–mouse antibodies (Molecular Probes).

2.3. Mosquito infections and related techniques

2.3.1. Mosquito rearing

Mosquito infection experiments were done with the susceptible G3 strain of *Anopheles gambiae.* The mosquitoes were reared in an insectary maintained at 28°C, with a relative humidity of 80% and a 12/12 h light/dark cycle. Larvae were raised in distilled water and fed with powdered cat food. Adults were fed on 10% (w/v) sucrose solution (through cotton soaked in sucrose solution). Female

mosquitoes were allowed to feed on anesthetized BALB/c mice to maintain the culture.

2.3.2. Mosquito infections

Gametocyte feeding was performed as follows: adult mosquitoes 4–7 days post-emerged were starved of sucrose for a period ranged 6–16 h, and then fed on infected anaesthetized mice for 15 min. Care was taken to use mice with matched parasitemias.

In ookinete feedings, mosquitoes were fed on *in vitro* cultured and purified ookinetes for 40 minutes as described (Sinden, 1997). Purified ookinetes were mixed with naïve mouse blood in a concentration ranged 3000-5000 ookinetes/µl and were provided to mosquitoes through home made artificial membrane feeders. During the feeding, the ookinete mixed blood meal in the feeders was maintained at 37–39^{o}C.

In both cases, unfed mosquitoes were removed from the cages 24 h following the feed. The remaining mosquitoes thereafter were maintained at 19°C, 70-80% humidity to allow parasite development until midgut dissections. In most of the experiments, ookinete to oocyst transition was used to estimate parasite infectivity. Mosquito midguts were dissected on day 10 post-feeding and fixed for 45 min to 1 hour before visualized under fluorescent or light microscope. Both intensity (number of oocysts seen on a whole mosquito midgut) and prevalence (% of mosquito infection) have been used as parameters of transmission. Each experiment was repeated at least twice.

2.3.3. Statistical data analysis

Independent-Sample *t*-test comparisons were used to compare the oocysts means of the transgenic strains with the parental strain. Statistical analyses were performed using SPSS 11.5 and statistical differences were considered significant at a probability of $P < 0.001$ and 0.05 for NcPI-S and LGI expressing strains respectively.

2.3.4. Midgut Immunofluorescence (IFA) staining

Infected midguts were dissected in ice-cold phosphate buffered saline (PBS). The tissue was prefixed for 90 seconds in ice-cold 4% formaldehyde in PBS, transferred to ice-cold PBS and cut open longitudinally with micro-dissection scissors. The luminal contents (blood meal) were carefully removed and the epithelium fixed for another 45–60 min in 4% formaldehyde at room temperature. The samples were washed for 3x10 minutes in PBS, blocked and permeabilized for 1 h 30 minutes in PBT (1% bovine serum albumin, 0.1% Triton X-100 in PBS) at room temperature.

For detection of ookinetes and the invasion process, tissues were incubated overnight at 4 °C with primary antibodies diluted in PBT, followed by 3 washes of 10 minutes each with PBT and by incubation with secondary antibodies for 1 hour at room temperature during which the nuclei were counterstained with TOPRO 3 (Molecular Probes). Midguts were washed three times in PBT for 10 minutes before being flattened and mounted in vectorshield mounting medium (Molecular Probes). All samples were analyzed using a Zeiss

confocal microscope with BioRad lasers under 100 and 630X magnification. Primary antibody dilutions: 1:333 SRPN10; 1:100 P28; secondary antibodies; Alexa 488-, Alexa 555-conjugated anti-mouse, anti-rabbit IgGs (Molecular Probes).

2.3.5. Isolation of midgut and salivary gland sporozoites

For the dissection of midgut and salivary gland sporozoites, infected mosquitoes were maintained at 19°C till day 14 and 20 respectively. Dissections were performed in RPMI-1640 supplemented with Hepes (25mM) and glutamine (2mM, Sigma) and 3% BSA. Dissected midguts/salivary glands were mechanically crushed, spun down for 3 min at 1000rpm, at 4°C and the supernatant enriched with sporozoites was transferred to clean tubes. The pellet was resuspended in the RPMI-1640, 3% BSA and crushed once more to liberate some sporozoites trapped in the debris. The two supernatants were combined, diluted 1:10 and sporozoites counted on a hemocytometer using a light microscope.

2.3.6 Bite back infections

Approximately 20 mosquitoes infected with either transgenic or wt parasites on day 20 post infection were allowed to bite naïve anaesthesized Balb C mice for 15-20 minutes. The mice parasitemia was monitored everyday on Giemsa stained thin blood films. Blood was drawn from the infected mice by

cardiac puncture, genomic DNA extracted from the resulting parasites and analyzed by diagnostic PCR for the presence of wild type of transgenic parasites.

2.4. Protein analysis

2.4.1. Western analysis of protein extracts

In most of the experiments, *in-vitro* cultured ookinetes purified by erythrocyte lysis were the main protein source. Purified ookinetes were usually lysed in cold RIPA buffer (50Mm Tris-HCl Ph 8.0, 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate and 0.1% SDS) on ice for 30-40 min. In the experiment reported in section 4.4.2, 24 hrs infected mosquito midguts were crashed in PBS, and purified ookinetes resuspended in cold RIPA buffer and sonicated. In both cases, lysates were spun in a microfuge for 10 minutes at 10000 rpm, 4°C and the supernatant was further boiled in 1X sample buffer for 5 minutes. In the experiment reported in section 3.5 in order to minimize contamination from schizonts, *in-vitro* cultured ookinetes were further purified using 13.1 monoclonal antibody against P28 coupled to magnetic beads (Dynabead®). In this case ookinetes were boiled directly into protein loading buffer for 5 minutes, the extract resolved in 10% SDS-PAGE gel. Isolated sporozoites boiled directly in protein loading buffer were the starting material for the experiment reported in section 4.5.3.

All the above extracts were fractionated on gels of appropriate acrylamide concentrations according to the sizes of the proteins of interest for detection: 12% (LGI), 9% (CTRP) and 15% (NcPI-S), 15 % (SOAP), 15% (WARP), 10%

(CSP), 10% (SUB2). Upon electrophoresis, proteins were transferred onto nitrocellulose membranes by electroblotting following standard procedures. Blotted membranes were blocked in PBS-T (PBS containing 0.05% Tween 20) containing 5% skim milk, and incubated with primary antibodies diluted in 1% milk. Primary antibodies used: SRPN10/LGI (1: 1000; Danielli *et al*., 2003), anti-CTRP (1:1000), NcPI-S (1: 1000, Morris *et al.,* 2004), anti-V5 (1:3000; Invitrogen), SOAP serum (1: 1000; Dessens *et al*., 2003), P28 (1: 1000), CSP (1:8000; Potocnjak *et al*., 1980), SUB1 (1: 500; unpublished, gift from Dr. J. C. Barale). Specific signals were detected, following incubation with the respective secondary horseradish peroxidase-conjugated secondary antibodies (Invitrogen) appropriately diluted in PBS-T, by using a chemiluminescence kit (ECL detection system, Pierce Biotechnology Inc. or the more sensitive Femto ECL detection system, Pierce Biotechnology Inc.) according to manufacturers instructions.

2.4.2. Fractionation of whole ookinetes

The fractionation procedure was adopted from (Patra *et al*., 2008) with some modifications. Briefly, five million ookinetes freshly purified from the *in vitro* ookinete culture were resuspended in 50 µl of 10 mM Tris-HCl pH 8.5, incubated for 1 hour on ice to lyse the ookinetes, centrifuged for 30 minutes at 10^5 rpm at 4°C and the supernatant was transferred to a new tube (soluble lysate). The pellet was resuspended in 50 µl of 0.1 M Na_2CO3 pH 11.5, incubated on ice for 1 h, centrifuged at 10^5 rpm for 30 min at 4°C, the supernatant was transferred to a new tube (salt-eluted membrane fraction). The final remaining pellet was

resuspended in 50 µl PBS (membrane insoluble fraction). Appropriate volume of protein loading buffer was added to each fraction, boiled for 5 minutes and resolved on 15% SDS-PAGE gel. All the remaining western blotting steps were carried as described earlier with NcPI-S detected with anti-V5 monoclonal antibody (Invitrogen).

CHAPTER 3: RESULTS AND DISCUSSION

PROMOTER EXCHANGE IN THE PbSUB2 GENE LOCUS

Plasmodium subtilisin-like 2 (Psub-2) expression was first reported in the late schizonts and merozoites and shown to facilitate the invasion process of erythrocytes by merozoites (Barale *et al*., 1999, Hackett *et al*., 1998, Uzureau *et al*., 2004, Harris *et al*., 2005). Transcripts of PfSUB2 were detected in gametocytes (Florens *et al*., 2002), while PbSUB2 protein is detected in the invading ookinetes (Hans *et al*., 2000). Moreover, according to personal communication, PbSUB2 is also expressed in sporozoites (J. C. Barale).

Efforts to disrupt SUB2 gene through gene knock-out technology in *P.falciparum* (Withers-Martinez *et al*., 2004) and *P.berghei* (Uzureau *et al*., 2004) have failed due to the vitality of SUB2 for merozoite invasion. Moreover, efforts to express PfSUB2 protein in an active form in heterologous systems, in order to establish *in vitro* activity assays have been proven fruitless (Dr. M.J. Blackman pers. com.). Up to date we miss important information about the exact role of SUB2 during parasite invasion, while putative SUB2 substrates have been only suggested based on indirect biochemical evidences.

Like in merozoite invasion, SUB2 may play similar critical roles in the invasion processes of midgut epithelium by ookinetes and salivary glands by sporozoites. Therefore, the study of its role in the later processes may aid us to uncover some common functions and similarities.

To investigate a putative implication of PbSUB-2 in the gametocyte to oocyst transition, we first generated transgenic parasites by inserting a linearized transfection vector, containing *Pbama-1* promoter and a modified *Toxoplasma gondii* dihydrofolate reductase/thymidylate synthase (*Tgdhfr/ts*) gene, (conferring

resistance to the antimalarial drug pyrimethamine), into PbSUB-2 locus by single cross-over homologous recombination (Figure 6) as described (Menard and Janse 1997; Waters *et al*., 1997). Transfection vector contained in addition a 2Kb fragment derived from the start of *Pbsub-2* ORF that served for homologous recombination. The vector was linearized within the 2Kb SUB2 sequence and inserted at nucleotide 879 of the Pbsub-2 ORF sequence, resulting into reconstitution of a complete Pbsub-2 gene and a truncated copy of the gene's ORF. In this way *Pbama-1* promoter was placed in frame with the complete copy of SUB2 ORF replacing the native *Pbsub-2* promoter (Figure 6C). Since *Plasmodium* apical membrane antigen-1 (ama-1) is exclusively expressed in salivary gland sporozoites (Silvie 2004, Srinivasan *et al*., 2003) and late schizonts/merozoites (Narum and Thomas, 1994; Triglia *et al*., 2000), by following this strategy we expected to restrict the expression of Pbsub-2 in merozoites where it is known to be vital (Uzureau *et al*., 2004) and abolish it in the later stages, particularly gametocytes and ookinetes. .

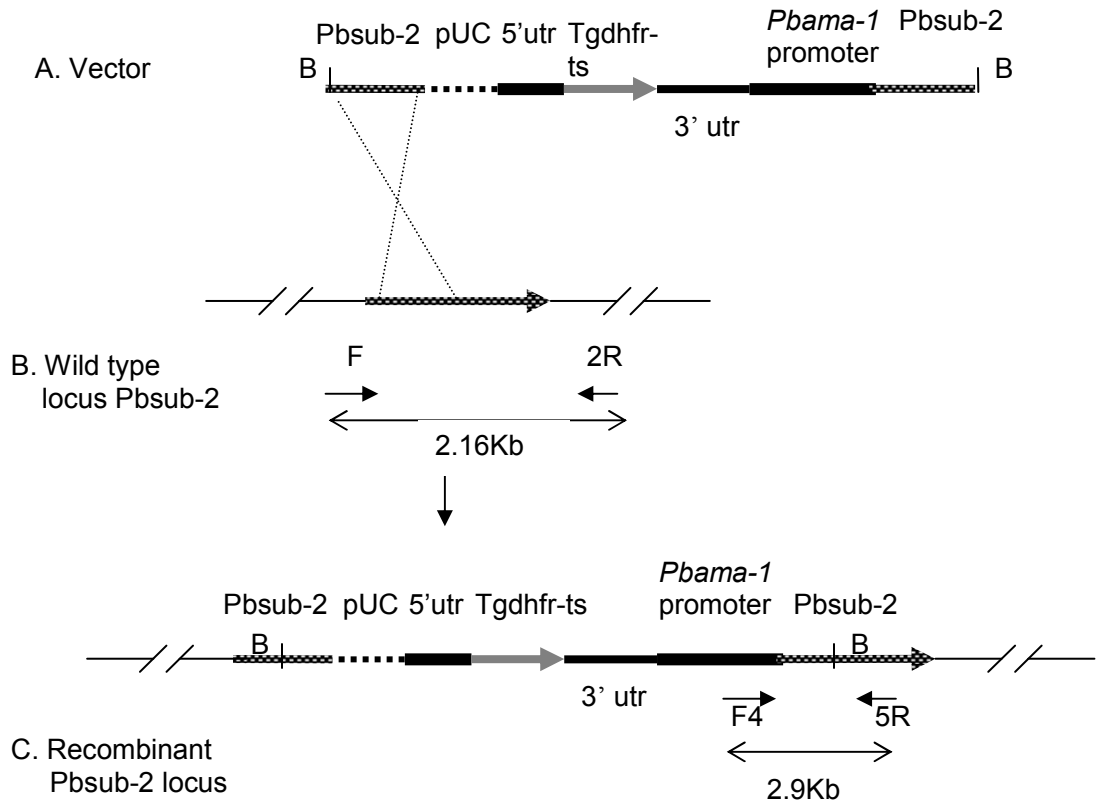

Figure 6: Replacement of Pbsub-2 promoter with that of Pbama-1 by a single cross-over homologous recombination

A. The targeting vector used to integrate Pbama-1 promoter into Pbsub-2 locus in frame with Pbsub-2 ORF, replacing the endogenous Pbsub-2 promoter. The vector contains pyrimethamine-resistant *tgdhfr-ts* selectable cassette for selection of transgenic parasites and Pbama-1 promoter. 2Kb of Pbsub-2 ORF was also incorporated into the transfection vector as homologous targeting sequence. The vector was linearized at a unique restriction site B (BlpI) within the 2kb of Pbsub-2 ORF.

B. Schematic representation of Pbsub-2 target wt locus. Arrows indicate primer combination for wt-specific product.

C. Schematic representation of the recombinant Pbsub-2 locus. A single cross over integration event resulted into reconstitution of Pbsub-2 gene in frame with Pbama-1 promoter. An incomplete copy of Pbsub-2 gene (the first 2Kb) in frame with the native Pbsub-2 promoter separated from the reconstituted Pbsub-2 gene by plasmid and tgdhfr-ts cassette sequences. Arrows denote the primers for integration specific product.

3.1. Genotyping of the transgenic parasites

The parasite population was cloned by limited dilution as described (Waters *et al*., 1997) to separate transgenic parasites from the wt, and the resulting pyrimethamine resistant clones were analyzed by diagnostic PCR. Using an integration specific primer combination, F4/5R, a characteristic 2.9kb product was readily amplified from the transgenic parasite gDNA but not from the wild type (Figure 7A). This clearly demonstrated not only the integration of the vector in the expected Pbsub-2 locus but in addition insertion of Pbama-1 in frame with the Pbsub-2 coding sequence as shown in the representative clone (Figure 7A). However, all the clones harbored a fraction of wild type parasites as demonstrated by the amplification of a 2.16kb wt specific fragment using a primer pair specific for the wild type Pbsub2 locus (Figure 7B). Even extended selection with pyrimethamine did not clear the wild type parasites.

The simplest interpretation for this persistent wt population in the parasite clones is reversion back to the wild type configuration of the locus. A consequence of the replacement strategy initially followed is that the region of

homology is duplicated in the recombinant locus, and the two copies can recombine resulting to the removal of the entire inserted plasmid or part of it through genomic rearrangement.

Reversions are commonly seen in transgenic parasites generated via a single cross-over homologous recombination in *P.berghei* model system (Menard and Janse 1997). Alternatively, the wild type product may derive from wild type parasites with unaffected Pbsub2 locus but harboring episomal DNA, and therefore resistant to pyramethamine selection. .

To exclude the later possibility, we conducted a plasmid rescue experiment. We transformed *E. coli* DH5α bacteria using total DNA derived from propagated transgenic parasites. In the case of episomal DNA presence, ampicillin resistant bacteria colonies should be recorded and such colonies were not obtained (data not shown).

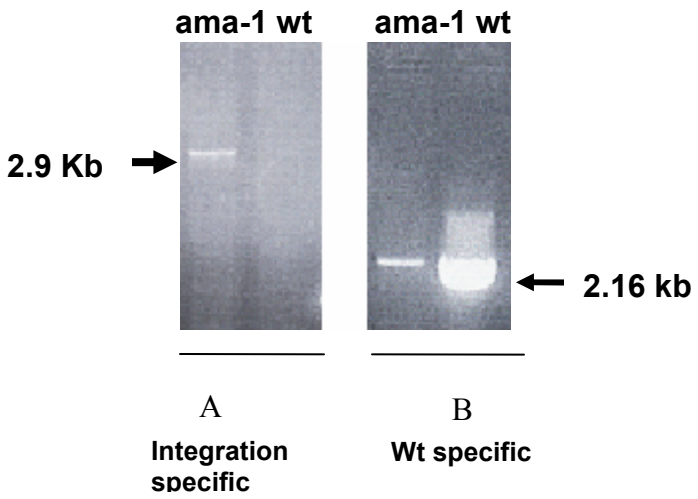

Figure 7: Integration-specific PCR genotyping of Pbama-1 transgenic parasites.

