MOLECULAR STUDIES ON *PLASMODIUM*
DURING DEVELOPMENT IN THE MOSQUITO

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A thesis submitted in accordance with the requirements of Imperial College London for the degree of Doctor of Philosophy

May 2011
DECLARATION

I confirm that this thesis entitled “Molecular studies on Plasmodium during development in the mosquito” and the research to which it refers is the product of my own work and that any contributions from the work of others have always been fully acknowledged and clearly referenced.

Rebecca Armson
ABSTRACT

Among the factors that regulate transmission of malaria are host-derived immune factors which can inhibit the progression of *Plasmodium* through the mosquito. This thesis addresses whether members of a family of LCCL/lectin Adhesive–like Proteins (LAPs) could act as feasible targets to elicit an antibody-mediated blockade in parasite transmission. The LAP family comprises six putatively secreted multidomain proteins which appear conserved among *Plasmodium* species and other apicomplexan parasites. Studies utilise the *Plasmodium berghei* rodent malaria parasite as an experimental system to evaluate the potential transmission-blocking activity of antibodies raised against LAPs. Regions of the proteins were selected for heterologous expression in *Escherichia coli*. Eight recombinant proteins were successfully expressed and were used as antigens to generate antisera in mice. Following immunogenicity tests, antisera against regions of LAP1, LAP3, LAP4 and LAP6 were selected for further characterisation and tested for antimalarial transmission-blocking activity. A combination of *in vitro* and *in vivo* assays revealed that the presence of anti-LAP antibodies did not inhibit parasite development. Whilst not conclusively excluding their potential, this work provided no further evidence to support the inclusion of LAPs as candidates for transmission-blocking vaccines. Immunolocalisation studies using anti-LAP1 antibodies revealed that the protein is expressed in cytoplasmic regions of female gametocytes and to a lesser extent in female gametes. Although it has been proposed that LAP expression may cease following fertilisation, PbLAP1 was detected in the cytoplasm of developing zygotes and intriguingly was subsequently found to concentrate in compartments of the *P. berghei* ookinete corresponding to the crystalloids. Antisera against regions of LAP1, LAP3, LAP4 and LAP6 were all found to similarly label the distinct electron dense cellular compartments. Furthermore, observation of *Pblap* mutant ookinetes by light microscopy indicated deficiencies in the formation of crystalloids. A transient cellular compartment, formed in the ookinete and subsequently fragmenting during early stages of oocyst development, roles of the crystalloid remain unknown. The association between LAPs and the crystalloids however leads to potential insights into the biological roles of the LAP family, the crystalloids, and the cellular processes of *Plasmodium* sporogony. Through microarray analysis, a comparison of the transcriptional profiles of *P. berghei Δlap1* and wild-type ookinetes was made, detecting 274 differentially expressed genes and thereby indicating that the absence of PbLAP1 and resulting deficiency in crystalloid formation may have several knock-on effects on cellular processes important to *Plasmodium* development in the mosquito.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ABS</td>
<td>Asexual blood stage</td>
</tr>
<tr>
<td>ACT</td>
<td>Artemisinin combination therapy</td>
</tr>
<tr>
<td>Ag</td>
<td><em>Anopheles gambiae</em></td>
</tr>
<tr>
<td>AMA</td>
<td>Apical membrane antigen</td>
</tr>
<tr>
<td>ANKA</td>
<td>Anvers/Kasapa</td>
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<tr>
<td>APL</td>
<td><em>Anopheles Plasmodium</em>-responsive leucine-rich repeat</td>
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<tr>
<td>APN</td>
<td>Aminopeptidase N</td>
</tr>
<tr>
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<td>Aspartyl protease</td>
</tr>
<tr>
<td>AT</td>
<td>Adenine-thymine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BAEBL</td>
<td>BA erythrocyte binding-like protein</td>
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<tr>
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<td>Bagg albino/c</td>
</tr>
<tr>
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<td>Base pair</td>
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<tr>
<td>EBA</td>
<td>Erythrocyte binding antigen</td>
</tr>
<tr>
<td>ECP</td>
<td>Egress cysteine protease</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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ECP  Egress cysteine protease
EDTA  Ethylene diamine tetraacetic acid
EGF  Epidermal growth factor
ELISA  Enzyme linked immunosorbent assay
EMP  Erythrocyte membrane protein
ER  Endoplasmic reticulum
F-actin  Filamentous actin
FBS  Foetal bovine serum
FN2  Fibronectin type 2 domain
FH  Formin homology
fHBP  Factor H-binding protein
GAP  Glideosome-associated protein
Gc  Gametocyte
GCS  Generative cell specific
GFP  Green fluorescent protein
GLURP  Glutamate-rich protein
GPI  Glycosylphosphatidylinositol
Gm  Gamete
GST  Glutathione S-transferase
HEPES  Hydroxyethyl-piperazine-ethanesulphonic acid
HSP  Heat shock protein
IFA  Immunofluorescence assay
IMC  Inner membrane complex
IMP  Intramembranous particles
i.p.  Intraperitoneal
IPT  Intermittent preventive treatment
IPTG  Isopropyl-beta-D-thiogalactopyranoside
iRBC  Infected red blood cell
IRS  Indoor residual spraying
ITN  Insecticide treated nets
LAP  LCCL/lectin adhesive-like protein
LB  Luria broth
LCCL  Limulus clotting factor C, Coch-5b2 and Lgl1
LCR  Low complexity region
LH  Lipoxygenase homology
LIC  Ligation independent cloning
LRIM  Leucine rich-repeat immune gene
LSA  Liver stage antigen
MACPF  Membrane-attack complex and perforin-related domain
MAEBl  Merozoite AMA1/erythrocyte binding ligand-like protein
MAOP  Membrane-attack ookinete protein
MAPK  Mitogen-activated protein kinase
MDV  Male development gene
ME  Multiple epitopes
MEOP  Microneme-enriched ookinete proteome
MIC  Microneme protein
MMLV-RT  Moloney murine leukaemia virus reverse transcriptase
mRNA  Messenger RNA
Mrz  Merozoite
MSP  Merozoite surface protein
MTIP  Myosin A tail domain interacting protein
MTOC  Microtubule organising centre
MTRAP  Merozoite thrombospondin-related adhesive protein
MudPIT  Multidimensional protein identification technology
MVA  Modified vaccinia virus Ankara
MyoA  Myosin A
NEC  Neurexin-like domain
NiNTA  Nickel-nitrilotriacetic acid
NOS  Nitric oxide synthase
NRE  Nanos response element
OB  Osmiophillic body
Ocy  Oocyst
Ook  Ookinete
OSEP  Ookinete surface-enriched proteome
PAGE  Polyacrylamide gel electrophoresis
Pb  *Plasmodium berghei*
PBS  Phosphate-buffered saline
PCR  Polymerase chain reaction
PEG  Proteins of early gametocyte
Pf  *Plasmodium falciparum*
PH  Phenylhydrazinium chloride
PLA  Phospholipase A
PLAT  Polycystine-1, lipoxygenase, alpha toxin homology
PPLP  Plasmodium perforin-like protein
PPO  Prophenoloxidase
PO  Phenoloxidase
PUF  Pumilio family
PV  Parasitophorous vacuole
PVM  Parasitophorous vacuole membrane
RAP  Rhooptry-associated protein
RB  Refractile body
RBC  Red blood cell
RBM  Roll Back Malaria
RESSA  Ring-infected erythrocyte surface antigen
Rs  Ricin domain
RNA  Ribonucleic acid
RNaseA  Ribonuclease A
RNAi  RNA interference
ROM  Rhomboid serine protease
RPMI  Roswell park memorial institute
RT-PCR  Reverse transcriptase PCR
SALSA  Sporozoite and liver stage antigen
SDS  Sodium dodecyl sulphate
SEM  Standard error of the mean
SERA  Serine repeat antigen
SM1  Salivary gland and midgut binding peptide 1
SMART  Simple Modular Architecture Tool
SOAP  Secreted ookinete adhesive protein
SRCR  Scavenger receptor cysteine-rich
SP  Signal peptide
Spz  Sporozoite
SRPN  Serine protease inhibitor
STARP  Sporozoite threonine-asparagine-rich protein
STEVOR  Subtelomeric Variable Open Reading Frame protein
TE  Tris-EDTA
TEM  Transmission electron microscopy
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>TEP</td>
<td>Thioester-containing protein</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with tween-20</td>
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<tr>
<td>TBV</td>
<td>Transmission-blocking vaccine</td>
</tr>
<tr>
<td>Tg</td>
<td><em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>TO</td>
<td>Theiler’s Original</td>
</tr>
<tr>
<td>took</td>
<td>Transforming ookinete</td>
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<tr>
<td>Tpz</td>
<td>Trophozoite</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombospondin-related adhesive protein</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane-hydrochloric acid</td>
</tr>
<tr>
<td>TSR</td>
<td>Thrombospondin type I repeat</td>
</tr>
<tr>
<td>TVN</td>
<td>Tubovesicular network</td>
</tr>
<tr>
<td>Tween20</td>
<td>Polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UIS</td>
<td>Upregulated in infective sporozoites</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WARP</td>
<td>von Willebrand factor A domain-related protein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>XA</td>
<td>Xanthurenic acid</td>
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1  GENERAL INTRODUCTION

The post-genomic era of malaria research is yielding an ever increasing number of genetic, transcriptomic and proteomic datasets for both malaria parasites (Plasmodium spp.) and their mosquito vectors (Anopheles spp.). Mining this information provides exceptional prospects for malaria control — facilitating the discovery of genes, proteins and mechanisms critical to the development of Plasmodium, and presenting extensive opportunities for the identification of targets for drugs, vaccines, insecticides and novel vector control strategies.