A. Integration specific PCR. AMA-1 and wt denote the transgenic line and the wt parental line respectively. Amplification of 2.9kb, the integration specific product using the primer combination shown in figure 6C indicates integration into the desired locus. This is supported by the absence of this product in the wt parasites.

B. Wild type specific PCR: 2.16 Kb denotes the wild type specific product amplified using the primer combination shown in figure 6B. Amplification of this product also in the Pbama-1 transgenic parasites indicates the presence of wt parasites in the clone. All the clones tested harbored the wt parasites.

3.2. Construction of promoter targeting vector

In some cases, the problem of reversions can be circumvented by using DNA vectors integrating via double cross-over homologous recombination. Therefore we generated new transgenic *P. berghei* parasites using a replacement vector. This vector contains two distinct regions homologous to the target Pbsub2 locus separated by a selectable marker. Integration occurs via a double cross-over between the vector inserted sequences and their pair homologous sequences within the *Pbsub-2* locus, leading to a replacement of the sequence delimited by the fragments incorporated into the vector. The absence of sequence duplication in the recombinant locus makes the transgenic line more stable. We used a transfection vector in which the *Tgdhfr-ts* gene cassette and the 1.65kb *Pbama-1* promoter were bordered upstream by a 1.14Kb fragment derived from the upstream region of the *Pbsub-2* putative promoter, and downstream by a 0.87kb sequence derived from the start of Pbsub-2 ORF (Figure 8A). Upon integration of this plasmid via double cross-over recombination into the Pbsub-2 locus, ≈1kb of the putative native Pbsub-2 promoter (Figure 8C) was replaced with the Pbama-1 promoter.

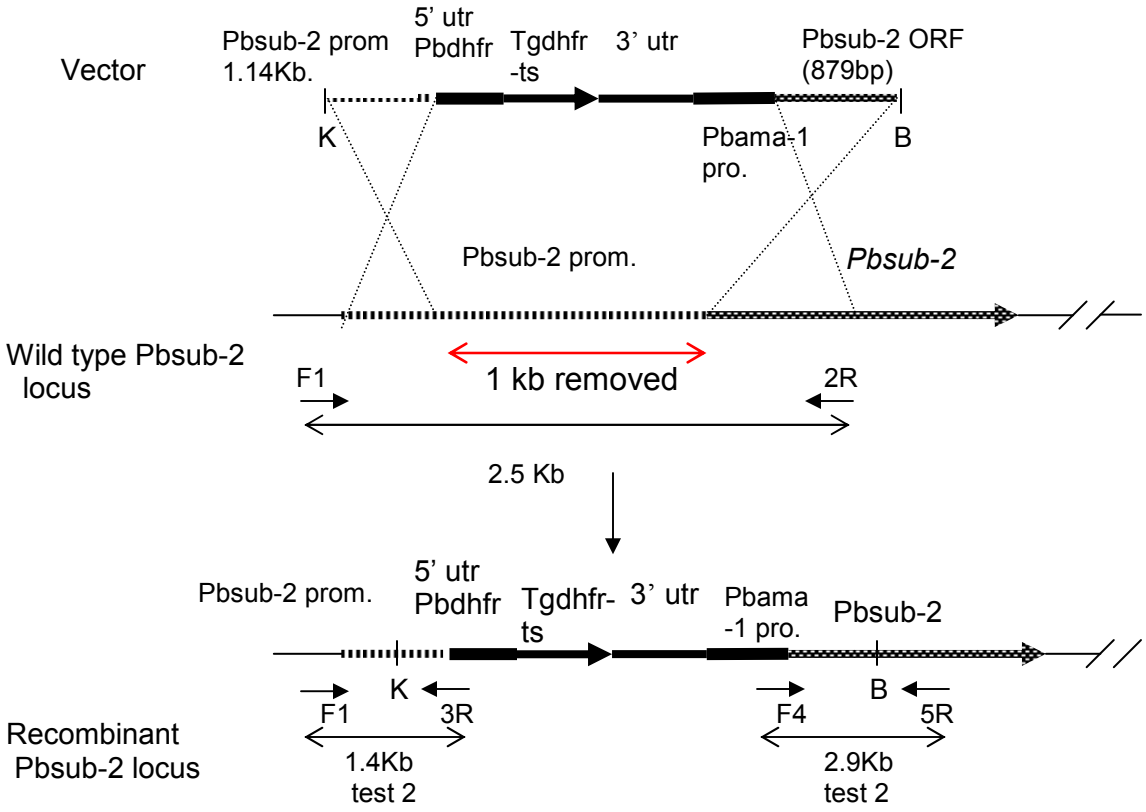

Figure 8: Targeted disruption and replacement of Pbsub-2 promoter with that of Pbama-1 in a double cross-over homologous recombination event

A. The targeting vector used to integrate Pbama-1 promoter into Pbsub-2 locus in frame with Pbsub-2 ORF, replacing the endogenous Pbsub-2 promoter. The targeting vector contains a selectable marker cassette, Pbama-1 promoter, and two distinct targeting sequences. The targeting cassette was released from the vector by digestion with unique restriction enzymes BlpI (B) within the first 2kb of Pbsub-2 ORF and KpnI (K) generating an upstream 1.14 kb of putative Pbsub-2 promoter and 879bp of the Pbsub-2 ORF on the downstream separated by the pyrimethamine-resistant *tgdhfr-ts* selectable cassette and the Pbama-1 promoter.

B. Schematic representation of WT Pbsub-2 locus. Integration of the targeting vector into the Pbsub-2 gene locus was by double crossover. Primers, F1 and 2R for the expected wt-specific product; the product size are indicated by arrows. The 1Kb excised from the locus is denoted by a red arrow.

C. Schematic representation of recombinant Pbsub-2 locus. This recombination event resulted in the disruption of the putative Pbsub-2 promoter, replacing it with that of Pbama-1 and introduction of the pyrimethamine resistance gene; Tgdhfr. Arrows denote the primers for the expected integration specific products and their sizes. Test 1 and test 2 indicate integration specific PCR products using the indicated primer sets F1/3R and F4/5R respectively. B and K denote the reconstituted restriction sites BlpI and KpnI respectively.

3.3. Genotypic analysis of the Pbsub-2 promoter KO parasites

Following limiting dilution of the transgenic population, six clones were selected and their respective gDNA was analysed by PCR. In all the 6 clones, integration specific products of 1.4kb and 2.9kb were amplified using the test 1 and test 2 primer sets respectively (Figure 9A). These products were not amplified in the wild type control gDNA demonstrating the generation of the expected transgenic parasites. The wt specific product was amplified only in the wt control (Figure 9A) confirming the purity of the clones.

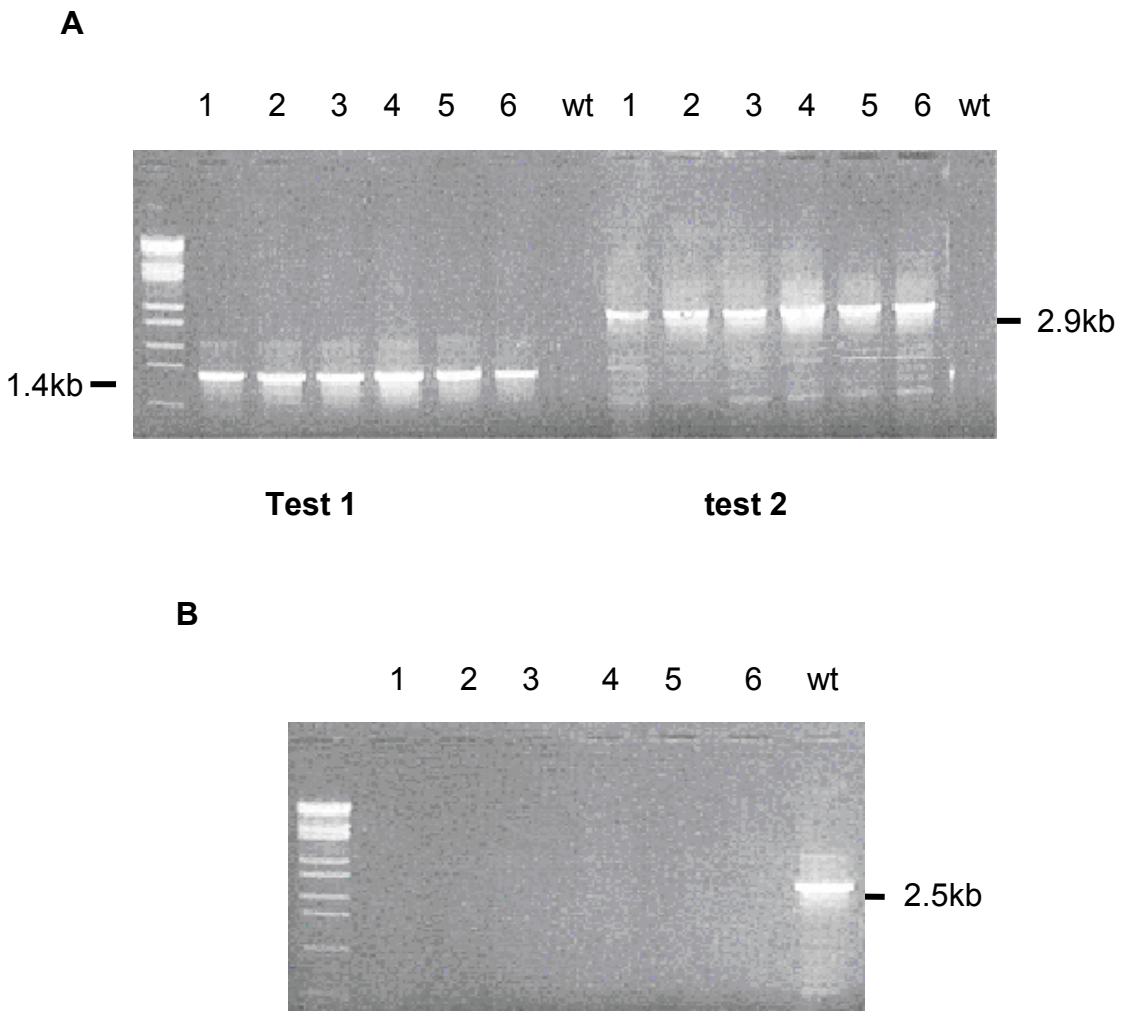

Figure 9: Integration-specific PCR analysis of Pbsub-2 promoter KO clones

One percent (1%) agarose gels showing successful integration event. A) test 1 and test 2 show integration specific PCR amplified products derived from the transgenic clones, the products are not amplified in the wt. B) Absence of the WT signal from the parasites clones 1-6 confirms the purity of the clonal population. All the products' sizes are indicated. 1-6 denote the clones and wt indicates wild type.

3.4. Analysis of the Pbsub-2 promoter KO parasites development

We examined the ability of the KO parasites to complete asexual cycle and the formation of ookinetes. Starting from equal parasitemia, Giemsa-stained blood smears revealed that the asexual erythrocytic development of the KO proceeds normally as the parasites attained comparable parasitemia to the wt at a similar rate. Furthermore, normal numbers of male and female gametocytes were formed. When cultured *in vitro*, gametocytes differentiated into gametes, fertilized, and the zygotes developed into morphologically normal mature ookinetes. The efficiency of ookinete production *in vitro* was indistinguishable from that of the wild type control.

3.5. Analysis of Pbsub-2 expression in ookinetes by western blot

We examined in parallel the expression levels of Pbsub-2 in ookinete extracts derived from the KO parasites. Since Pbsub-2 is expressed also in asexual stages (merozoites/schizonts), to minimize the contamination from the blood stages, pure ookinetes were isolated using 13.1 monoclonal antibody, against P28 ookinete surface protein, coupled to magnetic beads (Siden-Kiamos *et al*., 2000).

Using an anti-PbSUB2 polyclonal serum (provided by Dr. J. C. Barale), a signal corresponding to the mature PbSUB-2 form was detected in both wt ookinete extracts and those derived from mutant promoter-KO ookinetes (Figure 10). The signal was of the same intensity in both cases suggesting that PbSUB2 levels remained unaffected in the mutant promoter KO, despite the

clear genotypic demonstration of the native Pbsub-2 promoter replacement by Pbama-1 promoter.

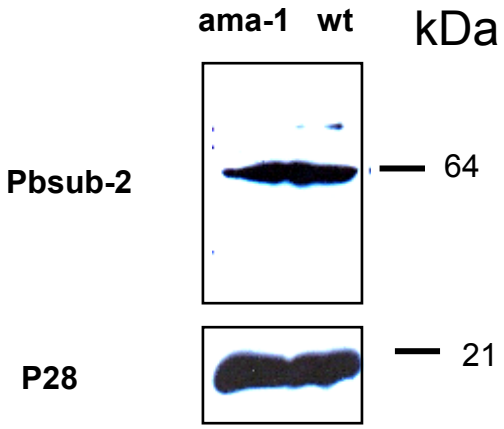

Figure 10: Analysis of Pbsub-2 expression in ookinete by western blot. Parasites were purified by erythrocyte lysis in 0.9% NH_4Cl followed by beads purification. The ookinetes were lysed in RIPA buffer on ice for 1 hr and boiled in sample buffer. The samples (~10^6 ookinetes per lane) were resolved in 10% SDS PAGE under reducing conditions and blotted onto nitrocellulose membrane for immunodetection using anti Pbsub-2 antibody followed by horseradish peroxidase-conjugated anti-rabbit IgG. P28, a 21kDa zygote-ookinete specific protein was used as a loading control using anti P28 mAb (lower panel). Molecular masses are indicated in kDa. Antibody dilutions: anti Pbsub-2, 1:1000, anti P28, 1:1000.

3.6. Infectivity of Pbsub-2 promoter KO parasites to mosquitoes

We further examined the infectivity of the KO parasites in the mosquitoes. Four to seven day-old post-emergence females mosquitoes were fed separately

on mice infected either with KO or wild type parasites and midgut oocysts were counted 10 days post infection. We observed indistinguishable numbers of oocysts between the transgenic and wild type parasites (Figure 11).

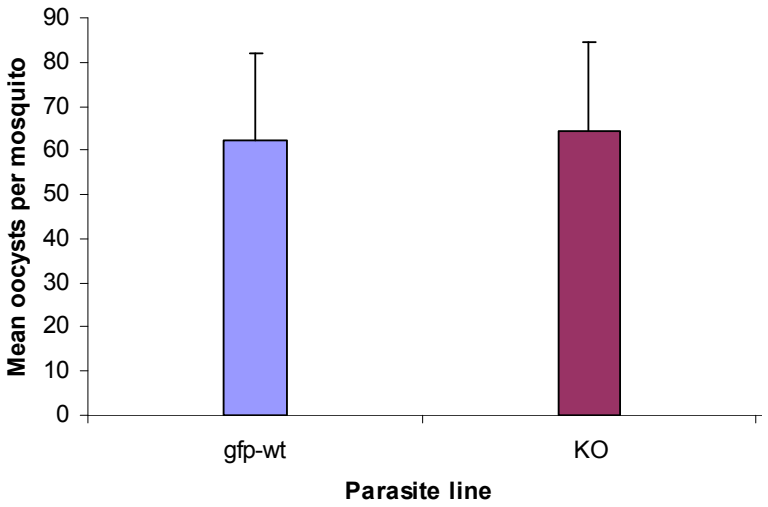

Figure 11: Development of the knockout parasites in the mosquito. Oocyst count showing indistinguishable oocyst formation of KO parasites to the wt. Adult female mosquitoes 4-7day old *A. gambiae* (G3 strain) were fed on mice infected with the KO or wt parasites with matched exflagellation and parasitemia and their midgut checked for oocyst 10 days post infection. No significant difference in oocyst formation was observed.

Discussion

In this part of the study, we aimed to generate a mutant *P. berghei* strain that potentially could aid our efforts to reveal PbSUB2 function in early sporogony. Previous studies have linked SUB2, a subtilisin like protease associated with the surface of the parasite, to the processing/maturation of MSP-

1 and AMA-1 adhesins, which are involved in successful invasion of erythrocytes by merozoites. Similarly to those, adhesins expressed in the mosquito stages could equally represent substrates of SUB2, and expression of the protease in mosquito stages may also contribute significantly to the invasion process. Therefore, SUB2 could also be an ideal target for transmission interventions. However, despite the evidence that PbSUB2 is present in invading ookinetes, we lack any further knowledge regarding its role in this particular stage.

Attempts to disrupt SUB2 gene via a classical gene knock-out have been fruitless, prompting us to adopt the alternative of stage specific silencing of SUB2 expression through a promoter swap approach. We attempted a replacement of the endogenous Pbsub-2 promoter with Pbama1, which potentially should restrict SUB2 expression in the schizonts and merozoites, (ensuring viability of the transgenic parasites), and abolish it in the subsequent stages.

In parallel to this work, the same promoter replacement strategy has been successfully applied on another *P. berghei* essential gene, PbMyoA, expressed throughout the parasitic life cycle. Pbama1 promoter was incorporated by a single cross-over into the PbMyoA locus leading to an efficient silencing of MyoA at the ookinete stage (K. Matuschewski and I. Siden-Kiamos, personal communication).

Pbama-1 promoter was efficiently and accurately incorporated into the Pbsub-2 locus as well, by two different ways (single and double cross-over recombination), which once more demonstrates that Pbsub-2 locus is not resistant to recombination, and therefore inability to KO the gene is due to the

vitality of SUB2. Importantly, in the case of double cross-over not only Pbama1 promoter was incorporated collinear with PbSU2 ORF, but in addition, ~1kb of the sequence upstream of the Pbsub-2 start codon, ATG, was eliminated.

Despite the successful Pbama1 promoter incorporation, in the transgenic parasites derived from both, single and double cross-over attempts, PbSUB2 protein was present at the ookinete stage in equal amount as in the wild type ookinete extracts (Figure 10).

The presence of wild type revertants in the clones derived from single cross-over was initially encouraging. Revertants could possibly indicate that levels of SUB2 protein in transgenic merozoites deviating from the normal due to different promoter strength, or absence of SUB2 activity from trophozoites or ring forms, (as ama1 is active in late schizonts and merozoites), might force reversion back to the normal. The fact that stable, and wild-type parasite free, transgenic clones derived from our second attempt that involved double cross-over, makes this possibility very weak. However, it is intriguing to follow comparatively the timing, as well as, the levels of SUB2 expression in asexual stages of mutant and wild type parasites.

Alternative promoters are often known to be responsible for transcriptional regulation mechanism of tissue-, time- or stage-specific expression. There is evidence demonstrating that single copy genes with multistage expression profile in *Plasmodium* are regulated at transcriptional level by distinct, independent and stage specific promoters. This is best represented by *Plasmodium set* locus*,* which encodes for a conserved nuclear protein involved in chromatin dynamics

that is expressed in both asexual and sexual stages, and over-expressed in male gametocytes (Pace *et al*., 2006). Two alternative transcripts of different sizes, derived from the *set* locus, one in asexual and the other in sexual stages have been identified, with identical sequence in their coding regions and differing only in their 5' untranslated regions (5' UTRs) (Pace *et al*., 1998). The size difference between asexual and gametocyte specific set-mRNA has been attributed lately to the use of alternative transcription initiation sites, as two distinct promoters regulate the timing and the level of *set* transcription (Pace *et al*., 2006).

Other genes in *Plasmodium* exhibiting differences in transcript size between asexual and sexual stages have also been described (Ridley *et al*., 1991, Delves *et al*., 1989). Moreover, the use of alternative promoters is a common theme in eukaryotes in response either to different stimuli and/or developmental changes (Ayoubi and Van de Ven 1996, Arpaia *et al*., 1995). Recently, an ookinete specific transcription factor, AP2-O, was characterized in *P. berghei*, controlling the expression of stage specific genes including those reported to be required for midgut invasion (Yuda *et al.,* 2009). AP2-O mRNA is pre-synthesized by intraerythrocytic female gametocytes and translated during ookinete development in the mosquito. The presence of developmental stage specific transcription factors, in combination with alternative promoter usage, point to a complex regulation of gene expression in *Plasmodium*.

We cannot exclude thus the possibility that *Pbsub-2* gene expression is subjected to alternative promoter usage, one active in the asexual and the other in mosquito stages. It should be noted at this point that sequence information

upstream of the transcription start of PbSUB2 is limited (J. C. Barale personal communication). In this case, we might just exchange a proximal promoter active in the asexual stages with Pbama1, without interfering at all with the expression of SUB2 in the ookinete stage. Size variation between the asexual and sporogonic stage transcripts should be an indication of alternative promoter usage, but unfortunately PbSUB2 mRNA has been only analyzed by Northern in asexual stages (Uzureau *et al*., 2004).

Another option is that we generated a hybrid promoter with characteristics similar to those of the endogenous Pbsub2 promoter. This would imply that the remaining Pbsub2 upstream sequences, which may contain controlling elements switching on SUB2 expression in sexual stages, is influencing the activity of an heterologous promoter like Pbama1, even from a distance (as it is separated from the later by 5kb of the selection marker). Although this is quite weak as possibility, in higher eukaryotes cis-acting elements controlling promoter activity are sometimes found even 30.000 bp upstream of the transcription start.

In addition to the critical role played by 5' upstream regions in the regulation of gene expression, 3' untranslated regions (UTR) of genes are equally important for mRNA stability and thus for the regulation of gene expression, not only in developing metazoan cells (Decker and Parker 1995, Bashirullah *et al*., 1998) but also in the *Plasmodium* (Cann *et al*., 2004, Golightly *et al*., 2000, Braks *et al*., 2008). Recent studies have established translational repression as a key mechanism for gene regulation in asexual-sexual developmental transition (Golightly *et al*., 2000, Braks *et al*., 2008). A DNA

binding protein belonging to the high mobility group, HMGB2, is indispensable in *Plasmodium yoelii* for the transcription of a set of genes that although transcribed in the gametocyte stage, are stored and translated in the ookinete stage (Gissot *et al*., 2008). This translational repression (TR) mechanism is widespread among *Plasmodia* and as it was shown in *P. berghei* involves both cis- and trans- acting translational repression elements (TR elements). U-rich TR elements acting in cis were identified in the 3' or even in the 5' UTR regions of transcripts (Braks *et al*., 2008), while DOZI , a protein with homology to DDX6 family of RNA helicases, has been found involved in the silencing of many gametocyte mRNA species (Mair *et al*., 2006).

Unfortunately, due to limited sequence availability from the PbSUB2 locus the 3' UTR of PbSUB2 is presently scrutinized for the presence of putative cis-TR elements. In any case, however, since the already described mechanism of translational repression may not be the only one, replacement of the Pbsub-2 3' UTR with that of a schizont/merozoite stage specific gene such as the *Plasmodium* MSP-1 or AMA-1, or deletions/point mutations in the Pbsub2 3' UTR would probably reveal any putative involvement of 3' UTR in regulating PbSUB2 expression.

CHAPTER 4: RESULTS and DISCUSSION

GENERATION AND CHARACTERIZATION OF TRANSGENIC PARASITES EXPRESSING SERPIN GENES AT THE OOKINETE AND SPOROZOITE STAGE

4.1. Construction of the CTRP-NcPI-S targeting vector

In order to avoid any position effect on transgene expression, we chose to target gene fusions into the same locus; the non essential *c-ssu* locus. In order to achieve this, we used the vector system pDEFSSUToxo (RV) (Figure 12-i). This vector system contains a selection marker cassette comprised of the *dhfr-ts* gene of *T. gondii*, under the transcriptional control of the *eef1a* promoter of *P. berghei,* which confers resistance to pyrimethamine. To restrict the expression of the serpins in sexual stage of the parasites, they were put under the control of CTRP promoter and 3'utr of P28. Both CTRP and P28 are abundantly expressed in ookinete stage. The introduction of linearized pDEFSSUToxo (RV) vector into the *P.berghei* genome was achieved through targeted integration of a *d-ssu-rrna* sequence also present in the vector (Figure 12-i), by a single cross over homologous recombination generating an incomplete and a complete copy of *ssu* separated by the plasmid backbone, the selectable cassette (*tgdhfr*) and the introduced sequences of interest (Figure 12-ii & iii),. However, since the sequence of the *c-ssu-rrna* and *d-ssu-rrna* units are 95.9% identical, integration can take place either into the *c-* or *d-rrna* gene unit (van Spaendonk *et al*., 2001).

The serpins were placed under the control of PbCTRP promoter and P28 3' UTR. The same promoter fragment was used before to drive the expression of GFP. As it was shown, GFP was expressed not only in the zygotes, and ookinetes, but also in the late oocyst stage (Vlachou *et al*., 2004). CTRP-serpin-P28 transgenes were transferred in the unique EcoRV site of pDEFSSUToxo(RV), as EcoRV fragments. The serpins were tagged amino-

terminally with V5 epitope tag in order to allow their detection, and subsequently fused to the putative signal peptide sequences of Pbsub-2, in order to be sorted into the secretory pathway.

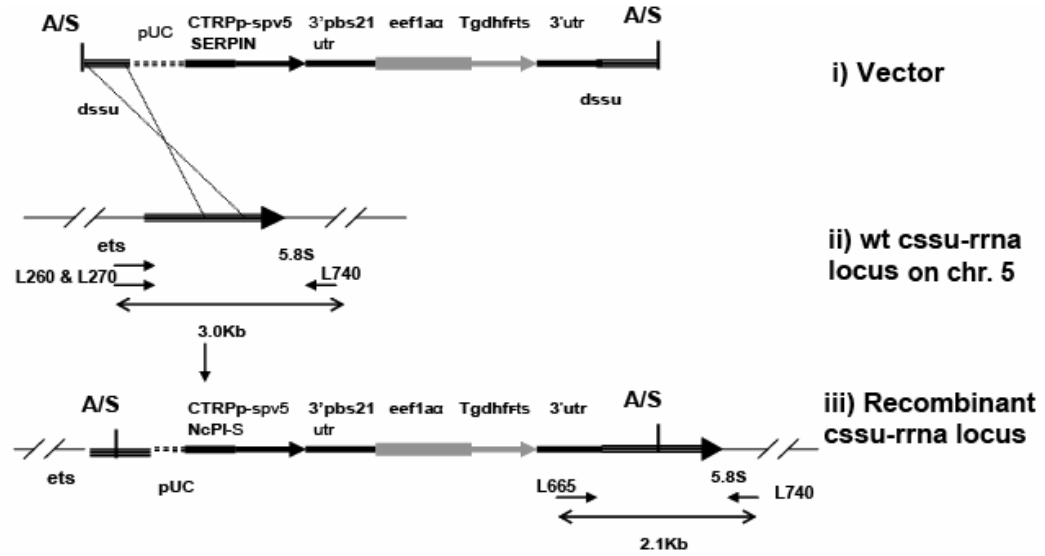

Figure 12: Strategy for the generation of transgenic serpin-expressing *Plasmodium berghei* parasites in zygote/ookinete stages

(i) The targeting vector: The vector used to integrate the serpins under CTRP promoter into *c- rrna* unit was linearized at a unique restriction site, ApaI (A) or SacII (S). The vector contains an incomplete copy of the *d-ssu-rrna* as a targeting sequence sufficient for homologous recombination, and a pyrimethamine-resistant *tgdhfr-ts* selectable cassette for selection of transgenic parasites. Serpin cDNAs were fused N-terminally with the signal sequence of Pbsub-2 (sp), and V5 epitope tag and were flanked by CTRP promoter and 3' UTR of P28 sequences. (sp) signal peptide of Pbsub-2, (V5) V5 epitope tag. Dotted line indicates pUC19 plasmid sequences.

(ii) Schematic representation of the integration target locus, the *c-rrna* unit in chromosome 5. Ssu-rrna, 5.8s, and the 5 external transcribed spacer regions (ets) are indicated.

iii) Schematic representation of the integrated serpin carrier-vector into the *c-rrna unit*. The integration results into an incomplete and a complete copy of the *cssu* gene separated by pUC19 sequences, the selectable (*tgdhfr-ts*) and the serpin expression cassette. Arrows indicate the expected products size upon integration into the correct target locus (*c-* or/and *d-rrna* unit) by PCR analysis (see also Figure 14).

4.2. Transgenic parasites establishment and genotyping

Linearized vectors were introduced by electroporation using the Amaxa device, into blood stage parasites of the original and a transgenic GFP-expressing ANKA strain (507cl1) (Janse *et al*., 2006b). Recombinant, pyrimethamine- resistant parasites were selected. Integration of the vector into the expected locus was verified by Pulsed Field Inversion Gel Electrophoresis (PFIGE) followed by Southern analysis of the chromosomal DNA. PFIGE allows separation of parasite chromosomes according to their sizes (Figure 13-i). Upon transfer onto Hybond-N+ nylon membrane, the resulting blots were probed with radiolabelled DNA corresponding to *Pbdhfr/ts* 3' UTR. The probe hybridized to the endogenous *dhfr-ts* locus on chromosome 7 (chr.7), as well as to the integrated vector on chromosome 5, which cannot be separated from chromosome 6 (chr.5/6) (Figure 13-ii).

Transgenic parasite populations of both lines were subsequently cloned by limiting dilution. Correct insertion/integration of the plasmid DNA into the *c-rrna,* or the *d-rrna* unit of the resulting clones was verified by PCR using as template, genomic DNA from the clones. In two LGI clones and two NcPI-S clones derived from independent transfection experiments (Figure 14 ia & iia), a 2.1kb product was readily amplified using integration specific primers (see primers in Figure 12). A fragment of 3kb was amplified using wild type specific *dssu-rrna* primers in all the clones and the wt (Figure 14 ib & iib). This product is expected in the case of an intact *dssu-rrna* unit. In contrast, when *cssu-rrna* specific primers were used, a 3kb fragment from wt genomic DNA was amplified but notably absent from all four clones (Figure 14 ic & iic). These results not only confirmed the homogeneity of the clones but in addition demonstrated that integration took place at the *cssu-rrna* unit. Due to the fact that the clones had identical genotypes, one representative clone from each transgenic line (NcPI-S cl3 and LGI cl2) was selected for further investigation.

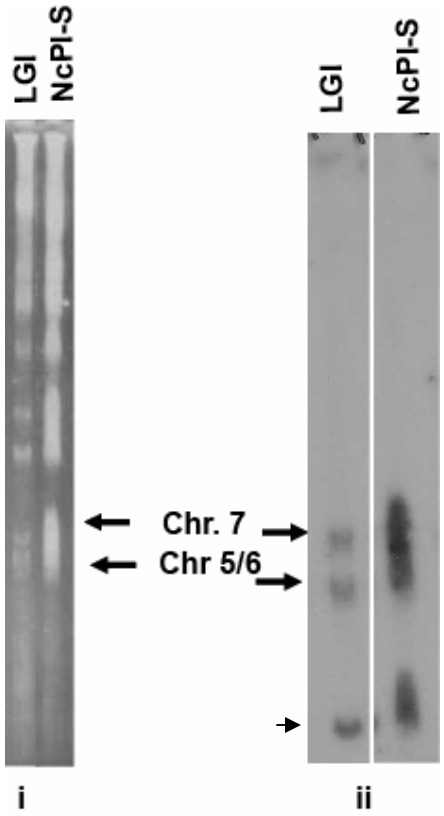

Figure 13: Integration analysis by PFIGE

Parasite chromosomes were separated by PFIGE followed by Southern analysis in order to confirm integration of the plasmid DNA construct into the desired chromosome and also the possible presence of wild-type parasites.

i) Chromosomal DNA from transfected parasites run on 1% agarose gel under PFIGE running conditions, showing chromosomes separated according to their molecular sizes, chromosome 5/6 and 7 are indicated by arrows. Chromosomes 5 and 6 (Chr. 6/5) migrate very closely thus cannot be separated from each other.

ii) The blot from the same PFIGE gel showing chromosome hybridization with the 3' UTR sequence of Pbdhfr/ts. The probe detects the 3′-UTR of the selectable marker (integrated vector on chromosome 5), and the 3′-UTR of the endogenous

dhfr gene of *P. berghei* on chromosome 7 (Chr. 7). The lowest band (arrowhead) could derive from episomal plasmid DNA.

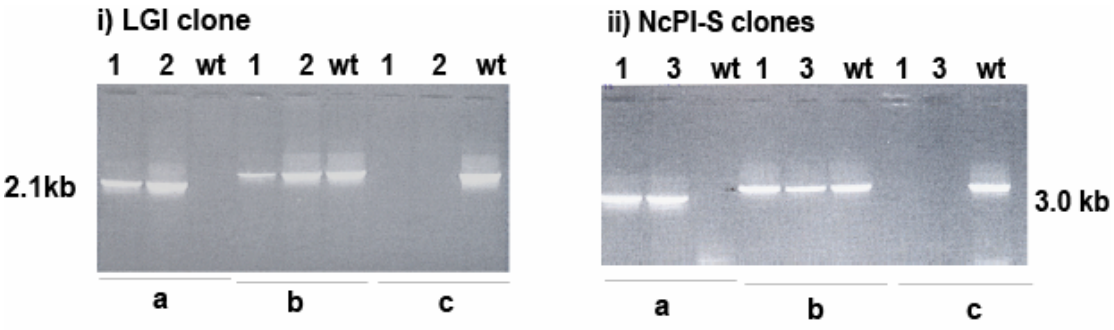

Figure 14: Integration-specific PCR analysis

Correct integration of the transfection vectors carrying NcPI-S or LGI serpin into the *cssu* locus shown by diagnostic PCR. i) 1 and 2, ii) 1 and 3 indicate PCR product derived from genomic DNA of LGI and NcPI-S expressing clones respectively. Genomic DNA from wild type parasites is indicated by wt. Both i and ii; reveal the presence of an integration specific product, while b and c wild type specific *dssu* and *cssu-rrna* respectively, using specific primers L665/L740 for a, L260/L740, for b and L270/740 for c. Primers and sizes of the respective predicted PCR products; 2.1kb and 3kb are indicated (also in figure 12).

4.3 Expression of LGI and NcPI-S serpins in the respective transgenic lines

4.3.1 Expression and subcellular localization of LGI

In the case of LGI-cl2 line a previously reported anti-SRPN10 purified serum (Danielli *et al*., 2003) was used in order to detect the protein in western blots of ookinete-derived total homogenates. SRPN10 serum recognizes the common backbone of all four isoforms derived from the *Anopheles* SRPN10

locus; among them serpin CAM, from which LGI serpin derived. Besides a major band of an expected size of approximately 45 kDa, which corresponds to the intact LGI serpin, a minor band of about 37 kDa is also detected. This minor band may represent a proteolytic product of the LGI serpin. Comparable amounts of P28 protein were detected in both, WT and the LGI ookinete samples with an anti-P28 monoclonal antibody (Figure 15, lower panel). P28 is a surface protein abundantly expressed in *P. berghei* zygotes and ookinetes (Winger *et al*., 1988).

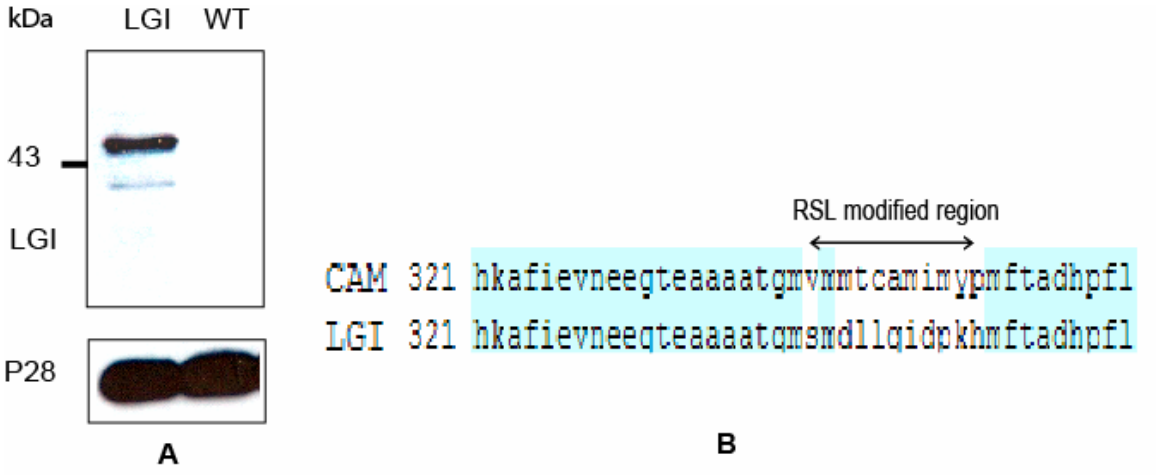

Figure 15: Generation and expression of LGI serpin in ookinetes.