With recent estimates of the annual malaria burden in the region of 247 million malaria cases, around 1 million deaths (WHO World Malaria Report 2008) and a direct cost of at least $12 billion in Africa alone (Gallup and Sachs, 2001), the potential health and economic benefits of effective malaria control and elimination programmes are obvious. A worldwide effort to control malaria — driven by the Roll Back Malaria (RBM) consortium (Nabarro & Taylor, 1998) and backed by improved economic and political support — has enabled increased implementation of three chief malaria control measures: antimalarial drugs, insecticide-treated nets and indoor residual spraying with insecticides. Although these interventions can be remarkably effective (see for example review by Wakabi, 2007), the long-term success of malaria control and elimination programmes crucially depends on the effective deliverance of interventions to target reservoirs of transmission and counteract the extensive capacities of the Plasmodium parasite and its mosquito vector to adapt and resist control. Given the complex nature of the parasite’s life cycle, there are numerous possibilities for expanding the available repertoire of malaria control measures. Transmission-blocking strategies, which aim to disrupt transmission by preventing the establishment or progression of Plasmodium through the mosquito, offer a promising approach for improving prospects for long-term malaria control and elimination programmes.
1.1. Basis of malaria transmission and considerations for control

1.1.1. Life cycle features of malaria parasites

Malaria parasites (Plasmodium) belong to a large, diverse protozoan phylum of obligate intracellular parasites, the Apicomplexa, which includes pathogens responsible for a variety of diseases of medical and veterinary importance, such as Babesia, Cryptosporidium, Eimeria, Theileria and Toxoplasma. The apicomplexan life cycle typically involves transformation through phases of invasion (mediated by motile stages with a specialised apical complex), asexual replication (the production of daughter cells by division of sessile replicative stages) and sexual reproduction (the formation of gametes and subsequent fertilisation to form a zygote). Plasmodium and other genera of haemosporidian parasites have a complex (two–host) life cycle in which phases of development occur in the tissues and blood of a vertebrate host and transmission is mediated by haematophagous dipteran such as mosquitoes, hippoboscid flies or culicoides species (Valkiunas, 2005). The general life cycle of the Haemosporidia comprises three major phases: i) pre-erythrocytic development, initiated by the injection of sporozoites into the skin and culminating in the release of thousands of merozoites following asexual replication (schizogony) in vertebrate host tissues, ii) intraerythrocytic development in the blood of the vertebrate host, and iii) sexual reproduction and sporogony in a haematophagous vector (Valkiunas, 2005). The occurrence of a phase of intraerythrocytic schizogony in blood stages and the presence of malaria pigment (haemozoin), formed as a result of haemoglobin digestion, distinguish Plasmodium species from other haemosporidian parasites (Valkiunas, 2005). It is the repeated cycle of red blood cell invasion, asexual replication, and subsequent release of progeny (merozoites), which is associated with all pathological symptoms of malaria (reviewed by Miller, 2002).

So far around 200 species of Plasmodium have been described, each infecting specific mammals, birds or reptiles. Those that infect humans: Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae and (by zoonotic transmission) the simian malaria parasite Plasmodium knowlesi, are transmitted exclusively by female Anopheles mosquitoes. Of more than 400 known anopheline species, around 35 are considered potentially important malaria vectors (Kiszewski et al., 2004). The role of mosquitoes as hosts for Plasmodium became established by Ronald Ross towards the end of the 19th century. Inspired and guided by Patrick Manson (Bynum and Overy, 1998; Ross, 1902), Ross achieved two critical breakthroughs: in August 1897,
he discovered that parasites from the blood of a malaria patient could establish development in certain (*Anopheles*) mosquitoes to undergo growth on the outer wall of the mosquito gut (Ross, 1897) and in July 1898 — through further studies with an avian malaria species — he discovered that development of *Plasmodium* in the mosquito culminates in the invasion of mosquito salivary glands, and subsequently demonstrated transmission to a new host via infective mosquito bites (Manson, 1898).

An overview of the life cycle of *Plasmodium falciparum* — the most virulent of the human malaria parasites, and the species responsible for the majority of malaria-related mortality — is shown in Figure 1.1. Species-specific features exist, but all malaria parasites follow the same general life cycle.Whilst repeated rounds of asexual replication in the vertebrate red blood cells maintains infection and causes pathology, a proportion of intraerythrocytic parasites differentiate into gametocytes: these are sexual stages that provide the essential link for transmission to mosquitoes upon their uptake in a blood meal. Developmental progression within the mosquito can be envisaged as four phases: i) sexual replication leading to formation of an ookinete, ii) ookinete invasion of the mosquito gut epithelium resulting in oocyst formation, iii) oocyst maturation and release of sporozoites into the mosquito haemocoel, and iv) sporozoite invasion of salivary glands. The phase of parasite development in the mosquito is known as the extrinsic period and takes approximately 10–28 days, depending on species and temperature. Sporozoites residing in salivary glands are injected alongside saliva into the skin of a host when the infected mosquito takes a blood meal. In all mammalian species, sporozoites progress via the bloodstream to the liver where they invade hepatocytes. Intrahepatic parasites grow and replicate asexually by schizogony, resulting in a massive amplification in parasite numbers over a period of around a week. Each mature schizont contains around 10,000-40,000 uninucleate merozoites, which exit in packages termed merosomes and are released into the bloodstream where they invade red blood cells. *P. vivax* and species of *P. ovale* are known to produce hypnozoites, a life cycle stage referring to dormant stages of apicomplexan parasites (as coined by Markus in 1978; see Markus 2010). A subset of invading *P. vivax* and *P. ovale* sporozoites may develop into hypnozoites, enabling the parasite to survive in the liver indefinitely and periodically initiate erythrocytic infections (known as relapse).
Figure 1.1 Life cycle of *Plasmodium*

Transmission to mosquitoes is mediated by uptake of male and female gametocytes in a blood meal (1). Male microgametes and female macrogametes form in the gut of the mosquito and subsequent fertilisation results in the development of a zygote (2). The zygote differentiates into a motile ookinete, which leaves the blood meal, migrates through the midgut epithelium and comes to lie just beneath the basal lamina (3). The ookinete forms an oocyst which grows for several days and undergoes multiple rounds of asexual replication to form sporozoites (4). Upon oocyst rupture, sporozoites spill into the haemocoel of the mosquito, a proportion of which enter the mosquito’s salivary glands (5). Mosquito blood feeding induces a proportion of salivary glands sporozoites to enter the salivary duct, from where they are inoculated with saliva into the skin of a new host each time the mosquito takes a blood meal (6). Sporozoites migrate through skin cells to reach the bloodstream where they circulate until reaching the liver sinusoids (7). Invasion of hepatocytes is mediated via traversal through Kupffer cells (8). Parasites grow and replicate asexually (hepatocytic schizogony), reaching maturity in around a week (9). Merozoites are released into the bloodstream and invade red blood cells (10). Intraerythrocytic parasites grow and replicate asexually (erythrocytic schizogony) to give rise to blood-stage schizonts (11). Mature schizonts rupture, releasing a new progeny of merozoites into the bloodstream and the asexual cycle continues. A proportion of intraerythrocytic parasites differentiate into gametocytes (12).
1.1.2. Malaria transmission potential

The basic determinants of malaria transmission are expressed in the Ross-MacDonald equation for the basic reproductive number ($R_0$), a term which denotes the theoretical number of new infections that will arise from a single infection in the absence of pressure from control or immunity.

$$R_0 = \frac{ma^2bp^n}{r - (\log p)}$$

(MacDonald, 1956)

The expression comprises three components:

i) **Infectivity of the human reservoir** ($1/r$); the inverse of the recovery rate from infection.

ii) **Vectorial capacity** ($ma^2bp^n/\log p$); the number of infective bites that can arise from a vector after feeding on an infectious host. This depends on multiple parameters:

- $m$, the number of vectors per human;
- $a$, the average number of blood meals a vector takes from humans in one day;
- $p$, the expected daily survival probability of a vector (lifespan expectancy = $1/\log p$);
- $n$, the number of days required for the parasite to complete sporogony.

iii) **Vector competence** ($b$); the physiological capability of a mosquito species as a vector (may be included as an additional component of vectorial capacity).

This basic model for $R_0$, and derivatives of it, are fundamental to the design of malaria control programmes, indicating the prospective impact on transmission of applying different interventions and the reduction in transmission necessary for eventual elimination of the disease. Crucially, for the number of infections in a given population to decrease and for disease to be eliminated, the reproductive rate (which under the pressure of control is termed $R_c$) must be sustained at a value of <1.

The application of environmental, chemical and biological vector control interventions to reduce vector density ($m$), the number of blood meals taken from humans ($a$), and most influentially, vector longevity ($p$), have formed the basis of malaria control since the early 1900s. Initial efforts focused on the practices of draining mosquito breeding sites, filling in stagnant pools of water or applying oils to destroy larvae populations, as advocated by Ronald Ross (Ross, 1902). Following their development and introduction in the 1940s, insecticide-based interventions have had the most dramatic effect on reducing transmission. Use of the long-lasting insecticide dichloro-diphenyl-trichloroethane (DDT)
together with the antimalarial drug chloroquine formed the basis of the Global Malaria Eradication Programme (1955-1969), which contributed to the successful elimination of malaria from much of the temperate world. Chemical attack of adult mosquitoes with insecticides reduces vector longevity \( (p) \), which since it is raised to the power \( n \) is considered as the most important parameter contributing to transmission. Indoor residual spraying (IRS) with insecticides and the use of insecticide-treated nets (ITNs) have accordingly become central to control.