A. Western blot analysis of ookinetes purified from 22-24 hrs ookinete cultures. Parasites were purified by erythrocyte lysis in 0.9% NH_4Cl, lysed in cold RIPA buffer on ice, and boiled in sample loading buffer. The samples (~10^6 ookinetes per lane) were resolved in 12% SDS PAGE under reducing conditions and blotted onto nitrocellulose membrane. As a loading control, monoclonal antibody (13.1) was used to detect ≈21kDa band corresponding to P28, a zygote/ookinete specific protein (lower panel). Molecular masses are indicated in kDa. **B.** Molecular engineering of CAM to generate LGI. A region corresponding to the

modified 10 amino acids within the CAM Reactive Site Loop, RSL is not highlighted.

In our next step, we used immuno-fluorescence (IFA), in order to reveal the sub-cellular localization of the expressed LGI serpin. *In vitro* cultured ookinetes were clearly stained with anti-SRPN10 purified serum, whereas, control WT ookinetes, treated under similar conditions, were not (Figure 16). SRPN10 immuno-reactive material in the LGI-expressing ookinetes was concentrated around the nucleus, with some weaker staining observed across the whole ookinete body (Figure 16-top panel a). This type of staining has been reported previously, and has been linked to secreted, membrane associated proteins (Yuda *et al*., 2001). Therefore, it is reasonable to speculate that the perinuclear staining may indicate a localization of LGI in the secretory pathway (ER and/or Golgi). This in turn implies that the signal peptide of Pbsub-2 is adequate to sort the serpins into the secretory pathway of the ookinete. However, our efforts to detect secreted LGI in the ookinete culture or upon ookinete stimulation were fruitless.

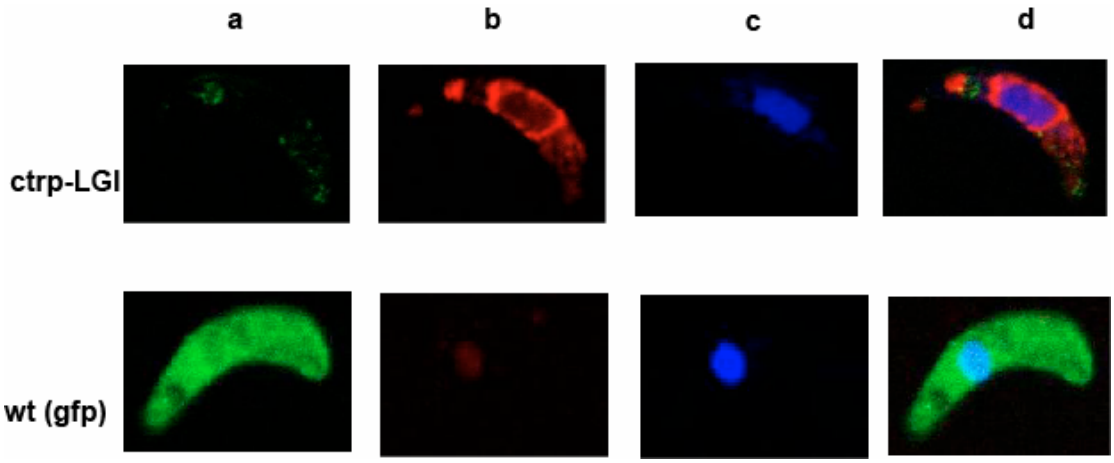

Figure 16: Subcellular localization of LGI serpin in ookinetes

Upper panel shows confocal microscopy analysis of LGI expressing ookinete stained with anti-SPRN10 antibody (b-red). The ookinetes were purified using NH_4Cl lysis, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-100 and stained with anti-SPRN10 primary antibody. Specific signal was detected with Alexa 555 conjugated anti- rabbit IgG. The nucleus is labeled with topro-3 (c-blue); d shows merged pictures. The lower panel shows wild type control ookinete treated similarly as the LGI. Samples were scanned using Leica confocal microscope under 630X magnification. Anti-SRPN10 dilution 1:333, rabbit anti IgG A555 conjugated, 1:1000.

4.3.2. Expression and subcellular localization of NcPI-S

In order to confirm NcPI-S expression in transgenic NcPI-S parasites, homogenates of *in vitro* cultured ookinetes were resolved in 15% SDS-containing polyacrylamide gels under reducing conditions, and transferred onto nylon membranes for Western analysis. A commercially available anti-v5 monoclonal antibody (Invitrogen), or/and a mouse anti-NcPI-S serum (kind gift from Dr.

Carruthers) were used to detect NcPI-S serpin. A band corresponding to ≈16 kDa protein was detected in extracts derived from NcPI-S line, which was absent from the wild type control. The same band is detected with either anti-V5 mAb (data not shown), or anti-NcPI-S serum (Figure 17), and may well correspond to NcPI-S dimers. Endogenous NcPI-S in *Neospora caninum* tachyzoites tends to form homodimers as it was shown with MALDI-TOF analysis (Morris *et al*., 2004). Moreover, when we expressed and purified NcPI-S from *E. coli*, the bulk of the purified NcPI-S runs as an SDS stable dimer (see figure 33). However, this recombinant product retains its activity when tested in an *in vitro* assay against bacterial subtilisin Carlsberg. Homodimerization of serpins has been previously demonstrated to be required for their inhibitory activity against their target proteases (Eggers *et al.,* 2001, Nar *et al*., 2000).

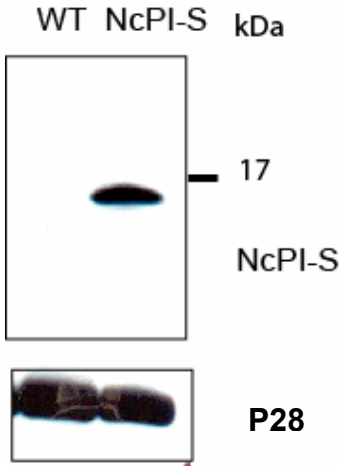

Figure 17: Expression of NcPI-S serpin in ookinetes.

Western blot analysis of ookinetes purified from 22-24 hrs ookinete cultures. Parasites were purified by erythrocyte lysis in 0.9% NH_4Cl, lysed in cold RIPA

buffer on ice, and boiled in sample loading buffer. The samples (~10^6 ookinetes per lane) were resolved in 15% SDS PAGE under reducing conditions and blotted onto nitrocellulose membrane. NcPI-S serpin was detected with anti-NcPI-S mouse serum followed by horseradish peroxidase-conjugated anti-mouse IgG. As a loading control, monoclonal antibody (13.1) was used to detect ≈21kDa band corresponding to P28, a zygote/ookinete specific protein (lower panel). Molecular masses are indicated in kDa.

Both anti-V5 mAb and mouse anti-NcPI-S serum failed to stain specifically *in vitro* cultured NcPI-S ookinetes. The antibodies stained both NcPI-S and wild type ookinetes with the same intensity. Since both antibodies are of mouse origin, it is possible that they recognize mouse blood-derived material associated with the ookinetes. Neither pre-incubation of the antibodies with excess of *in vitro* cultured wt ookinetes, nor alternative fixation methodologies solved the problem.

Alternatively, in order to reveal the subcellular localization of NcPI-S, we carried out a stepwise fractionation of NcPI-S expressing ookinetes and we analyzed the resulting fractions by western blot. Five million fresh ookinetes from NcPI-S-cl3 line and wt control were subjected to a stepwise fractionation protocol adopted from Patra *et al*., 2008, with minor modifications. The fractions were classified as follows, soluble: the supernatant after 1 hour incubation on ice in 10mM Tris-HCl pH 8.5, membrane salt-eluted fraction: wash of the pellet with 100mM Na_2CO3 pH 11.5, and membrane fraction: the remaining pellet.

NcPI-S specific signal of ≈16kDa was detected in all NcPI-S ookinete-

derived fractions, however, signal intensity varied for each fraction. The strongest signal was detected in the soluble fraction (Figure 18a). This suggests that approximately three quarters of the NcPI-S expressed in the ookinetes remains in the cytosol. A less strong NcPI-S signal (Figure 18c) was detected in the membrane fraction, while the signal was much weaker in the salt-eluted membrane fraction (Figure 18b). Since Na_2CO3 treatment releases soluble peripheral membrane proteins (Fujiki *et al*., 1982), the stronger and salt persistent signal in the membrane pellet indicates that most of the NcPI-S in the membrane is in a tight association with it, either directly or indirectly through other membrane associated proteins. We have also noticed an additional signal of unknown protein of approximately 26kDa in the membrane fraction in both the NcPI-S and wt control ookinetes (Figure 18c). This additional signal is never detected with non-mouse derived antibodies. Most likely the signal corresponds to a mouse-derived protein associated with the ookinete surface, which may cause the problems we encountered in the immuno-staining referred earlier.

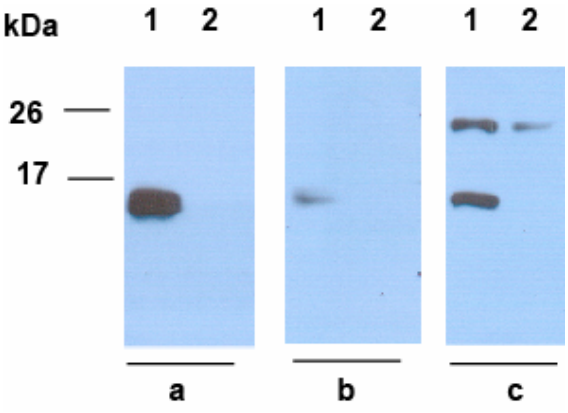

Figure 18: Subcellular localization of NcPI-S as revealed by western blot analysis of ookinete fractions

Five million ookinetes were subjected to stepwise fractionation as described in the materials. Sub-fractions of freshly purified ookinetes (~ five million) were classified as follows: a) Soluble fraction b) Membrane salt-eluted fraction c) Membrane fraction: (the remaining pellet). The lysates and the pellet Fractions were resolved in 15% SDS-PAGE under reducing conditions and NcPI-S presence in each fraction was detected by anti-V5 mAb, followed by secondary anti-mouse HRP conjugated IgG, and detection using a super signal chemiluminescence kit (ECL detection system, Pierce Biotechnology Inc.). Lane 1 and 2 in all panels denote transgenic NcPI-S cl3 and wt extracts respectively.

4.4. Phenotypic characterization of the transgenic parasites

4.4.1. Infectivity of the parasites to mosquitoes

Both LGI and NcPI-S transgenic lines showed normal asexual and sexual blood stage development, and were able to form ookinetes *in vitro* in numbers similar to, and morphologically indistinguishable from wild type parasites (data

not shown). To assess the effects of NcPI-S and LGI expression on the transgenic parasite infectivity to mosquitoes, mice infected with NcPI-S-cl3, or LGI-cl2 or WT parasites were fed to *P. berghei* susceptible *Anopheles gambiae* females (G3 strain). Ten days later, the mosquitoes were dissected and the numbers of oocysts determined. This constitutes a routinely applied assessment of a parasite's ability to survive and develop normally in the mosquito.

Whereas a weaker and insignificant reduction in oocysts numbers was observed in the case of LGI parasites, a drastic reduction (97-98% ($p<0.001$)) was consistently recorded in all the experiments in which mosquitoes from the same cohort were infected with NcPI-S transgenic parasites (Table 1 and Figure 19).

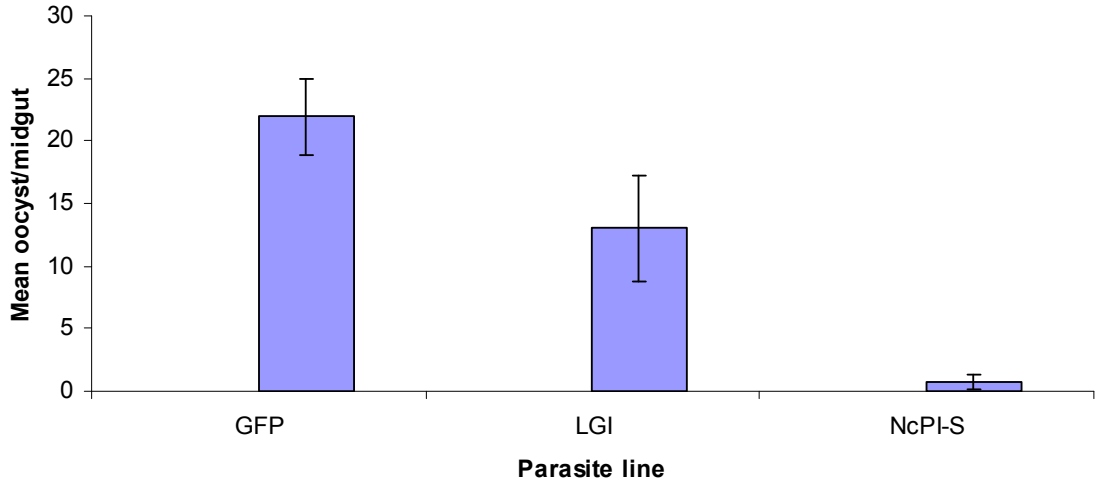

Figure 19: Oocyst numbers in mosquitoes infected with serpin expressing or wt (GFP-ANKA) parasites. Histogram showing the reduction in oocyst formation of LGI cl2 and NcPI-S cl3 strains compared to the wt represented as the mean number of oocysts per midgut (see numbers also in table 1 below)

Development of LGI cl2 and NcPI-S cl3 expressing parasites in *A. gambiae*

Expt. A	Parasite	*n*	%Prevalence	Mean oocysts (range)	±SEM	*p-value*
1	WT-GFP	13	92	14.85 (0-55)	20.5	_
	NcPI-S cl3	27	22	00.33 (0-2)	0.13	p<0.001
	LGI cl2	5	60	08.00 (0-30)	14.87	p>0.05
2	WT-GFP	12	75	24.33 (0-120)	9.8	_
	NcPI-S cl3	27	0	00.00 (0)	0	p<0.001
	LGI cl2	11	64	80.10 (0-310)	39.5	p>0.05
3	WT-GFP	22	77.2	25.59 (0-125)	6.45	_
	NcPI-S cl3	32	6.25	00.06 (0-1)	0.04	p<0.001
	LGI cl2	17	64.7	21.53 (0-96)	7.17	p>0.05
4	WT-GFP	19	78.95	25.37 (0-126)	9.17	_
	NcPI-S cl3	27	48.14	01.85 (0-8)	0.43	p<0.001
	LGI cl2	17	76.5	09.53 (0-40)	2.77	p>0.05

Table 1: Oocyst numbers in infected *A. gambiae* mosquitoes (G3 strain) 10 days post infection. Four to seven day old females were fed on NcPI-S (clone3), LGI, or GFP expressing ANKA *P. berghei* strain (control) infected mice (with 12-15% parasitemia and >10 exflagellation centre per field) for 15 minutes. Mosquitoes were dissected on day 10 post infection and oocysts counted under fluorescence microscope at 200 and 400X magnification. Expt: number of experiment; parasite: parasite line; n: number of mosquitoes; prevalence: percentage of mosquitoes infected with oocysts; Mean: mean number of oocysts per mosquito; ±SEM, standard error of the mean; P-value as determined by *t-test.*

In order to exclude the possibility that this is a unique characteristic of the selected clone for study, reflecting a genotypic variation, we generated new transgenic lines expressing NcPI-S, through an independent transfection experiment, using as parental strain the original *P.berghei* ANKA strain. One of the derived clones (cl1) was selected for further investigation. A similar

transmission phenotype as in the case of NcPI-S-cl3 was observed in the case of NcPI-S clone 1 (NcPI-S-cl1) that derived from this second experiment (Table 2 and Figure 20).

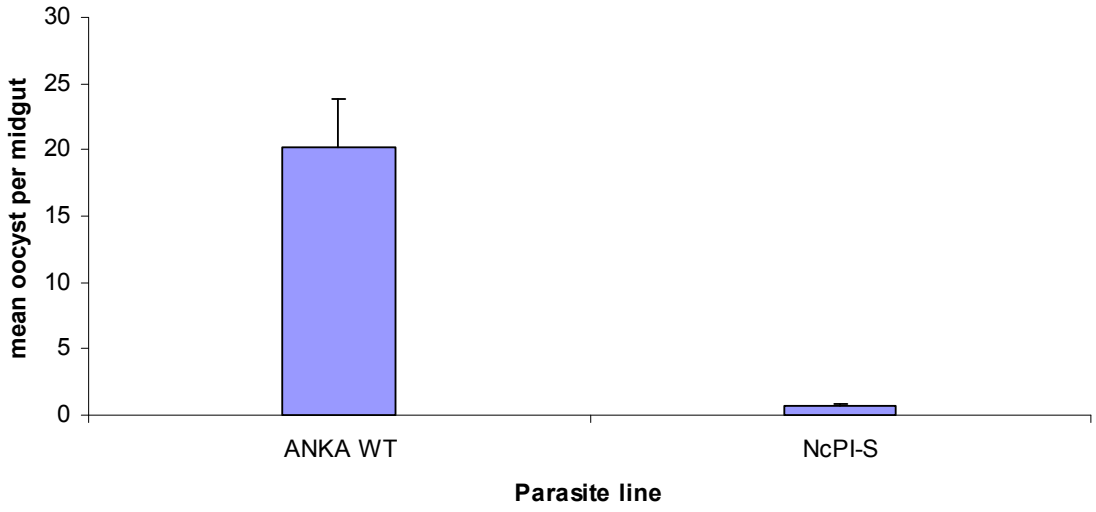

Figure 20: Oocyst numbers in mosquitoes infected with NcPI-S expressing or wt-ANKA parasites. Histogram showing the reduction in oocyst formation of NcPI-S cl1 strain compared to the wt represented as the mean number of oocysts per midgut (see numbers also in table 2 below).