More than a century after the introduction of targeted vector control, around half of the world’s population remains exposed to malaria (Hay et al., 2004); prevailing in tropical and subtropical regions of the world, where higher magnitudes of \( R_0 \) and logistical problems associated with implementing and sustaining control sustained interruption of transmission make elimination of the disease far more challenging. In regions of sub-Saharan Africa — where the overwhelming burden of malaria falls most heavily — estimates of \( R_0 \) often reach >100 (Smith et al. 2007). Elimination is therefore far more difficult to achieve and the capacity for resurgence of transmission much greater in regions of sub-Saharan Africa than anywhere else in the world. High transmission potential can be largely attributable to ecological factors; most notably the existence of highly efficient malaria vectors in environments where conditions are ideal for the survival of both parasite and vector. The world’s most efficient malarial vectors are endemic to sub-Saharan Africa: *Anopheles gambiae sensu stricto* (hereafter referred to as *An. gambiae*) and *Anopheles funestus* almost exclusively take blood meals from humans, are characterised by a short gonotrophic cycle of around 48 hours (therefore require frequent blood meals) and have a high probability of daily survival (Coetzee, 2004). Combined with environmental conditions, these properties confer a high potential stability of transmission (see Figure 1.2.).
Figure 1.2. Global representation of the potential stability of malaria transmission

The stability index depicted is calculated based on components of vectorial capacity, taking into account rate of blood feeding on humans, vector longevity and the extrinsic incubation period. Malaria has been eliminated from North America, Europe and Northern Asia where indices of stability were low, but remains prevalent across sub-Saharan Africa, Asia, Latin America and the Pacific Islands. Taken from Kiszewski et al. (2004).

1.1.3. Capacities for parasite and vector adaptations

The dynamic nature of malaria transmission, the large extent of vector biodiversity and parasite genetic variability, and the notorious abilities of both parasite and vector to adapt to selective pressures, leading for instance to mosquito resistance to vector control, parasite resistance to antimalarial drugs, parasite immune evasion strategies, and changes in host-parasite relationships pose fundamental limitations on malaria control interventions.

1.1.3.1. Mosquito susceptibility/resistance to vector control

The success of vector control, which depends foremost on the ability to effectively target susceptible mosquito populations, is considerably limited by the large extent of vector biodiversity and adaptive abilities. Firstly, a range of physiological and behavioural factors may render certain species and strains less susceptible to intervention by. For example, the two main transmission control measures, insecticide-treated nets (ITNs) and indoor residual spraying (IRS), inherently rely on endophilic mosquito feeding behaviour: whilst effective against mosquitoes that feed and rest indoors, mosquitoes which exhibit outdoor feeding and resting behaviours are not well targeted. Similarly, although targeting mosquito larvae can be a successful vector control approach where breeding sites are easy to identify and low in abundance, its application is limited where vectors are opportunistic
breeders and use diverse and abundant small temporary pools of water for oviposition. Furthermore, genetic-based resistance to insecticides, for example by mutations that confer amino acid changes on the insecticide target site, can enable the mosquito to survive a dose of insecticides that would normally be lethal. Knockdown resistance (kdr) conferred by mutation in the *Anopheles* voltage-gated sodium channel gene is of particular concern since it elicits cross resistance to both DDT and pyrethroids. ITNs are particularly vulnerable to the impact of resistance since they depend on pyrethroids, which, due to their low mammalian toxicity, are the only suitable choice of insecticide for treatment of bednets. The limitations of existing vector control interventions are discussed by Ferguson et al. (2010) who highlight that constraints imposed by vector ecology limit vector control strategies to such an extent that current measures are insufficient to interrupt the transmission of *P. falciparum* in areas where transmission is most intense.

1.1.3.2. Parasite resistance to antimalarial drugs

A number of antimalarial drugs have been developed for treatment of the disease and/or for prophylaxis (reviewed by Greenwood, 2010). Yet with large intra–host population sizes and extensive genetic diversity, the strong selective pressure exerted by the use of antimalarial drugs inevitably leads to the emergence and selection for resistant mutants. This has been well documented in the case of the global spread of *P. falciparum* strains resistant to the antimalarial drug chloroquine: used widely over the final half of the 20th century, the spread of chloroquine resistant *P. falciparum* strains was a leading factor in the collapse of the Global Malaria Eradication Programme (1955-1969) and the subsequent resurgence in malaria. Chloroquine-resistant *P. falciparum* strains have been associated with not only a survival advantage under drug pressure, but also increased infectivity to mosquitoes compared with non-resistant strains (Sutherland et al., 2002).

The use of combination drug therapy has become essential in order to stem the development of drug resistant parasite strains. Several drug combinations are possible, but constraints imposed by resistance, costs and side-effects pose considerable limitations on availability and use. Artemisinin combination therapy (ACT), in which an artemisinin derivative is used in combination with a longer acting drug, e.g. artesunate-mefloquine, and artemether-lumefantrine, is the prime option for treatment, yet due to expense and availability only reaches a small proportion of the African population (Marsh and Snow, 2010). The anti-folate combination sulphadoxine-pyrimethamine (SP) is currently recommended for use in intermittent preventive treatment (IPT): for prevention of disease during pregnancy (IPTp) or for prevention of disease in infants (IPTi), and also remains
important for treatment. Resistance to SP — conferred by mutations in the genes encoding dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes of the parasite’s folate synthesis pathway (Plowe et al., 1997) — and growing resistance to artemisinins (Dondorp et al., 2009; Noedl et al., 2009) pose critical threats to the success of current malaria control programmes.

1.1.3.3. Parasite virulence and immune evasion strategies

Host immune responses are counteracted by numerous parasite immune evasion strategies, enabling the parasite to maintain chronic infections in the vertebrate host and continue to contribute to transmission. Key evasive mechanisms are mediated by the expression of parasite adhesive proteins on the surface of infected RBC (iRBCs). The best characterised parasite iRBC surface protein is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which mediates adherence to a range of different host tissue receptors (reviewed by Rowe et al., 2009). Resulting adherence to the endothelial lining of the microvasculature (cytoadhesion), to uninfected erythrocytes (rosetting) and to other parasitised erythrocytes (autoagglutination); reviewed by Rowe et al. (2009), initiates sequestration of iRBCs. This acts to enhance parasite survival and replication since sequestered intraerythrocytic parasites escape from the circulating bloodstream and thereby circumvent passage through the spleen. In addition to facilitating enhanced parasitemias, a combination of obstructed blood flow caused by sequestered cells and the induction of inflammatory processes due to the interaction of parasites with leukocytes or vessel endothelium sequestration, contributes to severe complications for the host, such as cerebral malaria, respiratory distress, and fatal multi-organ failure due to (Miller et al., 2002). Immune recognition of parasite adhesive proteins exposed on the surface of iRBCs is counteracted by antigenic variation: the expression of antigenically distinct variants of parasite iRBC surface proteins, encoded by multigene families, enables evasion of the host immune response (reviewed by Kyes et al., 2001). For example, approximately 60 different PfEMP1 variants are encoded by the multigene family *var*. The existence of antigenic variation is linked to the slow and incomplete acquisition of immunity against malaria. With repeated exposure to parasite antigens, a state of semi-immunity develops that can suppress parasitemia and disease but is insufficient to clear infections (Butcher, 1989; Molineaux et al., 2002; Snow and Marsh 2002).
1.1.3.4. Changes in host-parasite population interactions

Changes in the dynamics of host-parasite population interactions — caused for example by environmental change — can enhance transmission and/or disease severity. Recently identified changes in transmission dynamics include increased zoonotic transmission of the simian malaria parasite *P. knowlesi*. Most commonly found in macaques, the transmission of *P. knowlesi* to humans is thought to be enforced by alterations in the behaviour of the vector, *Anopheles leucosphyrus*. This species is increasingly found in close association with humans as a consequence of deforestation, thereby contributing to the growing public health importance of *P. knowlesi* (Cox-Singh and Singh, 2008).

It has recently been recognised that *P. vivax* infections, long established as being limited to individuals positive for the RBC Duffy glycoprotein due to the dependence of *P. vivax* merozoite invasion on the Duffy antigen (Miller et al., 1976), may be capable of infecting Duffy-negative individuals. Where Duffy-negative and Duffy-positive ancestry co-exists, it is thought that frequent opportunities to attempt infection of Duffy-negative blood cells may select for strains capable of causing blood stage infections in Duffy negative individuals, as has been demonstrated in Madagascar (Menard et al., 2010). Therefore, whilst the proportion of populations infected by *P. vivax* has previously been limited, there are indications that Duffy-negative individuals may no longer be refractory to infection.

These examples of the extensive capacities for adaptation highlight how each individual malaria control intervention has fundamental limitations. The use of multiple interventions and a continuous pipeline of new control measures, including transmission-blocking strategies, are therefore crucial to the prospects of malaria eradication (discussed by Mendis et al., 2009).
1.2. Antimalarial transmission-blocking strategies

Malaria transmission can be reduced by both targeting the mosquito (vector-targeted strategies) and by targeting the parasite (antimalarial strategies). Vector control has provided the most effective means of reducing transmission to date and the development of vector-targeted interventions (e.g. insecticides, biological control and novel vector control strategies) continues to be a key area of research. The application of antimalarial strategies have so far focussed on reducing disease rather than transmission, but there exists considerable potential for antimalarial transmission-blocking strategies. The malaria life cycle comprises three phases of asexual replication, resulting in massive amplification in parasite numbers, and two population bottlenecks: upon transition from sporozoite-to-liver stage and gametocyte-to-oocyst (Figure 1.3). These bottlenecks represent key targets for interrupting the parasite’s life cycle.