Development of NcPI-S cl1 expressing parasites in *A. gambiae* (G3)

Expt.	Parasite	*n*	%Prevalence	Mean oocysts (range)	±SEM	*p-value*
1	WT-ANKA	19	89.47	20.47 (0-99)	5.97	
	NcPI-S cl1	22	40.91	00.50 (0-3)	0.16	p<0.01
2	WT-ANKA	37	78.38	18.68 (0-111)	4.06	
	NcPI-S cl1	24	25.00	00.88 (0-7)	0.41	p<0.01

Table 2: Oocyst numbers in infected *A. gambiae* mosquitoes (G3 strain) 10 days post infection. Four to seven day old females were fed on NcPI-S (clone

1) or native ANKA *P. berghei* WT (control) infected mice (with 12-15% parasitemia and >10 exflagellation centre per field) for 15 minutes. Mosquitoes were dissected on day 10 post infection and oocysts counted under light microscope at 200X and 400X magnification. Expt: number of experiment; parasite: parasite line; n: number of mosquitoes; prevalence: percentage of mosquitoes infected with oocysts; Mean: mean number of oocysts per mosquito; ±SEM, standard error of the mean; P-value as determined by *t-test*.

These results demonstrate that most NcPI-S ookinetes lost their infectivity to the mosquito host as a result of NcPI-S expression, which potentially interferes with essential biological events during parasite development in the mosquitoes. In addition, the similar transmission deficient phenotype observed in the two independent NcPI-S clones (NcPI-S-cl1 and NcPI-S-cl3) indicates that the drastic drop in infectivity is unlikely to be a result of clonal phenotypic variation, and demonstrates that the parasite loses its virulence towards the mosquito vector as a result of NcPI-S expression. Based on these results, NcPI-S was an obvious promising molecule and hence its selection for further investigation.

4.4.2. NcPI-S expression does not interfere with *in-vivo* gametocyte to ookinete transition

Development and differentiation of the parasite in the mosquito midgut, and the subsequent traversal of the peritrophic matrix and the midgut epithelium is a very complex procedure. In the hostile environment of the blood meal, and

against antagonistic factors derived from the mosquito and/or the blood, the parasite must differentiate successfully into a mature ookinete that will cross efficiently the peritrophic matrix, and subsequently the microvilli-associated network and the midgut epithelial cells, in order to form an oocyst at the basal side of the midgut epithelium. Failure in the sexual differentiation and sporogonic development, susceptibility of the parasite to antagonistic factors derived from mouse blood or mosquito midgut, inability of the ookinete to move and invade the midgut may be the cause of a reduction in oocyst numbers.

According to what was shown previously the “window” of expression of an exogenous gene (GFP) placed under the CTRP promoter, starts at late zygote, continues at the ookinete stage and ends in the 10 day-old oocyst (Vlachou *et al*., 2004). Thus, NcPI-S expression at late zygote may affect its normal transformation into a mature motile ookinete *in vivo*. Alternatively, expression of NcPI-S at late zygote and ookinete may increase their susceptibility to the antagonistic factors of the blood and the midgut. Both of these two reasons could lead to fewer ookinetes.

In order to exclude these possibilities, we checked the presence of mature ookinetes in the mosquito blood meal. Mosquitoes were fed on mice infected with either NcPI-S-cl3 or wt parasites with matched parasitemia. Midguts were dissected 22-24 hrs post infection and their contents checked for ookinete presence. Giemsa staining of the content smears derived from the NcPI-S infected mosquitoes revealed the presence of ookinetes in numbers and morphology indistinguishable from that observed in the midgut smears derived

from mosquitoes infected with wt parasites (Figure 21a & b).

In order to have a quantitative indication of normal differentiation of NcPI-S ookinetes *in vivo* we performed a western blot of midguts (derived from equal numbers of NcPI-S and wt infected female mosquitoes), isolated 24hr post infection. Western blots were hybridized with anti-PbCTRP monoclonal antibody. A strong signal of ≈215 kDa corresponding to CTRP protein was detected in both blood meal extracts, indicating a normal ookinete differentiation of NcPI-S parasites (Figure 21c).

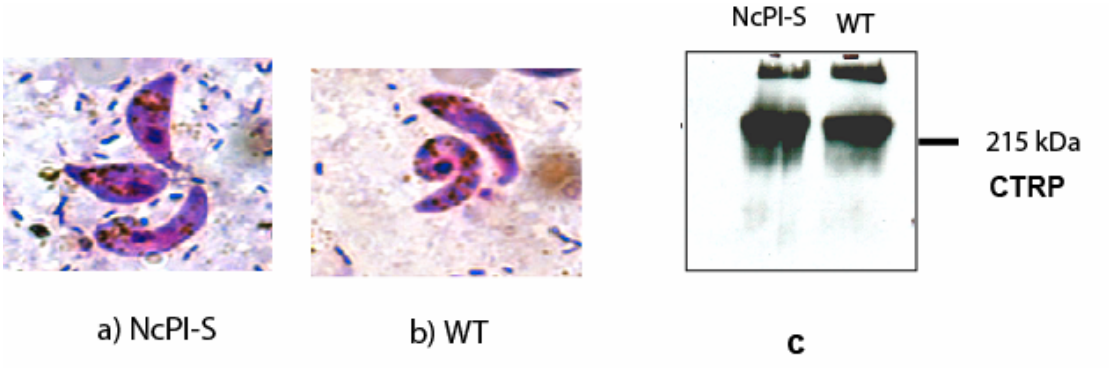

Figure 21: *In vivo* development of ookinetes. Giemsa staining of smears made from the content of *An. gambiae* (G3) midguts, 24 hours after an infective blood meal. (a) NcPI-S-expressing ookinetes and (b) wt ookinetes (1000X magnification). Similar numbers of morphologically normal ookinetes were recorded in both cases.

C. Western blot analysis of ~60 *An. gambiae* (G3) female midguts, 24 hrs upon an infected blood meal. The midguts were crashed and ookinetes were purified by erythrocyte lysis in 0.9% NH_4Cl, sonicated and boiled in sample buffer. The samples were resolved in 9% SDS PAGE under non-reducing conditions and

blotted onto nitrocellulose membrane. A signal corresponding to CTRP (215kDa) was detected in both ookinete extracts, indicating normal ookinete differentiation in both cases. Molecular masses are indicated in kDa; anti-CTRP antibody dilution, 1:1000.

4.4.3. NcPI-S-expressing ookinetes exhibit normal motility

The next reasonable hypothesis to test is, if expression of NcPI-S leads to an ookinete motility defect, possibly by inhibiting proteases needed for maturation/processing of proteins involved in this process (Dessens *et al*., 1999, Templeton *et al*., 2000, Yuda *et al*., 1999). In an *in vitro* assay, we assessed the NcPI-S ookinete motility on matrigel. The ookinetes were mixed with matrigel and the mixture allowed to set at room temperature on a glass slide. Individual ookinetes were then examined under the microscope and photographed at 10 second time intervals, as they glide through the solidified matrigel for five minutes. NcPI-S expressing ookinetes exhibited normal motility behavior compared to the WT (Figure 22), which excluded that a defect in motility is the basis of NcPI-S phenotype.

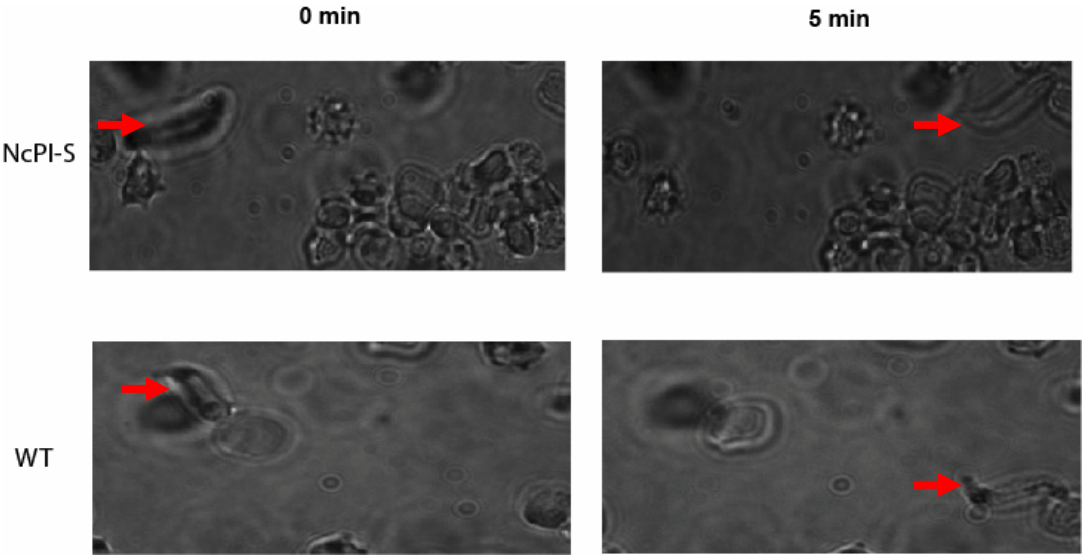

Figure 22: ***In vitro* ookinete motility.** *In vitro* ookinetes derived from NcPI-S cl3 (upper panel) and wt (lower panel) performing normal gliding motility through solidified matrigel. Arrows indicate the ookinete positions in pictures taken at 0 seconds and after 5 minutes. The experiment was repeated at least 3 times.

4.4.4. The majority of NcPI-S expressing ookinetes are lost 24hrs post infection

P. berghei ookinetes maturation in the *Anopheles gambiae* midgut takes between 18-24hrs upon ingestion of an infected blood meal. At the end of this period the majority of mature ookinetes has also penetrated the peritrophic matrix and can be found in close association with the midgut epithelium in the process of invasion. Mosquito midguts were dissected 22-24 hrs upon an infected blood meal, and the blood bolus was carefully removed, after a brief fixation. After intensive washing, the remaining ookinetes in the midgut epithelial sheets were stained with anti-P28 mAb and topro3.

Strikingly, NcPI-S expressing ookinetes found in association with the

midgut epithelium were reduced by 95.34% (Figure 23B & 24) compared to wild type control ookinetes (Figure 23A & 24). Moreover, the midgut epithelium appeared intact. Extruding invaded epithelial cells that over-express SRPN10 serpins, which usually mark the sites of active invasion, and which were massively recorded in the case of the control WT infections, were very rare in the case of NcPI-S-cl3 infections (data not shown). These results are in a perfect agreement with the oocyst reduction. Collectively, they show that the NcPI-S expressing ookinetes lost their ability to efficiently invade the mosquito midgut. The few remaining NcPI-S ookinetes seem to be tightly associated with the midgut epithelium, and might be the “founders” of the small number of oocysts that we observed in the case of NcPI-S strain. However, it should be stressed out that this type of experiment (gametocyte feeding) does not distinguish between an inability of ookinetes to penetrate the peritrophic matrix and their possible failure to associate with the midgut epithelium.

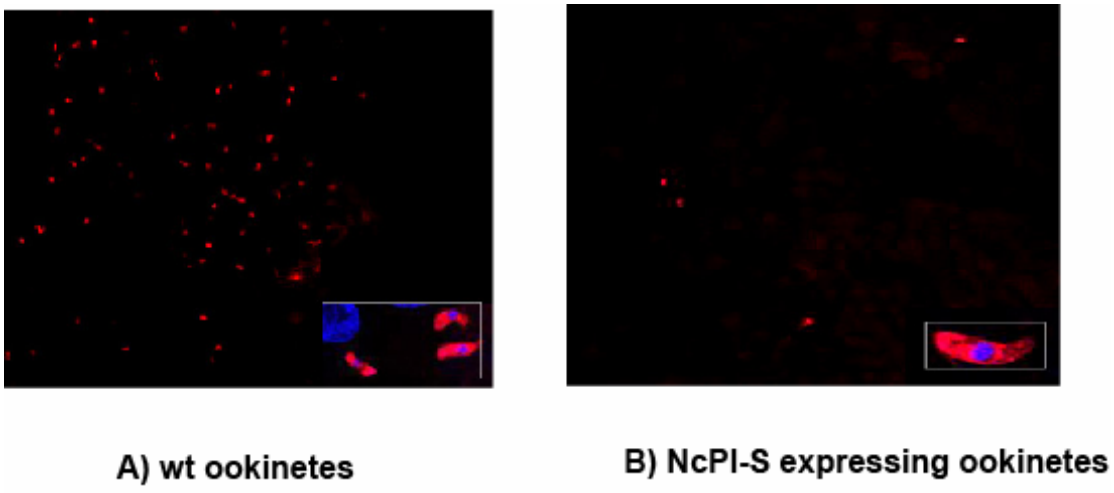

Figure 23: Association of ookinetes with the midgut epithelium 24hrs post infection

Midguts of NcPI-S expressing or wt parasites infected mosquitoes were dissected 24hrs post infection and ookinetes in the epithelium (red dots) were revealed by staining with anti P28 monoclonal antibody (13.1 mAb) and their nuclei counterstained in blue with TO-PRO 3. P28 staining, in combination with the typical ookinete banana shape and a well stained round nucleus (as shown in the representative magnified ookinetes in the insets), were defining properties used to identify and count real ookinetes. Six midguts from each parasite line were examined and the experiment was repeated 3 times. A) WT strain showing more numerous ookinetes associated with the epithelium compared to, B) NcPI-S expressing strain, in which case, handful ookinetes are associated with midgut epithelium.

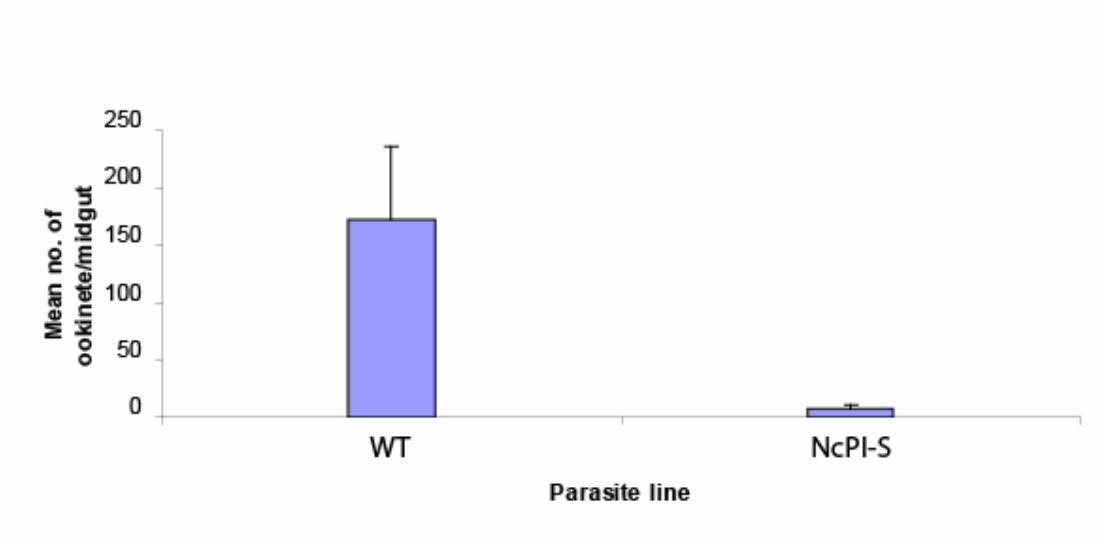

Figure 24: Ookinetes association with midgut epithelium.

Histogram showing the number of ookinetes associated with midgut epithelium represented as the mean number of ookinetes per dissected midgut. The ookinetes were counted on a Zeiss fluorescence microscope at 400X magnification.

4.4.5. Bypassing Peritrophic Matrix (PM) does not rescue NcPI-S ookinetes

Concurrently with parasite differentiation in the midgut lumen, the ingested blood meal triggers the formation of peritrophic matrix (PM), a thick (1–20 μm) chitin-containing layer (Ponnudurai *et al*., 1988, Tellam *et al.,* 1999), separating the gut lumen from the epithelial cells lining the midgut. The PM is thought to protect the midgut epithelium from mechanical damage and attack from pathogens and toxin (Lehane 1997, Tellam *et al*., 1999). As the ookinetes emerge must actively traverse the PM in order to gain access to the midgut epithelium. Since PM is a strong physical barrier, in order to disrupt it, ookinete secretes either inactive or partially active chitinase, which is further activated by

mosquito derived trypsin-like serine protease (s) (Huber *et al*., 1991, Shahabuddin *et al*., 1993). Active chitinase possibly is also required in the subsequent invasion steps, as chitin presence is suggested in the microvillar network (Dessens *et al*., 2001). We postulated that NcPI-S expression may interfere with the activation of the pro-chitinase, and that this could be the molecular basis of the "NcPI-S phenotype". In this case bypassing the peritrophic matrix should theoretically rescue the NcPI-S ookinetes. Persistence of the "NcPI-S phenotype", (without excluding the possibility that NcPI-S ookinetes are unable to penetrate the PM), would indicate a more severe defect in invasion. The experimental way to assist the parasite to bypass the PM is by feeding mosquitoes with controlled numbers of *in vitro* formed mature ookinetes, through artificial membrane feeders. Under these conditions, the preformed ookinetes rapidly associate with the midgut epithelium prior to the formation of PM.

When *in vitro* developed ookinetes were provided to female mosquitoes through membrane feeding, a severe reduction in oocyst numbers, in the range of 97-98%, was again observed (Table 3). Moreover, in experiments that parallel the ones reported in 4.4.4, a very small number of ookinetes were found associated with the mosquito midguts, 6hrs upon the ingestion of preformed ookinetes, compared to the wild type control (data not shown). These results, without excluding an additional possible defect of NcPI-S ookinetes in penetrating PM, strongly indicate that their ability to interact with the midgut epithelium is severely impaired.