![Figure 1.3. Schematic representation of the Plasmodium life cycle depicting targets for interruption of transmission](image)

The malaria parasite life cycle comprises three phases of asexual replication: intrahepatic schizogony (liver stages), erythrocytic schizogony (asexual blood stages) and sporogony (oocysts). Each results in massive amplification in parasite numbers (represented as black arrows, as by Gandon et al., 2001). Since natural bottle-necks occur upon transition from sporozoite-to-liver stage and gametocyte-to-oocyst, these stages represent ideal targets for a complete blockade in parasite development, whilst targeting the asexual blood stages can reduce disease.

Sporozoites are prime targets since blocking their progression has the advantage of directly preventing disease, yet transmission-blocking strategies — which intend to
reduce parasite infectivity to mosquitoes, or attack parasite development in the mosquito — represent an attractive approach for interrupting the parasite’s life cycle, particularly in the absence of a 100% effective preventative intervention.

An effective transmission-blocking approach may not only prevent infections, but also have the potential to prevent the spread of drug and vaccine resistance by inhibiting transmission of resistant parasite populations (Carter, 2001). In addition, it is predicted that transmission-blocking interventions would not impose a selective pressure on increased virulence, and may actually select for reduced virulence (Gandon et al., 2001; Mackinnon et al., 2008). Three main antimalarial transmission-blocking approaches are under development: transmission-blocking drugs, genetic control of vector competence, and transmission-blocking vaccines.

1.2.1. Transmission-blocking drugs

Despite providing effective treatment, antimalarial drugs available to date are inefficient at reducing transmission. Almost all currently used antimalarial drugs have been introduced based on their activity against the pathogenic asexual blood stages for treatment of the disease. They inhibit for example various synthetic or metabolic processes of the parasite detoxification of haem in the parasites food vacuole, folate synthesis, the electron transport chain of the mitochondria, protein synthesis in the chloroplast-like apicoplast (reviewed by Greenwood et al., 2008). Whilst effective against asexual blood stages and often also against young gametocytes, mature gametocytes remain insusceptible to the majority of antimalarial drugs. Treated individuals therefore remain infectious to mosquitoes and are able to contribute to transmission. Treatment using certain antimalarial drugs (e.g. Chloroquine) has in fact been shown to be capable of having the effect of enhancing transmission by increasing gametocyte production (Hogh et al., 1998).

Mature gametocytes are halted in development, with their continued life cycle progression taking place only upon removal from the bloodstream. Their reduced metabolism and state of cell cycle arrest renders them refractory to the effects of many antimalarial drugs (Sinden and Smalley, 1979). Some 8-aminoquinolines (e.g. Primaquine) are able to kill mature gametocytes, eliciting a cytotoxic effect by interfering with the electron transport chain in the mitochondria, yet largely due to side effects and costs, a transmission-blocking drug that can be widely used is yet to be developed. The
development of further drug combinations that can effectively reduce gametocyte populations or inhibit sporogony are thereby among the aims of future antimalarial drug approaches. Prospective targets include kinases, proteases, electron transport in the mitochondria, or DNA/protein synthesis in the parasite’s endosymbiont-derived apicoplast (a non-photosynthetic plastid homologous to the chloroplasts of plants). Drugs that are active against both sexual and asexual stages may be beneficial, although a transmission-blocking drug could be used in combination with drugs targeting asexual stages. Administered purely to individuals requiring treatment, however, impacts on gametocyte numbers at a population level may be limited in areas of high transmission since many of the individuals in a population may be semi-immune, carrying infective parasites and contributing significantly to transmission, yet are asymptomatic for disease, so are not treated with drugs (Drakeley et al., 2000). Transmission-blocking drugs may thereby be more suited to areas of low transmission intensity, although the addition of a drug with transmission-blocking activity to any treatment regime would bring the added major advantage of helping to limit the spread of drug resistance.

1.2.2. Genetic control of vector competence

The possibility of vector-targeted genetic control has emerged following the ability to achieve stable germline transformation of mosquitoes through transposable element-based transfections (Catteruccia et al., 2000) along with the capacity perform functional RNA interference studies by injecting double-stranded RNA into the body cavity of mosquitoes (Blandin et al., 2002). These advances have given rise to the opportunity to develop novel ways to reduce vectorial capacity: for example by the release of genetically engineered sterile male mosquitoes (to reduce vector populations) or by genetically altering mosquito olfactory behaviour (to reduce mosquito blood feeding). It also provides the opportunity of targeting vector competence, since mosquitoes may be rendered refractory to infection by the genetic manipulation of mosquito immune responses. With this in mind, considerable progress has been made towards the identification of mosquito refractory mechanisms (discussed in section 1.6). Yet numerous obstacles exist between the progression from laboratory studies and application of the technology in the field (discussed by Collins and Besansky, 1994; Curtis, 1994; Spielman, 1994; Carlson, 1996; O’Brochta and Atkinson, 1997). The development of a practical mechanism to drive mosquito transgenes through the natural vector population is perhaps the major technical challenge, particularly since genetic alteration often confers reduced fitness and a competitive disadvantage against wild mosquitoes. Furthermore,
considerable logistical barriers associated with the release of transgenic vectors must also be overcome.

An interesting alternative to the genetic alteration of mosquitoes is the use of paratransgenesis, involving the genetic modification of symbiotic bacteria of the mosquito midgut such that they express and secrete molecules that kill the parasite or block parasite invasion of the midgut (Riehle et al., 2007). Such an approach was developed as a potential means of controlling the transmission of *Trypanosoma cruzi* (the causative agent of Chagas disease) by *Rhodnius prolixus* (triatomine bug). A bacterial symbiont of the vector was genetically transformed to secrete the antimicrobial peptide cecropin A, or a single chain antibody, to render vectors carrying the genetically altered bacteria refractory to *T. cruzi* infection (Durvasula et al., 1997; Durvasula et al., 1999). Similar approaches to target *Plasmodium* in *Anopheles* mosquitoes are under investigation (Riehle et al., 2007). The success of a paratransgenic approach requires a symbiont closely associated with the vector and in contact with the targeted pathogen, which is cultivable and amenable to genetic transformation whilst retaining its fitness, plus a suitable method for its introduction and spread in the vector population. As with approaches based on transgenic mosquitoes, much further research is required into vector population dynamics and how interventions could be integrated into existing control strategies.

1.2.3. Transmission-blocking vaccines

The development of transmission-blocking vaccines (TBVs) has been initiated based on the recognition that a proportion of parasites developing in a mosquito blood meal are killed by vertebrate immune components (e.g. complement, leukocytes and antibodies) from the sera of a malaria-infected individual (Sinden and Smalley 1976; Grotendorst et al., 1986; Margos et al. 2001). Leukocytes and components of the complement system remain active in the blood meal for a few hours after ingestion (Kaslow, 1997; Ranawaka et al., 1994) whilst antibodies remain undigested in the blood meal for 24 hours after ingestion (Beier et al., 1989; Carter, 2001). There is therefore the opportunity to induce transmission-blocking immunity during the entirety of the 24 hours over which *Plasmodium* develops in the mosquito midgut by provoking the production of anti-parasitic antibodies against parasite surface antigens.
The development of vaccines against ticks (Gavac, TickGARD and TickGARDPlus) — based on a recombinant form of a *Boophilus microplus* tick midgut protein, Bm86 (Rand et al., 1989; Fragoso et al., 1998; de la Fuente et al., 2000) — provides proof-of-concept for the development of a vaccine in which antibodies are produced in an immunised subject, with effects in the vector, and furthermore demonstrates the possibility of developing a vaccine based on a single recombinant protein (see 1.3.1). As with the anti-tick vaccine, potential exists to induce an antibody response which is lethal against the mosquito vector, but attempts to develop a mosquitocidal vaccine have not been fruitful (Jacobs-Lorena and Lemos 1995) and targeting the parasite remains the focus of research. Possible mechanisms of antibody-mediated antimalarial transmission blockade include parasite agglutination, steric interference of crucial parasite interactions, or lysis of parasites conferred by complement or cellular factors (Kaslow, 1997; Tsuboi et al., 2003). For example, a block in fertilisation may be mediated by limiting gamete motility through agglutination, preventing cell-cell contact through surface coating, promoting lysis/phagocytosis by interactions between antibodies and leukocytes, or by initiating complement-dependent lysis. The developing zygote/ookinete may be a target of lysis for as leukocytes and complement remain active in the blood meal. Thereafter, the continued presence of antibodies in the blood meal enables ookinete interactions with the mosquito midgut to be targeted, for example by steric interference of receptor ligand interactions to block ookinete invasion (targeting either parasite or mosquito-derived antigens).
1.3. Overview of antimalarial vaccine development

1.3.1. Vaccination strategies

Vaccination is traditionally achieved by immunisation with whole, killed/inactivated or live attenuated forms of a pathogen to induce an adaptive immune response that elicits protection from infection. For example, the Salk polio vaccine comprises formalin inactivated poliovirus and the Bacillus Calmette-Guérin (BCG) vaccine for tuberculosis is prepared from live attenuated *Mycobacterium bovis*. In the case of malaria, the promise of a vaccine has long been demonstrated based on experimental immunisations with whole parasite preparations:

- Injection of sporozoites attenuated by radiation conferred sterilising immunity that fully protected against challenge with live intact sporozoites in experimental immunisations of mice (Nussenzweig et al., 1967), monkeys (Nussenzweig et al., 1970) and humans (Clyde et al., 1973).
- Immunisations with preparations containing merozoites protected against RBC infection and clinical disease in monkeys (Mitchell et al., 1977a; Mitchell et al., 1977b).
- Immunisation with gametocytes/gametes induced the production of antibodies that suppress infectivity to mosquitoes, thereby eliciting transmission-blocking immunity (Huff, 1957; Gwadz, 1976; Carter and Chen, 1976).

Despite these experimental achievements, antimalarial vaccines using a whole vaccine approach have been generally regarded as infeasible — principally considering the difficulties and impracticalities of growing and purifying parasites in large enough quantities. A more cost-effective sub-unit vaccine approach is possible by the application of recombinant DNA technology, through which antigens may be produced as heterologously-generated recombinant proteins. Whereas it is costly and difficult to produce and isolate natural parasite antigens, recombinant antigens can be produced relatively cheaply in large quantities, particularly using bacterial expression systems such as *Escherichia coli*. Furthermore, whereas considerable variation may exist among natural antigens, recombinant proteins can be produced consistently without genetic and biological variation. Heterologously-produced proteins can be extracted and purified from the host cells used for expression in a form suitable for immunisations. Recombinant vaccines can also take the form of viral vectors (e.g. attenuated vaccinia virus strains can be engineered to express recombinant proteins) or DNA vaccines, in which the immunogen is a DNA plasmid encoding an antigen, inducing expression of the antigen in cells of the immunised host.
Pre-erythrocytic development, erythrocytic development and infectivity to the mosquito can all be inhibited by targeting various life cycle stages:

Table 1.1. Malaria vaccine strategies and candidate antigens

<table>
<thead>
<tr>
<th>Target</th>
<th>Candidate antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre-erythrocytic</strong></td>
<td></td>
</tr>
<tr>
<td>Sporozoite surface proteins:</td>
<td>CSP, TRAP, SALSA, STARP</td>
</tr>
<tr>
<td>- Sporozoites inoculated by a mosquito are exposed to antibodies for a short time as they drainate in the bloodstream before reaching the liver.</td>
<td></td>
</tr>
<tr>
<td>Liver stage antigens:</td>
<td>LSA180, EXP1</td>
</tr>
<tr>
<td>- Parasites can be targeted during the roughly seven days of development within hepatocytes by MHC presentation of parasite-derived epitopes.</td>
<td></td>
</tr>
<tr>
<td><strong>Asexual blood stage</strong></td>
<td></td>
</tr>
<tr>
<td>Merozoite surface antigens:</td>
<td>MSP1, 2, 3, AMA1, EBA-175, BAEB1, NAG6, PAP2, RESA, PBM1</td>
</tr>
<tr>
<td>- Merozoites released from liver cells or infected erythrocytes are briefly exposed to antibodies before invading other erythrocytes.</td>
<td></td>
</tr>
<tr>
<td>Surface of infected RBC:</td>
<td>GPI, SERAS, GLURP</td>
</tr>
<tr>
<td>- Antibodies against parasite-derived proteins on the surface of the iRBC can prevent binding to the endothelium to reduce disease and control parasitemia.</td>
<td></td>
</tr>
<tr>
<td>Schizont-associated antigens and exotoxins:</td>
<td></td>
</tr>
<tr>
<td>- Upon rupture of RBCs, the release of exotoxins induces proinflammatory cytokine response, antibodies against exoantigens could reduce disease.</td>
<td></td>
</tr>
<tr>
<td><strong>Transmission-blocking</strong></td>
<td></td>
</tr>
<tr>
<td>Surface antigens of gametocyte-infected RBC</td>
<td>Pre-fertilisation: Px230, Px46/45, HAP2</td>
</tr>
<tr>
<td>- Surface proteins of parasites developing in the blood meal:</td>
<td>Post-fertilisation: Px25, Px28, CHT1, CTRP, SDFP, WARP, MACR, PPLP4, PPLP5</td>
</tr>
<tr>
<td>- Extracellular gametocytes, gametes, zygotes and ookinetes exposed to human immune factors in the mosquito blood meal.</td>
<td></td>
</tr>
<tr>
<td>Mosquito midgut antigens</td>
<td>AgAPN1, Caimnucin, Annessin</td>
</tr>
</tbody>
</table>


1.3.2. Summary of antimalarial vaccines in development

Approximately 100 candidate antimalarial vaccine formulations were in development in 2008, around 1/3 of which reached clinical trials. As depicted in Figure 1.4, more than half of all candidates were based on just three antigens: circumsporozoite protein (CSP), merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1). The vast majority of candidates fail to reach Phase IIb endemic-country trials, reflecting the difficulties of vaccine development and highlighting the need for new candidates.

Figure 1.4. Representation of the status of malaria vaccine development

A representation of the status of malaria vaccine development in 2008 depicting the number of pre-erythrocytic vaccine candidates (yellow), asexual blood-stage vaccine candidates (red), and transmission-blocking vaccine candidates (purple) in clinical development. Candidate antigens are CSP: Circumsporozoite protein, TRAP: Thrombospondin-related anonymous protein, LSA1&3: Liver stage antigen 1&3, AMA1: Apical membrane antigen 1, MSP1&3: Merozoite surface protein 1&3, GLURP: Glutamate-rich protein, EBA-175: Erythrocyte binding antigen-175, SERA5: Serine repeat antigen 5, Ps25: 25 kDa zygote/ookinete surface protein. The number of vaccines are indicated in brackets. Data is taken from the World Health Organisation (WHO) table of vaccines in clinical development (2008).
1.3.3. Progress and challenges in antimalarial vaccine development

1.3.3.1. Pre-erythrocytic vaccines

The first large-scale Phase III clinical trials of a malaria vaccine candidate (RTS,S) — currently ongoing at 11 sites in Africa — represents a major landmark in the development of a vaccine against malaria. RTS,S is a pre-erythrocytic vaccine based on the major sporozoite surface protein, circumsporozoite protein (CSP), and was designed in the 1980s in view of the successful hepatitis B vaccine. A fragment of CSP (amino acids 207-395) that includes B-cell epitopes within the central conserved repeat region (R) and the c-terminal containing T-cell epitopes (T) is fused to the amino-terminal of hepatitis B surface antigen (S) to form RTS, which is expressed simultaneously with S in Saccharomyces cerevisiae to form virus-like particles. Delivered with an adjuvant termed AS02A, clinical trials have demonstrated that the vaccine provides partial immunity (Stoute et al., 1997; Bojang et al., 2001) associated with around a 50% suppression in the rate of infection and delay in the onset of disease (Alonso et al., 2004). It has been hypothesised that by limiting the number of hepatic schizonts, levels of pathological blood stage infection are reduced, and vaccinated individuals may be able to acquire asexual blood stage immunity (Guinovart et al., 2009).

The partial protection provided by immunisation with RTS,S appears to be mediated by humoral immune responses, yet sterile immunity — as induced by radiation attenuated sporozoites — involves induction of both antibody and cell-mediated immune responses (Hoffman et al., 1998; Hoffman and Doolan, 2000; (Weiss et al., 1990; Doolan and Martinez-Alier, 2006). Accordingly, an emphasis of pre-erythrocytic vaccine development is on the induction of broadened immune responses, for example by incorporating multiple antigens (both sporozoite surface antigens and liver stage antigens) in one vaccine and by utilising heterologous prime-boost immunisation strategies. Among the vaccines under investigation are those based on ME-TRAP, a polypeptipe construct which encodes multiple liver stage epitopes (ME) fused to P. falciparum TRAP (Moorthy et al., 2003). ME-TRAP may be delivered in the form of a DNA vaccine, or by the use of attenuated virus vectors such as modified vaccinia virus Ankara (MVA) or attenuate poxvirus FP9. Heterologous prime-boost immunisation regimens, involving sequential administration of different antigen delivery systems (reviewed by Dunachie and Hill, 2003) have successfully enhanced cellular immune responses (Wang et al., 2004). An ME-TRAP DNA prime immunisation followed by an MVA ME-TRAP recombinant viral vector boost has been shown to elicit a degree of protection against sporozoite challenge.
An alternative approach to induce the strong and broad immune responses necessary for protection has been re-investment in whole sporozoite vaccines. Previous trials demonstrated that a radiation attenuated sporozoite vaccine can elicit sterile protection, but required bites from 1000 mosquitoes (Hoffman et al., 2002). As a result the approach has been deemed infeasible, but current endeavours aim to meet the challenges associated with large-scale production of purified sporozoites, purification from salivary glands, cryopreservation of sporozoites, injection of parasites of undefined genetic composition, and establishing a vaccine delivery route. Sporozoites attenuated by radiation are able to invade hepatocytes but are incapable of nuclear division and therefore arrest during liver-stage development (Hoffman et al., 2002). The application of parasite genetic manipulation techniques has enabled the production of genetically attenuated sporozoites, rendered defective in liver-stage development by gene disruption. Immunisation with such genetically attenuated sporozoites deficient in liver-stage development has been shown to elicit protection in *Plasmodium berghei* rodent models. For example, targeted disruption of *P. berghei* genes named upregulated in infective sporozoites (*Pbuis3* and *Pbuis4*) are unable to develop into liver stage schizonts (Mueller et al., 2005a; Mueller et al., 2005b). Immunisation with $\Delta Pbuis3/\Delta Pbuis4$ double knockout sporozoites conferred complete protection in mice for six months after the last immunisation (Jobe et al., 2007). Sporozoites deficient in the surface protein, P36p, also elicit long-lasting protection against *P. berghei* infection in rodents. It is thought the mutant parasites are unable to prevent host cell apoptosis, leading to parasite clearance (Van Dijk et al., 2005; Douradinha et al., 2007). A vaccine based on genetic attenuation would overcome the concerns associated with unknown genetic composition, yet the main technical challenges associated with manufacture and delivery still remain.