To exclude the possibility that the phenotype is exclusively due to the inability of the NcPI-S expressing parasites to traverse the midgut, we bypassed the midgut by injecting preformed ookinetes into the mosquito haemolymph. Ookinetes injected this way can develop virtually anywhere in the mosquito hemocoel as ectopic oocysts (Paskewitz and Shi 2005). Due to technical difficulties involved in such experiments, both ectopic oocysts and salivary gland sporozoites in both, the mutant and wt parasites were not observed.

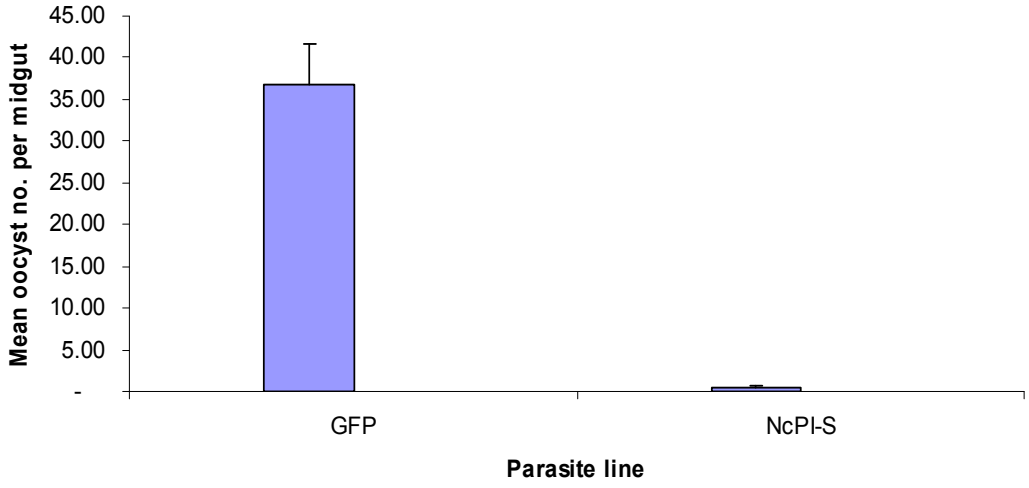

Figure 25: Oocysts formation in mosquitoes infected with NcPI-S expressing or wt (GFP-ANKA) ookinetes. Histogram showing the reduction in oocysts formation of NcPI-S cl3 parasites following mosquito infection with preformed ookinete via membrane feeders. (See also the numbers in table 3).

Development of *in vitro* preformed NcPI-S-expressing ookinetes in *An. gambiae* (G3 strain)

Expt.	Parasite	*n*	%Prevalence	mean (range)	±SEM	*p-value*
1	WT	9	88.9	83.00 (0-277)	31.82	_
	NcPI-S cl3	26	15.38	00.30 (0-3)	0.16	<0.001
2	WT	36	83.33	22.56 (0-117)	4.52	_
	NcPI-S cl3	32	9.38	00.28 (0-7)	0.22	<0.001
3	WT	25	92	30.88 (0-119)	6.5	_
	NcPI-S cl3	14	14.29	00.29 (0-3)	0.22	<0.001

Table 3: Oocyst numbers in infected *A. gambiae* mosquitoes (G3 strain) 10 days post infection with ookinetes. Four to seven day old female mosquitoes were fed on experimental membrane feeders with a mixture of purified *in vitro* developed ookinetes and naive fresh mouse blood for 30-40 minutes (final concentration 4000 ookinetes/µl). Oocyst numbers were determined 10 days upon feeding. Expt: experiment number; parasite: parasite line; n: number of mosquitoes; prevalence: percentage of mosquitoes infected with oocysts; Mean: mean number of oocysts per mosquito; ±SEM: standard error of the mean; P-value as determined by *t-test.*

4.4.6. NcPI-S is a cell autonomous phenotype

According to the initial design, due to the fact that NcPI-S was fused to the Pbsub-2 signal sequence, it should be secreted from the NcPI-S expressing ookinetes. Therefore, it is reasonable to test if possibly secreted NcPI-S may affect the invasiveness of wild type ookinetes, when fed together with NcPI-S ones in the same mosquito. Moreover, it is intriguing to test if, alternatively, wild

type ookinetes present in the same blood meal with NcPI-S ones, may provide the later with a rescuing activity.

In order to investigate these two options, we fed mosquitoes on a mixture of *in vitro* cultured wild type ookinetes labeled with red fluorescence protein (RFP expressing strain (Janse *et al*., unpublished)) and NcPI-S-cl3 ookinetes (which are labeled with GFP), in carefully controlled ratios. Two independent experiments were performed with mosquitoes fed with a mixture (2500:2500) of NcPI-S (GFP): RFP ookinetes. As a control, wild type ookinetes derived from GFP-ANKA parental line were mixed with wild type RFP ookinetes in the same ratio (2500:2500), and fed to mosquitoes. Mosquitoes were dissected at day 10 post infection and oocyst numbers determined. NcPI-S expressing ookinetes failed to form oocysts even in the presence of wild type RFP ookinetes (Figure 26A). In the control experiment, both differentially labeled wild type ookinetes were transformed into oocysts. It should be noticed however that in the control experiments higher number of green oocysts were formed than red, suggesting a better infectivity of the parental GFP-ANKA (WT) strain, than that of the new RFP-ANKA (WT) one (Figure 26B).

In any case, this experiment showed that the detrimental effect of NcPI-S on the infectivity of NcPI-S ookinetes cannot be neutralized by the presence of wild type ones. In addition, it excludes the possibility that NcPI-S expression in transgenic ookinetes may affect other aspects of invasion involving long-range soluble and/or secreted factors. This “cell autonomy” of the phenotype indicates that NcPI-S effect on the invasion process is restricted to the parasite that

expresses it, and that putative endogenous targets of NcPI-S should be first explored.

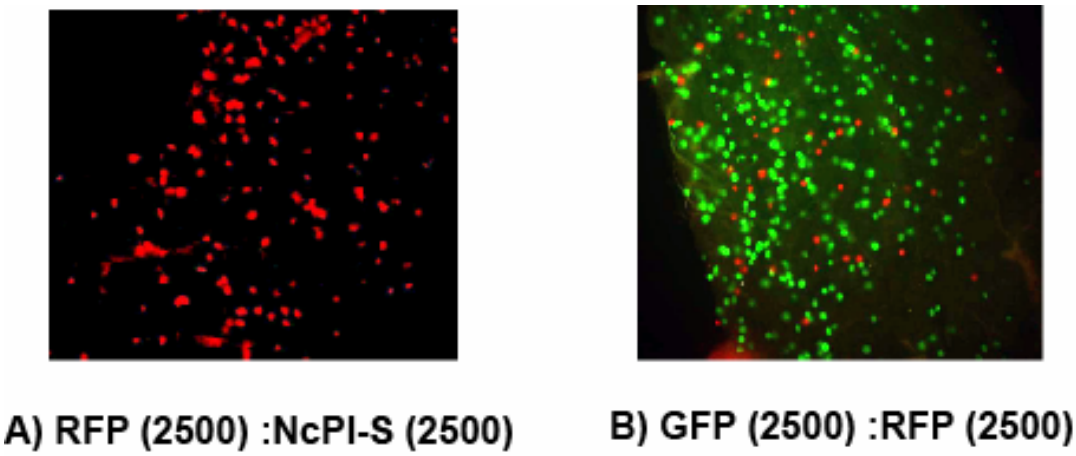

Figure 26: Co-infections of mosquitoes with preformed ookinetes through membrane feeding

RFP-expressing wild type oocysts are visualized as red spots; green spots reveal the presence of oocysts either from WT-GFP expressing parental strain or from NcPI-S line. **A)** Co-infection with wt-RFP and NcPI-S ookinetes. As is evident in this representative midgut, 10 days post infection, only RFP expressing oocysts develop. **B)** Control: Co-infection with wt-RFP and wt-GFP ookinetes. Both types of ookinetes develop into oocysts as it is evident by concomitant presence of both green and red dots in this representative midgut. All the midguts were examined using a Zeiss fluorescence microscope and pictures taken at 100X magnification.

4.4.7. Levels of secreted ookinete adhensive protein (SOAP) and WARP are significantly lower in NcPI-S expressing ookinetes

Among the prime candidates to test of proteins whose normal expression pattern might be altered by the co-expression of NcPI-S are micronemal proteins. Micronemes are the only secretory organelles present in ookinetes and their contents are considered essential for the invasion process. In fact, depletion of micronemal proteins through gene disruption, or their functional inactivation through the use of transmission blocking antibodies, results into a reduction in oocyst transformation. The severity of the phenotype depends on the micronemal proteins. Moreover, as it has been shown explicitly in the case of *Plasmodium* merozoites and *Toxoplasma* tachyzoites, targeted proteolysis by co-localized proteases, is a common mechanism to process and activate micronemal proteins prior to, or upon their secretion (Kim 2004, Blackman 2004).

Using antibodies against three essential proteins that have been reported to localize into the micronemes, (CTRP, WARP, SOAP), we carefully assessed their expression levels and processing. Among the three, SOAP and CTRP are essential for invasion and their disruption generates a severe reduction in oocyst formation (Dessens *et al*., 1999, 2003, Yuda *et al*., 1999, Templeton *et al*., 2000). Although motility of CTRP-depleted ookinetes is severely compromised, SOAP-depleted ookinetes are motile, and able to invade and form oocysts to a certain degree. However, a reduction in oocyst formation ranging from 60% to 85% has been reported for the later (Dessens *et al*., 2003).

The third micronemal protein, WARP is a multiple von Willebrand factor type A domain modular protein that is secreted as a high molecular weight complex, which as it has been reported for *Plasmodium gallinaceum* co-localizes with chitinase (*Pg*CHT1) and *Pg*CTRP in the same micronemal population (Li *et al*., 2004). Although WARP knock out parasites show no developmental defect, (Ecker *et al*., 2008), the fact that WARP localizes on the ookinete surface despite the absence of recognizable membrane-localizing motifs (Li *et al*., 2004), as well as its ability to elicit transmission blocking antibodies (Li *et al*., 2004, Abraham *et al*., 2004), imply an important role for WARP during invasion, and thus it is considered a transmission blocking target.

It was obvious from the experiment reported in 4.4.2 (and is again shown here in D) that the levels of expression and processing of CTRP is not affected, and this was further supported by the fact that the motility of NcPI-S ookinetes is normal. To asses the levels of WARP and SOAP in the NcPI-S expressing ookinetes, protein extract derived from equal numbers of NcPI-S expressing and wt ookinetes were resolved in SDS gel and resulting blots were probed with SOAP and WARP antisera. Compared to the wild type control extracts, the detected signals corresponding to these two proteins were significantly lower in the NcPI-S ookinete extracts, with more pronounced effect of NcPI-S expression on the levels of SOAP (Figure 27). This is a strong indication that exogenous expression of NcPI-S either affects SOAP and WARP expression or their stability.

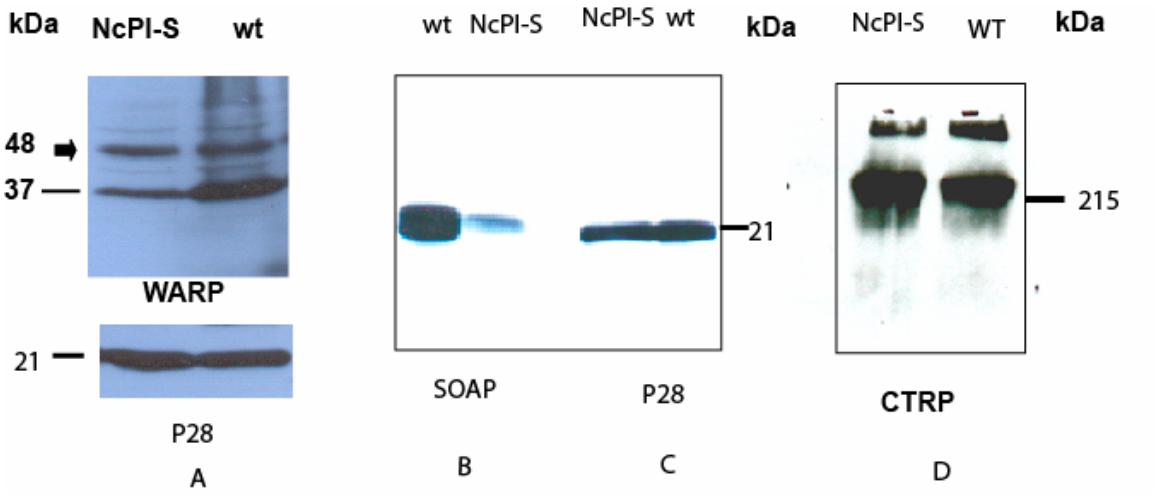

Figure 27: Western blot analysis of NcPI-S effect on SOAP, WARP and CTRP expression

Western blot analysis of protein extract from NcPI-S expressing and wild-type ookinetes probed with A) WARP B) SOAP antibodies. A non-specific band of about 48kDa is indicated an arrowhead in A. P28 used as a loading control in A) (lower panel) and in C). Extract of approximately 1.0 x10^6 ookinetes per lane was loaded. C) P28 specific antibody (13.1) was used as a loading control. D) Extract for assessing CTRP was derived from the in-vivo formed ookinetes (see also figure 17c) Molecular masses are indicated in kDa.

4.5. Generation of transgenic NcPI-S expressing *P. berghei* parasite in sporozoites

As already mentioned, expression of NcPI-S driven by the CTRP promoter is expected to culminate at the 10-day old oocyst. Our studies on the effect of NcPI-S expression on ookinete to oocyst transition, however, have convinced us that NcPI-S is a powerful molecular tool, which may possibly allow us to

molecularly dissect additional critical steps in the sporogonic development, more specifically sporozoite-egress from the mature oocyst and salivary gland invasion by sporozoites. In order to achieve a developmental time shift in NcPI-S expression we generated transgenic parasites in which NcPI-S was placed under the promoter of the CircumSporozoite (CS) gene. CS expression starts from 5-6 day old oocyst and terminates in the sporozoite stage (Simonetti *et al*., 1993). CS protein is also detected in infected hepatocytes (Coppi *et al*., 2005), but it is not clear if this presence reflects an active production, or a load carried by the sporozoites.

4.5.1. Construction of the CSP-spv5NcPI-S targeting vector and transfection

In order to obtain sporozoites that express the NcPI-S serpin, we employed a strategy that was used previously, in order to target transgenes of interest into the endogenous CS locus (Natarajan *et al*., 2001, Engelmann *et al*., 2006). A DNA fragment, corresponding to the NcPI-S open reading frame (fused N-terminally with signal sequence of Pbsub-2 and V5 epitope), was placed between a 1.5 kb fragment of the *CSP 5'UTR* including the *CSP* promoter (Amino *et al*., 2005, Natarajan *et al*., 2001), and a 1.2 kb fragment corresponding to the *Pbdhfr* 3'*UTR*. Upon linearization at a unique KpnI site within the CSP promoter, the expression cassette was integrated into the *CSP 5'UTR* via a single cross-over homologous event (Figure 28 A & B). Integration results into the full reconstitution of the *CSP* gene, while upstream of the locus is

incorporated the 5' UTR *CSP*-(sp-v5-NcPI-S)-3'Pbdhfr/ts UTR transgene separated by pUC19 sequences and the selectable marker cassette, *Tgdhfr-ts* from the *CSP* gene (Figure 28C).

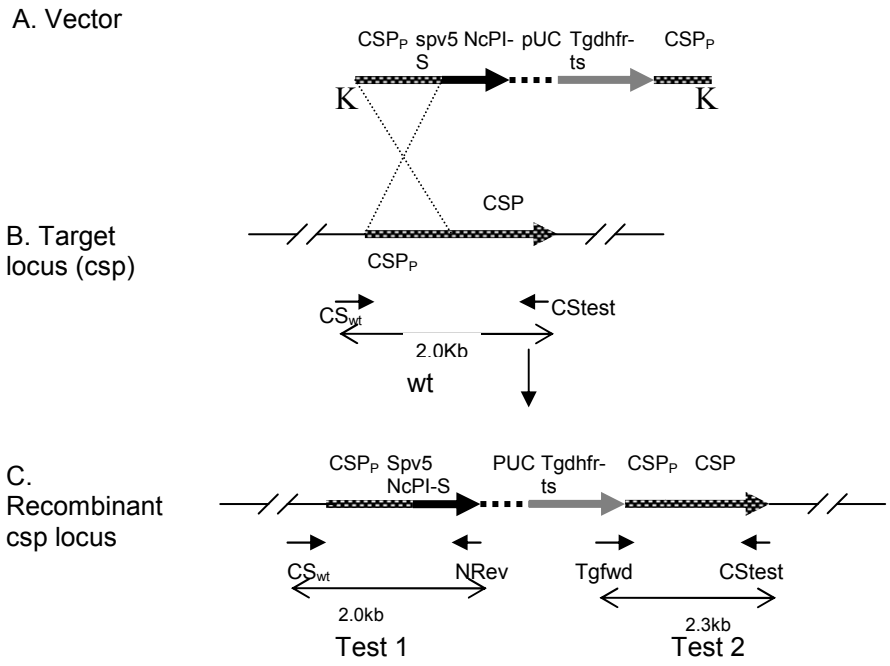

Figure 28: Generation of a transgenic *Plasmodium berghei* parasite expressing NcPI-S in sporozoite stage.

A. The plasmid vector used to integrate the NcPI-S expression cassette into the CS locus. The vector contains pyrimethamine-resistant tgdhfr-ts selectable cassette (tgdhfr-ts) for selection of transgenic parasites. Dotted line indicates pUC19 plasmid sequences. The vector was linearized at a unique restriction site K (KpnI) within the CSP promoter that provides sequences required for single cross over integration.