The study of sporozoite migration, liver infection, and hepatocytic schizogony utilising rodent malaria models and intravital microscopy have revealed that only a proportion of sporozoites enter the bloodstream after their injection into the skin – others may remain in the skin for some time or enter the lymphatic system (Vanderberg and Frevert, 2004; Amino et al., 2006). It has been estimated that ~35% of *P. berghei* sporozoites invade...
blood vessels, whilst ~50% remain in the dermis for up to seven hours after injection and ~15% enter lymphatic vessels (Amino et al., 2006). Parasites remaining in the skin and entering the lymph nodes have the potential to shape host immunity, with important implications for vaccine development. Parasites that enter the draining lymph nodes can surprisingly partially differentiate into exoerythrocytic stages and prime T-cells to recognise parasite peptides presented on the surface of infected hepatocytes (reviewed by Good and Doolan, 2007). The entry of parasites into lymphatic vessels can therefore make a vital contribution to the induction of a protective immune response, as is demonstrated by the removal of the draining lymph nodes from mice, which abolishes protective immunity (Chakravarty et al., 2007). It is also possible that the skin stage of infection could act to suppress vaccine-generated immune responses since it is suggested that the pre-existence of parasites in the skin may induce regulatory T-cells, providing a possible explanation as to why vaccines may fail in semi-immune individuals (Guilbride et al., 2010).

1.3.3.2. Vaccines against asexual blood stages
Asexual blood stages of malaria are naturally suppressed in semi-immune individuals, inhibiting clinical symptoms and protecting from severe forms of the disease (Butcher, 1989; Molineaux et al., 2002; Snow and Marsh 2002). Immunity develops with repeated exposure to parasite antigens and naturally acquired antibodies to various parasite antigens have been shown to be prevalent in residents of malaria-endemic areas and are capable of protecting from severe disease (see for example Riley et al., 1992). These antigens have formed the basis for development of a blood stage vaccine. Leading targets of protective antibody responses are merozoite surface protein 1 (MSP1; Blackman et al., 1991; Blackman and Holder, 1992), apical membrane antigen 1 (AMA1; Thomas et al., 1994; Bannister et al., 2003), merozoite surface protein 3 (MSP3) and glutamate-rich protein (GLURP); see Nebie et al. (2008).

Despite the promise for blood stage vaccine development (see for example Blackman et al., 1990; Chappel and Holder 1993; Pasloske and Howard, 1994; Daly and Long, 1995) several issues have compromised their development. These primarily include: i) the existence of antigen polymorphism (e.g. at least 50 polymorphic nucleotide positions have been identified in the gene encoding *P. falciparum* AMA1 (Marshall, 1996), which may enable rapid evasion of immune recognition (Gupta et al., 1994; Genton et al., 2002); ii) a lack of understanding of the mechanisms of natural acquired immunity (reviewed by
Doolan et al., 2009); and iii) the lack of translation from experimental systems (e.g. primate models) to humans, and again from non-immune to semi-immune human volunteers.

1.3.3.3. Transmission-blocking vaccine development

TBV development has lagged behind the development of pre-erythrocytic and blood stage vaccines, with only one candidate antigen (Ps25) reaching clinical trials. In clinical trials, immunisation with *P. falciparum* zygote/ookinete surface protein Pfs25 or its *P. vivax* homolog Pvs25 — expressed as recombinant proteins in *S. cerevisiae* — resulted in generation of transmission-blocking antibodies, but at a level too low for a practical vaccine (Malkin et al., 2005). Other TBV candidates in development include the Ps25-related zygote/ookinete surface protein Pfs28 or its *P. vivax* homolog Pvs28. As discussed further in section 1.5.5, antibody-mediated transmission blockade has also been demonstrated experimentally with antibodies against proteins secreted from ookinete micronemes, (Langer et al., 2002; Li et al., 2004) and with antibodies targeting mosquito midgut antigens (Kotsyfakis et al., 2005; Dinglasan et al., 2003; Dinglasan et al., 2007b)

It is thought that the low antibody titres elicited by immunisation with Pfs25 or Pvs25 recombinant proteins (Malkin et al., 2005) may be related to a combination of poor immunogenicity and lack of a boosting effect. Generating long-lasting antibody responses against proteins expressed exclusively in mosquito stages is a challenge since there is no natural boosting effect if the antigen is not expressed in vertebrate stages of the parasite. For this reason, antigens expressed in gametocytes may be more desirable as vaccine candidates than those expressed only in the mosquito. Established TBV candidates include the gametocyte/gamete surface proteins Pfs230 and Pfs48/45. Pre-synthesised in gametocytes in preparation for gametogenesis upon uptake in a mosquito blood meal, Pfs230 and Pfs48/45 are not exposed whilst the parasite resides in the RBC, but may be recognised by the vertebrate immune system following the death of gametocytes. Naturally-occurring antibodies against Pfs230 and Pfs48/45 have been identified in populations where malaria is endemic and correlate with reduced infectivity to mosquitoes (Rener et al., 1983; Vermeulen et al., 1985; Graves et al., 1988; Carter et al., 1989; Riley et al., 1994; Roeffen et al., 1995; Bousema et al., 2007; Bousema et al., 2006). The antibodies have their effect in the mosquito when the antigens become exposed on the surface of the parasite following RBC egress.
Considerable potential exists in the development and implementation of antimalarial transmission-blocking strategies, particularly since some of the main difficulties associated with pre-erythrocytic and blood stage vaccine may not be encountered with TBV approaches (Sauerwein, 2007; Saul, 2007; Carter et al., 2000). Amongst the main benefits of targeting mosquito-stage parasites rather than blood-stages are associated with the small number of parasites in the blood meal relative to the large number of parasites in the blood and the reduced selective pressures in the mosquito compared to within the vertebrate host. Genetic variation and antigenic polymorphism is accordingly considered to be lower and as such resistant mutants are less likely to emerge. The parasites are also more vulnerable to intervention simply since there are fewer targets to eliminate. Furthermore, mosquito-stage parasites are extracellular for an appreciable time, compared to only a few minutes per cycle for vaccines targeting the surface of the blood stage merozoites. The longer exposure time means that lower antibody affinities or concentrations can still be effective in targeting mosquito stage parasites, while ineffective in the host.

TBVs may also bring several advantages regarding the practicalities of developing a vaccine. Primarily, transmission-blocking immunity is expected to be entirely antibody-mediated, as has been suggested to be the case for Ps25 transmission-blocking antisera (Miura et al., 2007). This eliminates the need for a more complex vaccine strategy to drive both cellular and antibody based immunity. In addition, reliable assays to assess transmission-blocking activity both in vitro and in vivo already exist. Lastly, since malaria transmission is localised: distribution analysis predicts that 20% of host population may be responsible for 80% of infections (Woolhouse et al., 1997), appreciable reductions in transmission could be possible with relatively low TBV coverage, providing the ‘hotspots’ of malaria transmission can be identified and targeted and those individuals responsible for the majority of transmission can be vaccinated (Bejon et al., 2010; Bousema et al., 2010).

Exploiting the opportunity to target the parasite in the vector by transmission-blocking vaccination will be aided by both the identification and characterisation of novel candidates and an improved understanding of the development of Plasmodium in the mosquito in addition to the research on existing candidates.
1.4. Use of rodent malaria parasites in the study of transmission

The experimental analysis of human malaria parasites is very much restricted by the lack of compatible small animal models as a result of host specificity (Herrera et al., 2002). The advent by Trager and Jensen (1976) of a continuous in vitro culture system for P. falciparum blood stages revolutionised research on the disease (Trager and Jensen, 1997), but the experimental infection of mosquitoes with human malaria parasites remains wrought with technical difficulties and safety issues. Furthermore, there is no animal model for P. falciparum sporozoite infectivity. These limitations have led to the adoption of a number of experimental models based on the use of primate, avian and rodent malaria parasites.

Identified soon after the discovery of human malaria by Laveran in 1880, avian malaria parasites were commonly used as models for chemotherapeutic studies from the 1890s to the 1940s and have been fundamental to elucidation of the basic aspects of Plasmodium biology. The Plasmodium gallinaceum/domestic chicken system (initially described by Émile Brumpt in 1935) has been used extensively and remains a valuable model, particularly for studies regarding transmission (see for example Alavi et al., 2003; Vinetz et al., 1999) but also for the study of blood stages (e.g. Nagao et al., 2008). By far the most widely used experimental systems however are those that utilise rodent malaria parasites and laboratory mice.

Four closely related species of rodent malaria parasites (P. berghei, P. chabaudi, P. vinckei, and P. yoelii) naturally infect African thicket rats and can be maintained in laboratory rodents. P. chabaudi shows antigenic variation during long-lasting, non-lethal infections, making it invaluable in the studies of antigenic variation and drug resistance. P. yoelii is frequently used in the study of pre-erythrocytic stages, particularly since its sporozoites exhibit high levels of infectivity to laboratory rodents (Weiss, 1990; Khan and Vanderberg, 1991; Khushmith et al., 1991). P. vinckei, due to its synchronicity has proven useful in studying chronobiology of the asexual cycle in the blood (Gautret et al., 1994). P. berghei dominates investigations of the biology of mosquito stages of Plasmodium and provide an important experimental system for assessing transmission-blocking activity.

P. berghei was identified by Vincke and Lips in 1948 in the blood of Grammomys surdaster (a tree-dwelling rat) some years after it was initially found in its vector.
Anopheles dureni millecampsi (reviewed by Killick-Kendrick and Peters, 1978). It was the first rodent malaria parasite to be discovered. The natural vector proved difficult to maintain in the laboratory, but an experimental transmission system became established by Vanderberg and Yoeli in 1964 using the North American species Anopheles quadrimaculatus (Vanderberg and Yoeli, 1965). The Asian mosquito Anopheles stephensi was subsequently identified as a more effective vector (Yoeli et al., 1965). Since then, the P. berghei/An. stephensi and P. berghei/An. gambiae systems have become widely established. Parasite development in the mosquito is achieved by maintaining conditions reflecting those of the forested habitat of the natural environment from which the parasites were isolated (i.e. temperature of 18-22°C and relative humidity 90%). These systems provide the most attractive means for studying the biology of malaria in the mosquito:

- P. berghei can be reliably maintained in laboratory mice to attain gametocytes and mosquito infections can be efficiently achieved by allowing mosquitoes to feed directly on an infective mouse or by using an artificial feeding using a membrane feeding apparatus.
- Methods for the in vitro production of P. berghei zygotes and ookinetes from gametocyte-infected blood (initially described by Weiss and Vanderberg (1977)) have since been modified and established as routine (Janse et al., 1985; Janse and Waters, 1995; Rodriguez et al., 2002). More recently, a method for in vitro cultivation of oocysts and sporozoites has also been developed (Al-Olayan et al., 2002) meaning that it is possible for the entire mosquito phase of the parasite’s life cycle to be completed in vitro.
- The relative ease of genetic modification through the use of well-established transfection and selection techniques (Janse et al., 2006) and simple means of characterising transgenic parasites permits functional analysis of mosquito-stage genes.
- The system provides an amenable approach for studies of mosquito responses to infection and evaluation of transmission-blocking effects, which can be facilitated by the availability of well characterised clones and genetically modified mutant lines, such as the constitutively expressing GFP parasite line (Franke-Fayard et al., 2004).
Figure 1.5. *P. berghei* life cycle stages for the study of transmission

*P. berghei* blood-stage infections (i) may be induced in mice by inoculation with schizonts. A proportion of blood-stage parasites develop into gametocytes; male (M) and female (F). Gametocyte-infected blood removed from mice may be used to study exflagellation (ii), culture zygotes/ookinetes (iii) or infect mosquitoes using a membrane feeding apparatus. Mosquitoes can also be infected by allowing them to feed on an anaesthetised gametocyte-infected mouse. Oocysts can be observed on the wall of dissected mosquito midguts, preferentially 10-12 days post-infection (iv) and sporozoites can be extracted from dissected mosquito salivary glands, optimally approximately 21 days post-infection (v). The use of a transgenic parasite line expressing GFP can be used to aid visualisation of infections (iv and v).

The genomes of three rodent malaria parasites; *P. yoelii* (Carlton et al., 2002), *P. berghei* (Hall et al., 2005) and *P. chabaudi* (available through PlasmoDB) have been sequenced. Inter-species comparison of the genomes of these rodent malaria with that of the human malaria parasite *P. falciparum* (Gardner et al., 2002) revealed that around 4,500 genes out of the 5,268 encoded in the *P. falciparum* genome had an ortholog in at least one of the rodent malaria species (Hall et al., 2005), reflecting conserved biological features and validating the relevance of studies on rodent malaria parasites to human malaria (reviewed by Kooij et al., 2006).
1.5. Molecular basis of Plasmodium sexual & sporogonic development

Using experimental systems, together with molecular and post-genomic approaches, the cellular and molecular processes involved in parasite development are being elucidated, revealing the biological roles, expression and localisation of prospective TBV candidates, and identifying possible targets for immunological interventions.

1.5.1. Gametocyte development

Gametocytes, which are equipped to mature into gametes and initiate fertilisation in a mosquito blood meal, develop in a small percentage of infected red blood cells. As would be expected considering the functional differences between asexual blood stages and gametocytes, the transition from asexual to sexual development is associated with considerable cellular changes, reflected by transcriptomic and proteomic analyses confirming major differences in transcription and translation (Hall et al., 2005; Florens et al., 2002; Lasonder et al., 2002; Le Roch et al., 2003; Young et al., 2005; Silvestrini et al., 2005). The switch from asexual multiplication to sexual differentiation is likely to involve a combination of both parasite genetic factors and environmental cues. High parasitemias, anti-parasitic antibodies, depletion of fresh erythrocytes and chloroquine treatment are all associated with an increase in gametocyte formation (reviewed by Talman et al., 2004). The molecular mechanisms involved in the switch are not yet understood, but deciphering the associated signalling pathways represent a major goal of malaria research, with a view to prevent transmission (reviewed by Baker, 2010).

The gametocytes of most mammalian malaria parasite species are round or oval in morphology and develop over a time-frame just slightly longer than that of the asexual blood-stages (gametocyte development in P. berghei takes around 30 hours). There are however intriguing differences associated with the biology of P. falciparum and P. reichenowi gametocytes. P. falciparum and P. reichenowi comprise the sub-genus Laverania and are unusual among mammalian malaria parasites in that gametocytes are crescent shaped once mature, possessing a subpellicular complex. Furthermore, gametocytogenesis is prolonged (maturation of P. falciparum gametocytes takes around 9-12 days and can be divided as five stages: I-V).

All but mature (stage V) P. falciparum gametocytes sequester, most often in the bone marrow (reviewed by Day et al., 1998; Rogers et al., 2000; Alano, 2007). Proteins that are targeted to the gametocyte-infected RBC membrane could act as promising candidate antigens for a TBV (Sutherland, 2009). As in asexual blood stages, PfEMP1 is localised
to knob structures on the surface of stage I-II *P. falciparum* gametocyte-infected RBCs, but surface localisation of PfEMP1 appears absent beyond stage II, indicating that adherence, at least of stage III-IV gametocytes occurs by a different process to that exhibited by asexual stages (reviewed by Day et al., 1998). A possible involvement of STEVOR proteins, which like PfEMP1 are encoded by a multigene family has been proposed. During gametocyte maturation, STEVOR proteins are reported to be exported to the IRBC membrane (McRobert et al., 2004). Using flow cytometry, Saeed et al. (2008) analysed the sera of natural gametocyte carriers and identified the presence of naturally occurring antibodies with reactivity against antigens on the surface of gametocyte-infected erythrocytes.

Differences between male and female gametocytes begin to become apparent at mid-stages of maturity. Proteomic analysis of purified *P. berghei* male and female gametocytes highlights the divergence of the male and female gametocytes, with the male having 236 male specific proteins and the female 101, and only 69 proteins common in both (Khan et al., 2005). Mature male gametocytes are prepared for rapid DNA replication and mitosis in order to generate flagellated gametes for fertilisation of female cells. They are largely devoid of endoplasmic reticulum (ER) and retain only a low ribosome population sufficient for protein synthesis during gametogenesis (Sinden et al., 1978). Female gametocytes, on the other hand, are not only prepared for gametogenesis and fertilisation, but also for subsequent zygote development. Females continue rRNA synthesis and have abundant ER, and in common with metazoan oocytes, store a subset of mRNA for translation after fertilisation (Le Roch et al., 2003; Mair et al., 2006; Mair et al., 2010). An RNA helicase, named development of zygote inhibited (DOZI), is recognised as mediating storage of mRNA in cytoplasmic bodies of the female gametocyte, allowing for rapid translation after fertilisation (Mair et al., 2006). As gametocytes reach mid-stages of maturity, haemoglobin metabolism halts and mature gametocytes circulate in a state of developmental arrest, with their continued development taking place only upon removal from the bloodstream (Sinden and Smalley, 1979).
1.5.2. Gametogenesis and fertilisation

Environmental changes associated with the transition from the vertebrate bloodstream to the mosquito trigger the activation of mature gametocytes, resulting in differentiation into gametes (male microgametes and female macrogametes) and emergence from the host RBC. Despite differences in gametocyte biology, *P. falciparum* appears to undergo gametogenesis and fertilisation in a manner similar to other mammalian species of malaria.

Whereas the female gametocyte undergoes little change during gametogenesis, the male undergoes rapid and dramatic cellular differentiation — from a single immotile cell to eight flagellated gametes. These cellular changes are described by Sinden and Croll (1975). Differentiation depends on the completion of three mitotic divisions within a period of 8 minutes (Janse et al., 1986). Rapid DNA replication is associated with upregulation of the chromatin remodelling protein SET (Pace et al., 2006). Microtubule organising centres (MTOCs) organise the microtubules that make up the axonemes of the flagella. The flagella thrash as the gametes expel from the remnant of the gametocyte during the vivid process of exflagellation. *In vitro*, emerging microgametes draw in neighbouring erythrocytes, creating a wobbling clump of cells termed ‘exflagellation centres’. RBCs have been described to roll along exflagellating males and become retained by the residual body of the gametocyte as the gametes escape (Eksi et al., 2006). The formation of exflagellation centres is thought to involve binding to sialic acid on the surface of red blood cells (Templeton et al., 1998), but its significance is unknown.