B. Schematic configuration of the wild type target *CSP* locus. *CSP*$_p$ and *CSP* indicate the circumsporozoite promoter and the circumsporozoite open reading

frame (ORF) sequences respectively. The arrows indicate the primers and the expected wt specific PCR product.

C. Schematic representation of the recombinant *CSP* locus. The integration results in the duplication of the *CSP* promoter in frame with the sp-v5-NcPI-S cassette separated by pUC19 sequences and the selectable cassette (tgdhfr-ts) from a fully reconstituted *CSP* gene. Arrows indicate expected fragment-size upon integration into the correct target locus.

4.5.2. Genotyping of CS-NcPI-S transgenic parasites

Upon cloning by limiting dilution, 3 parasitic lines were obtained and genotyped by diagnostic PCR to confirm integration of the transgene and the reconstitution of the CS locus. Using two sets of integration specific primers, CS_{wt}/NRev and Tgfwd/CStest (Figure 28C), expected products of ≈2.0 Kb (test 1) and ≈2.3 Kb (test 2) were respectively amplified in the transgenic clones but not in the wild type parental strain (Figure 29). The homogeneity of the clones was further assessed for the presence of wt parasites using a wild type specific primer pair (Figure 28B). A diagnostic product of ≈2.0 Kb that indicates a wild type organization of the locus derives only from wt parasites (Figure 29), while is not amplified from the gDNA of the clones. One of the clones, NcPI-S cl.I3 was selected for further analysis.

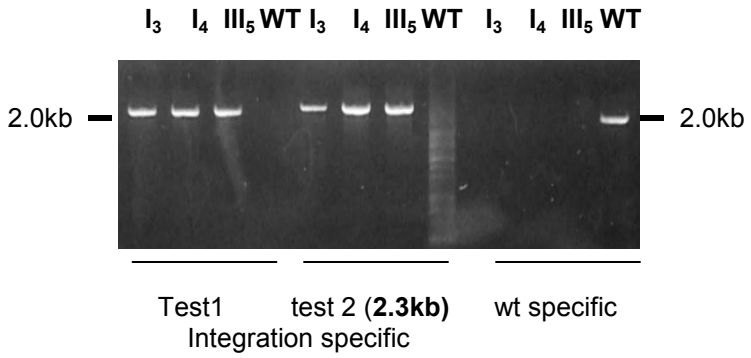

Figure 29: Integration specific PCR genotyping of transgenic parasites clones.

Correct integration of the vector, CS-spv5NcPI-S as revealed by PCR analysis of gDNA isolated from the clones. Lanes I_3, II_4 and III_5: indicate clones of the transgenic parasites and wt denotes wild type parasites. Primers and sizes of the different PCR products derived from respective gDNA are indicated by arrows in figure 28. Note that the primer combinations 'test 1 (2Kb right) and test 2 (2.3Kb middle) are indicative of an integration event whereas the absence of a wild type specific 2Kb fragment indicate the purity of the clones.

4.5.3. Analysis of NcPI-S expression in the transgenic sporozoites

Expression of NcPI-S serpin in the sporozoites was revealed by Western blotting. Equal amounts of extracts derived from $1x10^5$ transgenic or wild-type midgut sporozoites were resolved on two parallel gels 15% 10% and the blots probed with anti-V5 mAb or the CSP mAb respectively followed by peroxidase-coupled anti-mouse IgG. A specific signal of ≈16 kDa corresponding to NcPI-S was readily detected in extracts from transgenic sporozoites (Figure 30A), but

absent in the extracts derived from wild type sporozoites. Moreover, the levels of CSP protein expression were neither affected by the re-organization of the locus, nor infuenced by the co-expression of the NcPI-S (Figure 30B).

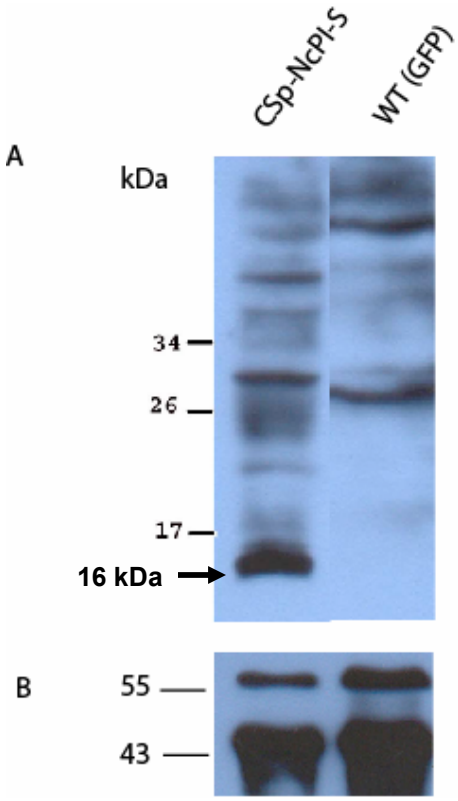

Figure 30: Western blot analysis of NcPI-S expressing sporozoites.

A) An ≈16kDa NcPI-S specific signal (indicated by an arrow) was detected only in the transgenic sporozoites extract using anti V5 mAb. B) As a loading control, extracts from similar numbers of sporozoites were run in a separate 10% SDS-PAGE gel and signal was detected with an anti-CSP monoclonal antibody (Potocnjak *et al*., 1980). The two bands, 44 and 56 kDa correspond to the fragments of a typical processed CSP protein. Antibody dilutions: anti V5, 1:2000, anti-CSP, 1:8000.

4.5.4. Characterization of NcPI-S cl.I3

The asexual and gametocyte-ookinete development of the NcPI-Scl.I3 is normal compared to the wt. To assess the effects of NcPI-S expression on mosquito infection, *Anopheles gambiae* (G3 strain) mosquitoes were fed on mice infected either with NcPI-S cl.I3 or wild type parasites. NcPI-Scl.I3 parasites developed into morphologically normal oocysts at a similar rate compared to the wild type. On day 14 post infection, the midguts of approximately 60 mosquitoes were dissected and sporozoites were mechanically released and counted on a hemocytometer. Salivary glands from the rest of mosquitoes were dissected three to six days later and sporozoites counted. NcPI-S cl.I3 formed midgut sporozoites in similar numbers as the wild type parasite (71,000 sporozoites per mosquito), while similar numbers of salivary gland sporozoites and the same sporozoite losses were recorded (10,000 sporozoites per mosquito). Moreover, the gliding motility of NcPI-Scl.I3 sporozoites as revealed through the trails of shed CS protein when sporozoites are left to glide on a solid support (Stewart and Vanderberg 1988) was indistinguishable from that of wild type ones (Figure 31A & B). These results indicate that neither the motility nor the salivary gland invasion was affected by NcPI-S expression.

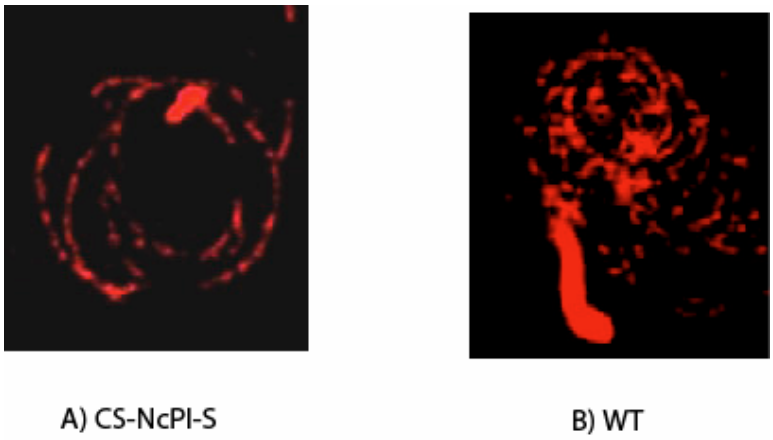

Figure 31: CS-NcPI-S transgenic salivary gland sporozoites perform gliding locomotion indistinguishable from WT sporozoites. Salivary gland sporozoites were allowed to glide in chamber slides at 37°C for 30 minutes and fixed with 4% paraformaldehyde, followed by 0.05% saponin permeabilization. The slides were stained with the primary antibody, anti-CSP and secondary antibody labeled with Alexa 555. Trails of CSP protein deposited on the slide during sporozoite gliding and detected by anti-CSP antibody both in the NcPI-S expressing and wt are indicative of normal sporozoites. In each case, the sporozoite is intensely stained (elongated shape).

In order to exclude the possibility that NcPI-S expression in sporozoites affects host infection, a bite-back experiment was performed. Equal numbers of mosquitoes infected with either NcPI-S cl.I3 or wild type parasites were allowed, on day 20 post infection, to feed on naïve Balb C mice. NcPI-S cl.I3 salivary gland sporozoites inoculated during the blood feeding were able to infect Balb C mice with prepatent periods (period necessary for apparent parasitemia) similar to wild type ones. NcPI-S cl.I3 parasites, as wild type ones, were first detected in

Giemsa stained blood smears 4–5 days after mice infections. In order to verify that infection established in the mouse was due to NcPI-S cl.I3 parasites and not to putative wild type revertants, PCR genotyping of blood stage parasites derived from NcPI-Scl.I3 infected mouse was performed. The diagnostic PCR products of ≈2.0 Kb and 2.3 Kb representative of NcPI-Scl.I3 parasites were amplified from the genomic DNA prepared from the resulting blood stage parasites whereas the wt specific product was never amplified (Figure 32). This confirmed that sporozoites inoculated into the mouse by the mosquito bites were of NcPI-Scl.I3 genotype. Contrary to NcPI-S expressing parasites in the ookinete stage whose ability to invade midgut epithelium is adversely compromised, this data indicate that the NcPI-S expressing sporozoites are not only normally formed but they are also fully infectious to the mosquito salivary glands and able to complete hepatocyte infection and liver stage development in the vertebrate host (mice).

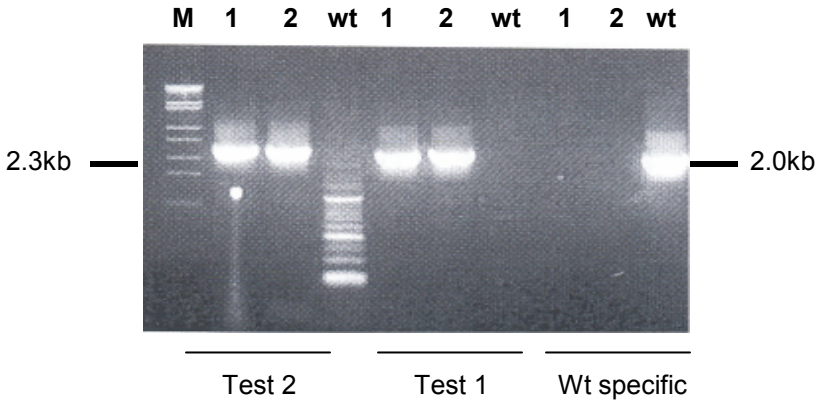

Figure 32: PCR analysis of two representative parasite clones derived from bite-back experiment. Infected mosquitoes on day 20 post infection were fed on naïve Balb C mice, inoculating sporozoites during the meal. gDNA resulting from the blood stages established 4-5 days after the feed was analyzed by PCR

as described earlier. All the tested clones retained their original NcPI-Scl.I3 genotype, as well as, their purity shown by the amplification of 2.0 and 2.3Kb integration specific and absence of 2.0Kb wt specific products. 1 and 2 denote clones of the transgenic parasites, wt denotes wild type parasites and M is the molecular marker. Primers and sizes of the different PCR products are as described in figure 28 and 29.

4.6. Preliminary validation of recombinant NcPI-S (rNcPI-S) purified from *E. coli* as transmission blocking agent.

A plasmid (pQE30-NcPI-S) for bacterial production of NcPI-S was constructed by amplifying the V5 epitope tagged NcPI-S ORF (excluding the Pbsub-2 signal sequence) from gDNA template derived from the transgenic *P.berghei* parasite NcPI-S cl3 rNcPI-S was produced by following established protocols and purified using Ni-NTA column (Qiagen) according to manufacturer's instructions. The purity of the rNcPI-S was assessed by Coomassie staining of the purified protein resolved by SDS-PAGE. rNcPI-S under reducing conditions runs as a ~16 kDa band, which most probably corresponds to a stable homodimeric form. Elution 2 (E2 Figure 33), which contained the bulk of the rNcPI-S, was dialysed against 0.1MTris-HCl pH 8.0, and its inhibitory activity against commercially available bacterial subtilisin Carlsberg tested (data not shown).

After Bradford quantitation, rNcPI-S was mixed with infected blood derived from mice infected with the wild type GFP expressing strain 507cl1, in three

different concentrations: 30, 90 and 160mg/ml. Exflagellation centres, a measure of male gametogenesis, were counted and compared to the controls 10-20 minutes later. There was no difference in the number of exflagellation events in the presence or absence of rNcPI-S. Subsequently, blood samples (with the three different concentrations of rNcPI-S) were mixed with RPMI medium and the cultures were maintained at 19-21°C. After 24 hours, Giemsa-stained smears of the blood pellets (containing ookinetes) were scored for ookinetes on a hemocytometer chamber. The ookinete yields of the treated parasites were indistinguishable from the untreated. The morphology of the rNcPI-S treated ookinetes was also comparable to the control. Two controls were used, cultures without rNcPI-S addition and addition of Tris-HCl, the buffer in which the rNcPI-S was diluted.

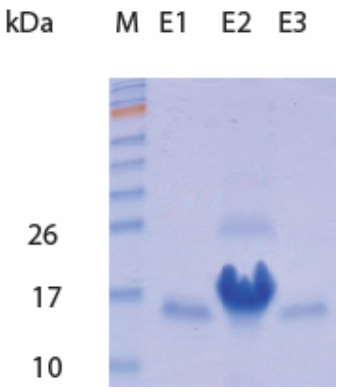

Figure 33: Coomassie blue stained gel showing purified rNcPI-S. Purified rNcPI-S was resolved in 15% SDS-PAGE and stained with Coomasie dye. E1, E2 and E3 indicate respective NcPI-S elutions 1, 2 and 3 obtained during the purification. M denotes molecular size marker.

To evaluate the rNcPI-S effects in the *in vivo* ookinete-oocyst transition, preformed wt (GFP 507 cl1) ookinetes were incubated for 5 minutes with varying concentrations of the rNcPI-S, 10, 30 and 120 mg/ml, mixed with naïve mouse blood and fed to mosquitoes through membrane feeders. The mosquitoes were dissected 10 days later and oocysts numbers determined. As controls, untreated ookinetes from the same batch were handled under similar conditions. In all rNcPI-S concentrations, the parasites formed oocysts in a range not deviating from the two controls (no rNcPI-S, and Tris-HCl buffer).

Discussion

Proteases are known for a long time to be critical mediators of essential processes across evolution. Dozens of proteases representing all the families have been identified in *Plasmodium* genomes with a number of them linked to essential roles during the parasite development both in the vertebrate host and the mosquito vector. In the vertebrate host, for instance, merozoite invasion, haemoglobin degradation that provides amino acid for the developing parasite, egress of merozoites from mature schizonts, are among the processes mediated by proteases. In the mosquito vector, involvement of proteolysis has been postulated so far for events such as gametogenesis, invasion of the mosquito midgut by ookinetes, and possibly for the egress of sporozoites from mature oocysts.

Importance of proteolysis in invasion was first uncovered through the use of broad range chemical inhibitors (Hadley *et al*., 1983). A set of serine protease

inhibitors prevented shedding of two merozoite surface proteins (MSP-1 and AMA-1), which drastically impaired merozoite invasion (Blackman 2000). Similarly to the use of serine protease inhibitors, cysteine protease inhibitors (CPIs), such as E64, unveiled roles of cysteine proteases (CPs) in *Plasmodium*. Interestingly, the use of E64 blocks circumsporozoite protein (CSP) processing, which results in inhibition of hepatocyte invasion *in vitro* and confers protection from malaria infection to pre-treated mice (Coppi *et al*., 2005). The metal chelator 1, 10-phenanthroline, other than erythrocyte invasion by merozoites, affects exflagellation by interfering with microgamete motility, indicating an implication of a metalloprotease activity in both processes (Rupp *et al*., 2008 Kitjaroentham *et al*., 2006).

Functional analysis of genes in *Plasmodium* species is conventionally achieved by applying a knock out strategy that depletes the parasite from the corresponding gene product. In the case of genes vital for merozoite survival, however, this strategy is unfeasible. Indeed, the classical knock out approach has been proven unrealistic in the case of SUB2 (Uzureau *et al*., 2004), and the same is likely for additional proteases expressed throughout the parasitic life cycle. SUB-2, a membrane-associated member of the subtilase superfamily, has been established through extensive genetic, molecular and biochemical analysis, as the primary candidate sheddase responsible for AMA1 and $MSP1_{42}$ processing (Harris *et al*., 2005). Importantly, SUB2 is among the shed parasite-derived proteins, during ookinete midgut invasion, although its involvement in invasion still remains unclear (Han *et al*., 2000). The inability to knock out the

corresponding gene hampers in depth investigation of SUB2 protease function not only in asexual but in sporogonic development too.

In this work, we validated an alternative strategy to functionally “switch off” proteases, and/or proteolytic machineries, at the early sporogony by using Macromolecular Protease Inhibitors (MPIs). MPIs control endogenous proteolytic pathways in almost every living organism. MPIs are classified into families and each family shows specificity to a certain class of proteases. For example, serpins, Serine Protease Inhibitors control serine proteases (Gettins 2002), while cystatins, regulate cysteine proteases (Bobek and Levine 1992). Members of a certain family show a certain degree of target protease preference, for example α1-antitrypsin inhibits trypsin.

Compared to their chemical counterparts, MPIs due to their protein-nature can be expressed *in vivo* in transgenic parasites, inducibly or in a stage specific manner as well as targeted into specific subcellular compartments. Moreover, MPIs can be molecularly manipulated to specifically inhibit desired target protease (s), or to enhance their inhibitory strength (Anderson *et al*., 1993, Watanabe *et al*., 1995). As a characteristic example, engineered alpha1-antitrypsin variants selectively inhibit subtilisin-like proprotein convertases PACE4 and PC6 (Tsuji *et al*., 2007).

Since at the start of this work our aim was to achieve an *in vivo* functional inhibition of SUB2 at the sporogonic stages, we chose two serpins, one a member of the well described ovalbumin family and the other of the non-classical Kazal (NcPI-S). We expressed them in a stage specific manner, under an

ookinete or a sporozoite promoter, in transgenic *P. berghei* parasites. Besides its inhibitory potency against bacterial subtilisins *in vitro*, the choice of the Kazal serpin NcPI-S was influenced by the fact that it is expressed by *Neospora caninum* (Bruno *et al*., 2004), which is phylogenetically related to *Plasmodium.* The exact role of NcPI-S in *N. caninum* remains unknown, however, a possible interaction with a *N. caninum* subtilisin-like serine protease (Louie *et al*., 1999, 2002) has been proposed (Bruno *et al*., 2004).

It should be emphasized at this point that no protease inhibitors have been so far identified in the *Plasmodium* genomes. However, non classical Kazal serpins have been previously reported in *T.gondii and N.caninum,* both members of apicomplexa family (Pszenny *et al*., 2000, 2002; Morris *et al*., 2002; 2004, Bruno *et al.,* 2004, Morris and Carruthers, 2003). Based on the conserved cysteine residues, a feature of Kazal inhibitors, our blast homology searches of the *Plasmodium* genome databases returned insignificantly low homology results.

The same rule, (inhibitory potency against bacterial subtilisins), influenced the selection of an *A. gambiae* serpin, the CAM serpin (Danielli *et al.,* 2003), as a scaffold in order to generate a molecularly engineered variant, LGI, ideally mimicking a substrate for SUB2. Considering robust biochemical evidence suggesting the merozoite $Msp1_{42}$ surface molecule as a substrate for SUB2, we changed 10 amino-acids, including the critical for serpin specificity, P1-P1' residues, according to the predicted cleavage site of $PbMsp1_{42}$.

Expression analysis in the derived transgenic parasites provided convincing evidence that both serpins, LGI and NcPI-S are properly expressed in the transgenic ookinetes. Importantly, NcPI-S is expressed in the form of SDS-stable homodimers similar to the expression of the endogenous NcPI-S in *N. caninum* tachyzoites (Morris *et al*., 2004). Based on this fact and in addition to our observation that bacterially produced, and active *in vitro* against bacterial subtilisins, NcPI-S also forms SDS-stable homodimers; we are almost convinced that NcPI-S expressed in ookinetes retains its inhibitory potential.

On the other hand, LGI is detected in ookinete extracts as a major ~45 kDa product. In the case in which LGI could function as a suicide protease inhibitor in ookinetes, formation of stable SDS complexes with the target protease(s) should be expected. However, such complexes were never observed. Ovalbumin serpin modification around the reactive site loop, a region that dictates its specificity, influences drastically its inhibitory activity (Irving *et al*., 2007) and may lead to unwanted results. In some cases the modifications render the serpin completely inactive or impair its suicidal function by promoting a substrate-like rather than a suicidal inhibitory mechanism (Laskowski and Kato 1980, Schulze *et al*., 1990, Anderson *et al*., 1993, Kojima *et al*., 2007). Replacement for example of a large portion of antichymotrypsin (ACT) RSL P10-P5' or P10-P10' with α1-antitypsin (AT) residues, generated a substrate instead of an inhibitor for neutrophil elastase (Rubin *et al*., 1990). In a similar way, CAM alteration to generate its variant, LGI may ultimately have produced a poorly competitive substrate. Although we favor this interpretation, as this should

explain the detection of a minor protein band of ≈37kDa (Figure 15), putatively corresponding to a LGI proteolytic product. We do not exclude the possibility that by modifying drastically the region around the P1-P1' residues of CAM to generate the LGI we completely abolished its inhibitory function.

Moreover, in addition to the serpin reactive site loop, the serpin scaffold is important in defining the inhibitory characteristics of a serpin (Bottomley and Stone 1998). Introducing the modification into different serpin scaffolds may generate a variant of desired inhibitory activity. The *Drosophila* serpins, sp4 and sp6, potent inhibitors of serine proteases, the subtilisin-like proprotein convertases (Osterwalder *et al.,* 2004) and trypsin (Han *et al.,* 2000b) respectively can serve as alternative scaffolds.

The initial phenotypic characterization of the derived transgenic parasites revealed a drastic effect of the ectopic NcPI-S expression on oocyst development. Instead the expression of LGI did not have any considerable effect. To identify the point of developmental arrest of NcPI-S expressing parasites we applied a combination of cell biology, *in vitro* ookinete cultures and mosquito infections. We checked the ability of the parasites to complete gametocyte-ookinete transition both *in-vitro* and *in vivo*, since this process was previously shown, through the use of chemical protease inhibitors to involve proteolysis (Torres *et al*., 2005, Rupp *et al*., 2008). NcPI-S expressing parasites transform into mature ookinete without any noticeable deviation in morphology or efficiency from the wild type both *in vitro* and *in vivo*. Moreover, motility of NcPI-S expressing ookinetes, which has been also suggested to be mediated by

proteases, appears to be normal. Infection experiments with ingested gametocytes revealed an inability of the NcPI-S expressing ookinetes to associate tightly with the midgut epithelium, a pre-requisite for successful invasion (Sinden and Billingsley 2001, Han *et al.,* 2000). This however, does not exclude the possibility that the NcPI-S expressing ookinetes may be in addition deficient in peritrophic matrix (PM) penetration.

As it has been suggested that PM penetration may involve proteolytic processing, which activates chitinase, the responsible enzyme for the hydrolysis of chitin, the major constituent of PM. Feeding mosquitoes on *in vitro* pre-formed ookinetes mixed with naïve blood allows the ookinetes to associate fast with the midgut epithelium. Since, bypassing the PM in this way still does not reverse the oocyst reduction (Table 3); we concluded that NcPI-S expressing ookinetes are defective beyond the PM traversal. They fail to associate tightly with the midgut epithelium.

A failure in the association with the midgut epithelium could be due to a blockage of critical proteolytic events taking place at the ookinete/mosquito cell interface. Several lines of evidence indicate that receptor-ligand type of interactions establish an efficient ookinete passage, and proteases of parasitic or even of mosquito origin may process molecules from both sides to facilitate and/or enhance these interactions (Shahabuddin *et al*., 1993, 1996, Srinivasan *et al*., 2009, Zieler *et al*., 2001, Ghosh *et al.,* 2001, Ito *et al*., 2002, Dinglasan *et al.,* 2007a, 2007b, Lavazec *et al*., 2007). As it is reported, SUB2 is shed during midgut invasion, which raises certain possibilities; its function may have either a

short or longer distance effect around the invading ookinete, and/or putative SUB2 targets may be of parasitic or mosquito origin. If actively secreted and diffused NcPI-S, as initially designed, could potentially have a longer distance inhibitory effect on such proteolytic processes. NcPI-S expressing ookinetes however, did not affect the passage of wild type ookinetes, when they were provided together in the same blood meal (Figure 26A). Looking at the same result in a slightly different way, one could assume that there is no “synergy” between ookinetes in traversing the midgut epithelium, as the presence of wild type ookinetes does not make the midgut epithelium more receptive for the NcPI-S-expressing ones.

We were unable to collect evidence indicating secretion of NcPI-S from NcPI-S expressing ookinetes, although we have tried to detect secreted NcPI-S in ookinete culture supernatants either without or upon stimulation by co-culturing ookinetes with insect cells (data not shown). Nevertheless, fractionation of ookinete extracts revealed a relatively tight association of NcPI-S with the membrane fraction despite the lack of a region that potentially could function as a membrane retention signal. Therefore we speculate that NcPI-S possibly associates with the membrane indirectly, e.g. through a partner/target membrane associated protein. However, it is not clear if NcPI-S association with the membrane is the only cause of the NcPI-S phenotype, since, a great amount of NcPI-S was detected in the soluble fraction. In other words, NcPI-S may target equally membrane associated and/or cellular proteases. It should be stressed out at this point that although recombinant NcPI-S inhibits *in vitro* bacterial subtlilisin

completely, it shows lower inhibitory capacity on human neutrophil elastase, animal trypsin, and chymotrypsin (Bruno *et al*., 2004). Therefore, the possibility that NcPI-S when expressed in ookinetes may inhibit a broader range of serine proteases should not be excluded, and should be further investigated. For instance, use of Differential In-Gel Electrophoresis (DIGE) technique may reveal altered patterns at expression level of proteins in whole ookinetes.

There are a couple of negative experimental results, which presently exclude SUB2 from our suspected targets of NcPI-S, despite the fact that NcPI-S was initially selected as a putative SUB2 inhibitor. First, we were unable to co-immunoprecipitate SUB2 in complex with NcPI-S from ookinete extracts, even after involving chemical cross linking (data not shown). In addition, only one band of ≈64 kDa was detected using a rabbit anti-PbSUB2 in total ookinete extracts, which corresponds to the mature form of the protease (Data not shown). Similarly to other subtilisins, in order to generate its mature active form, SUB2 undergoes an autocatalytic cleavage within the ER through which a prodomain that renders it inactive is removed (Withers-Martinez *et al*., 2004, Harris *et al*., 2005, Hackett *et al*., 1999). The prodomain often remains non-covalently attached to the catalytic domain and is competed out by the substrate. A blockage in autocatalytic maturation could possibly indicate functional inactivation of SUB2 by NcPI-S and such a blockage was not observed.

We considered that similarities in the phenotypic patterns may indicate putative NcPI-S targets. At least two proteases have been so far functionally linked to the process of gametocyte to oocyst transition. The cysteine protease

falcipain-1, is dispensable for erythrocytic growth, but is critical for gametocyte to oocyst transition (Eksi *et al*., 2004). An orthologue of Falcipain-1 is easily identified in *P. berghei;* however, we presently exclude the possibility that it may be a target for NcPI-S. There are few serpins reported in literature that show a cross-class inhibition, for instance, endopin (Hwang SR *et al.,* 2005) and SCCA/SQN5 (Al-Khunaizi *et al.,* 2002), both belong to the ovalbumin family and NcPI-S is a non classical Kazal serpin. However, comparison of SOAP and WARP expression levels in bergheipain-1/falcipain-1 knock out parasites should verify the exclusion of this protease as a potential NcPI-S target.

More recently, PbROM1, a member of the intramembrane serine protease family, the rhomboids, was also linked to the process of oocyst development (Srinivasan *et al*., 2009). Interestingly, PbROM1 (-) phenotype has many similarities with the NcPI-S phenotype. In both cases the point of developmental arrest is beyond the ookinete formation. In addition, PbROM1 (-) sporozoites are formed normally and are invading efficiently the mosquito salivary glands and the same is observed in the case of CS-NcPI-S expressing sporozoites. Moreover, it should be noticed at this point that when NcPI-S is constitutively expressed in transgenic *Toxoplasma gondii* tachyzoites, the later shows a delay at the first replication cycles (Zoe Tampaki unpublished), a phenotype very similar to the one reported for the TgROM1 conditional disruptants (Brossier *et al*., 2008). Based on these phenotypic analogies, the possibility that NcPI-S may target efficiently ROM1 emerges. In any case, biochemical evidence, (which we try

presently to collect), and use of DIGE techniques are necessary in order to further support this hypothesis.

The nature of NcPI-S phenotype, deficiency in ookinete attachment and invasion, implies a severe malfunction in micronemal proteins, as many of those have been directly implicated in the processes. Therefore we aimed to check the levels and processing of few micronemal proteins. The choice was random and depended on the availability of antibodies. We analyzed three micronemal proteins WARP, CTRP and SOAP known to be essential during ookinete-oocyst development (Blanco *et al*., 1999, Templeton *et al*., 2000, Dessens *et al*., 1999, 2003, Li *et al*., 2004). CTRP showed normal expression levels compared to the wt (Figure 27), while instead WARP and SOAP expression levels reduced in the NcPI-S expressing ookinetes to a varying degree (Figure 27).

The strength of the NcPI-S phenotype compared to that of the reported soap KO phenotype indicates that levels and/or secretion of additional micronemal proteins may be equally affected. Parasites completely depleted from SOAP show a ~60% reduction in oocyst formation, while in NcPI-S expressing parasites, in which SOAP is still detected this reduction reaches 97%. Therefore, it is possible that NcPI-S affects the levels of additional essential soluble secreted micronemal proteins of the ookinete in a similar manner, and thus their combined effects may generate a stronger phenotype. We can only speculate about reasons that could possibly connect NcPI-S expression and low levels of WARP and SOAP in transgenic ookinetes. For example the synthesis, assembling and maturation of micronemes, the only secretory organelles in

ookinetes (Li *et al*., 2004), could be affected and as a consequence proteins destined for micronemes follow alternative secretion pathways or are marked for degradation. We are presently checking microneme formation in NcPI-S expressing ookinetes by electron microscopy and our preliminary data suggest malformed micronemes.

Regulation of biogenesis of secretory organelles such as dense granules, rhoptries, micronemes and lysosome-related organelles is thought to involve proteolysis (Shaw *et al*., 2002, Binder and Kim 2004, Kim 2004, Kuliawat *et al*., 2000, Zhou *et al*., 1999, Berson *et al*., 2003, Turkewitz *et al*., 2004), however, proteases with a critical role in organellar biogenesis *per se* in apicomplexans are not yet identified. Due to our previous speculation that ROM1 may be a target of NcPI-S, it would be interested to investigate the levels of SOAP and WARP in ROM1 KO ookinetes. Alternatively, NcPI-S may affect SOAP and WARP levels in a totally different way, not involving a protease inhibition. It is established that soluble micronemal proteins form complexes with membrane protein partners in order to exit from the ER/Golgi and being trafficked into the micronemes (Reiss *et al*., 2001, Huynh *et al*., 2003). SOAP and WARP ability to form high molecular mass complexes via disulfide bonds (Dessens *et al.,* 2003) support the notion that it may associate with other microneme destined molecules and being trafficked into the microneme as a cargo protein. In this sense, cysteine-rich NcPI-S may function as competitor of those secreted micronemal proteins for the same carrier protein. This could potentially reduce significantly the chances of the later to form essential complexes necessary for their proper trafficking.

Contrary to the strong oocyst reduction phenotype, the NcPI-S expression in sporozoites neither interrupted sporozoites development nor affected their infectivity for both the vector and the vertebrate host. Ignoring other differences between the two mutant strains (i.e. different levels of NcPI-S expression), this could be explained if the target of NcPI-S either is not present or does not play the same critical role in sporozoites as in ookinetes. Such a putative target could be for example ROM1.

In addition, it should be stressed that despite the fact that both the ookinete and the sporozoite are invasive forms of the parasite, they differ significantly in their organellar structure. Sporozoites have both micronemes and rhoptries whereas micronemes are the only secretory organelle in the ookinete. If NcPI-S indeed affects the biogenesis of micronemes as we hypothesized in ookinetes, the presence of rhoptries in sporozoites, a second secretory organelle, may serve as an alternative destination for secreted micronemal proteins. Recently, DrpB, a member of Dynamin-Related Proteins conserved in apicomplexan, was shown to play a key role in the biogenesis of secretory organelles in *T. gondii* (Breinich *et al*., 2009). Conditional depletion of DrpB results into mature progeny that are devoid of micronemes and rhoptries. In the absence of these two organelles, invasion-related secretory proteins are mistargeted to the constitutive secretory pathway.

4.7. Concluding remarks

This study demonstrates for the first time that a “foreign” protein, and importantly a serine protease inhibitor, NcPI-S, ectopically expressed in a stage specific manner affects the development of mosquito stages of *Plasmodium* parasites. We speculate that interruption of microneme formation or trafficking of secreted/chaperone proteins into micronemes may be the cause of this detrimental effect. In any case, however, NcPI-S deserves further exploration as a novel tool for interventions against malaria, including transmission-blocking approaches. Important future steps to undertake are the identification of the target molecule and the development of a simple assay that would allow us a better understanding of the molecular nature of the interaction, by involving mutagenesis and structural studies. Such studies may aid the improvement of NcPI-S as inhibitory molecule and the design of novel highly selective inhibitory molecules.

Moreover, and as it was indicated by the present work, the simplicity of ookinete invasive form in which micronemes is the only secretory organelle, in comparison to the other two invasion forms, the sporozoite and the merozoite in which the organellar structure is more complex, allowing compartmentalization of proteolytic processes critical for the invasion, makes it ideal test tube system for a high throughput screening of additional macromolecular protease inhibitors. Such MPIs are already in the pipe line for testing in our laboratory, among which two multi-domain Kazal-like protease inhibitors, EPI1 and EPI10 from *Phytophthora infestans* which specifically inhibit subtilisin Carlsberg and tomato

P69B subtilisin-like serine protease *in vitro* (Tian *et al.,* 2004, 2005), as well as, cysteine protease inhibitors, cystatins. However, improvements of this approach are necessary in order to facilitate the whole screening procedure, and more specifically the introduction of proteomics analysis that will simplify the search for the upstream targets and the downstream affected molecules and molecular pathways.

In general discovery of molecules with inhibitory effect against vital proteases may substantially contribute to the efforts towards effective malaria vaccine development. When expressed in a stage specific and/or inducible manner (in merozoites or in infective sporozoites) such molecules may generate genetically attenuated parasites, which could be used as immunogens. A number of genetically attenuated parasites generated by disrupting essential genes (van Dijk *et al*., 2005, Ishino *et al*., 2005, Douradinha *et al*., 2007, Mueller *et al*., 2005a, 2005b) are currently tested, and proteolysis deficient strains could be added into this list. Alternatively, since mosquitoes are obligatory vectors for malaria transmission, MPIs with a potential inhibitory role of proteolytic processes taking place at the parasite/mosquito cell interface may provide valuable transmission blocking agents.

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