Key triggers for the onset of gametogenesis include a drop in temperature of more than 5°C and the presence of xanthurenic acid (XA), a by-product of the mosquito eye pigmentation pathway (Sinden, 1983; Billker et al., 1998; Billker et al., 1997; Garcia et al., 1998). *In vitro* studies using *P. berghei* demonstrate that XA acts to trigger a rise in cytosolic calcium levels in microgametocytes and a calcium-dependent protein kinase 4 (PbCDPK4) is involved in regulating differentiation into gametes by translating calcium signals (Billker et al., 2004).

The escape of gametes from RBCs involves the rupture of two membranes; the parasitophorous vacuole membrane (PVM) and the infected erythrocyte membrane. Egress is aided by osmiophilic bodies (OBs), which release proteins into the PV and may be considered as functionally equivalent to the electron dense exonemes of asexual
schizonts. OBs are present in both male and female gametocytes, but are much more abundant (4-5 fold greater in number) in females (Sinden et al., 1978). This corresponds with the absence of mechanical force to assist in female gamete emergence, whereas the movement of flagella facilitates escape of male gametes (Alano et al., 1995). Gene disruption studies indicate that Pf g377 is required for OB biogenesis and support the role of OBs in female gamete egress; ΔPfg377 female gametes have reduced numbers of OBs and emerge from their host erythrocyte significantly less efficiently (de Koning-Ward et al., 2008). A second OB-associated protein, known as protein of early gametocyte 3 (PEG3) (Silvestrini et al., 2005; Furuya et al., 2005; Ponzi et al., 2009) or male development 1 (MDV1) appears important for both male and female emergence, with a proposed role in destabilising the PV membrane (Silvestrini et al., 2005; Furuya et al., 2005; Ponzi et al., 2009; Lal et al., 2009a).

Following gamete release, fertilisation is usually efficiently completed within one hour. As yet, no microgamete-macrogamete recognition mechanisms have been identified, although cellular adhesion is predicted to increase the chances of gamete-gamete interactions. Upon fertilisation, the plasma membranes of the gametes fuse, the cytoplasm of the male gamete enters that of the female, and nuclei subsequently fuse to give rise to a zygote (Sinden and Hartley, 1985). Three members of a ‘6-cys motif protein family’, Ps230, Ps48/45 and Ps47, are known to be expressed on the surface of gametocytes and gametes and are key TBV candidates, with antibodies preventing fertilisation (Healer et al., 1997).

The 6-cys family consists of 10 proteins sharing a conserved arrangement of 6 cysteine residues (Gerloff et al., 2005). Ps230 is the largest representative of the family, with 7 cysteine-rich double domains, whilst Ps48/45 and Ps47 each have 1.5 double domains. Ps48/45 is a protein doublet of 45 kDa and 48 kDa produced by differential processing of the products of a single gene present on the surface of both male and female gametocytes and gametes (Kaslow, 2002). Gene disruption of Pfs48/45 and Pbs48/45 significantly reduced zygote formation, attributable to the impaired ability of ΔPfs48/45 and ΔPbs48/45 male gametes to bind female gametes (van Dijk et al., 2001). Furthermore, in ΔPfs48/45 male gametes, Pfs230 surface localisation is not retained (Eksi et al., 2006). Ps230 exists as a 360 kDa precursor protein (Pfs260) on the gametocyte surface (Vermeulen et al., 1986) which during gametogenesis is processed to a mature 310 kDa protein (Pfs230) and exists as a complex with Pfs48/45 (Kumar and Wizel, 1992).
Targeted disruption of *Pfs230* leads to inhibition of the formation of exflagellation centres due to reduced capacity of male gametes to bind erythrocytes and Δ*Pfs230* parasites are reduced in their capacity to produce oocysts in mosquitoes (Eksi et al., 2006). *Pfs47* is expressed on the surface of female gametes but does not appear crucial for female fertility (van Schaijk et al., 2006). *P. berghei* *Pfs47* in contrast is essential for fertilisation, with Δ*Pfs47* parasites producing infertile female gametes (Mair et al., 2006).

A male sterility gene, termed HAP2 (Liu et al., 2008; Khan et al., 2005) or generative cell specific (Hirai et al., 2008) has recently been identified as being conserved among higher plants and protists, including *Plasmodium*, and in *P. berghei* has been shown to be critical to male gamete fertility (Liu et al., 2008; Hirai et al., 2008). PbHAP2 localises along the length of the male gamete and is required for the fusion of male and female gamete membranes (Liu et al., 2008). In further experiments, antiserum against PbHAP2 has been shown to inhibit ookinete development and oocyst formation (Blagborough and Sinden, 2009), thereby identifying promise in the potential of HAP2 as a TBV candidate.

### 1.5.3. Zygote/ookinete development

The solitary phase of meiosis in the parasite’s life cycle occurs in the diploid zygote and the parasite remains as a tetraploid cell throughout; only at sporulation over a week later does cell division and separation into individual haploid genomes occur. Translation of gametocyte-derived mRNA, stored via the RNA-helicase DOZI, is essential for zygote development; Δ*Pbdozi* parasites abort zygote development before meiosis (Mair et al., 2006).

Leading TBV candidates Ps25 and Ps28 are among the proteins translated from DOZI-stored mRNA transcripts. The two structurally similar glycosylphosphatidylinositol (GPI)-anchored proteins, containing three and four epidermal growth factor (EGF)-like adhesive domains respectively, become the dominant surface proteins of the parasite. Observations in *P. gallinaceum* by cryofracture electron microscopy suggest that they form a bi-layered coating on the external surface of the plasma membrane (Raibaud et al., 2001). Crystal structures of yeast expressed Pvs25 recombinant protein show that the four EGF-like domains form a compact triangular prism that could tile the parasite surface (Saxena et al., 2004; Saxena et al., 2006). Pbs25 and Pbs28 have been shown to play multiple redundant roles in ookinete survival and ookinete-oocyst development, as demonstrated by individual and double gene disruptions in *P. berghei* (Tomas et al., 2001). In the
developing ookinete, the proteins appear to play a role in protease-protection, since ∆Pbs25/∆Pbs28 double knockout is more sensitive to trypsin type I digestion than single knockouts of either gene (Tomas et al., 2001). Accordingly, their expression coincides with the parasite becoming more resistant to proteases (Kaushal et al., 1983; Margos et al., 2001) in parallel with the increase in levels of mosquito digestive enzymes secreted into the blood meal (Gass and Yeates, 1979).

Transition into a motile, invasive ookinete begins with the formation of an apical end polar ring, which acts as a microtubule organising centre (MTOC) from which numerous microtubules extend to form the microtubular cytoskeleton and the elongate shape of the ookinete. The ookinete shares cellular features characteristic of all apicomplexan invasive-stage parasites (reviewed by Morrissette and Sibley, 2002); the definitive feature being the presence of an apical complex comprising secretory organelles which release proteins required for motility and invasion. Proteins are secreted from the specialised secretory organelles in a sequential manner: micronemes are essential for host cell adhesion and invasion (see 1.5.4); rhoptries are vital for formation of a parasitophorous vacuole (PV), within which the intracellular parasite resides (Dubremetz et al., 1998); and dense granules are required for later host cell modification. Consistent with these roles, ookinetes contain only micronemes and lack rhoptries and dense granules. Whilst sporozoites and merozoites come to reside within a cell (hepatocytes and erythrocytes respectively), ookinetes traverse the midgut epithelia resulting in destruction of the host cell and differentiate into oocysts extracellularly. The absence of rhoptries and dense granules is indicated by electron microscopy and predicted rhoptry proteins are absent from the ookinete proteome (Hall et al., 2005).

An inner membrane complex (IMC) composed of flattened vesicles forms a bi-layer beneath the outer plasma membrane to give a three-membraned pellicle. The IMC which is associated with cytoskeletal elements including actin, myosin and microtubules and contains a lattice of intramembranous particles (IMPs). Cryofracture electron microscopic analysis has also identified the presence of pores within the IMC of *P. gallinaceum* ookinetes. The function of these pores remains to be elucidated, but a role in allowing the transport of molecules has been hypothesised (Raibaud et al., 2001).
Figure 1.6. Representation of the pellicle and apical complex of ookinetes

A. Diagrammatic representation of a cross-section of the ookinete pellicle. Beneath the plasma membrane sits the inner membrane complex, comprising the outer alveolar membrane and the inner alveolar membrane and between these the alveolar lumen. The IMC is broken up by a suture and by pores. Beneath the IMC lies the subpellicular microtubules. Adapted from (Raibaud et al., 2001) and (Keeley and Soldati, 2004).

B. Diagrammatic representation of a Plasmodium ookinete depicting the apical complex and three-membraned pellicle. Adapted from (Baum et al., 2008). The apical region of the cell contains micronemes; small, electron dense organelles, with a high protein content that are restricted to the apical third of the cell.

1.5.4. Molecular basis of ookinete motility and invasion

Ookinete motility is driven by a substrate-dependent gliding mechanism common to all apicomplexan invasive stages (Kappe et al., 1999; Sibley, 2004; Baum et al., 2006). It relies on the apical secretion of adhesins followed by translocation of adhesin-substrate complexes along the cell surface of the parasite from the anterior to the posterior, overall resulting in a forward locomotion of the parasite. This motility is driven by an actin-myosin motor, comprising filamentous actin (F-actin) and an IMC-anchored myosin, located beneath the plasma membrane (Figure 1.7; reviewed by Keeley and Soldati, 2004). A structurally-related family of transmembrane adhesins secreted from the micronemes provides the link between the actin-myosin motor and the extracellular substrate. These adhesive proteins are identified as TgMIC2 in Toxoplasma tachyzoites (Wan et al., 1997), thrombospondin-related anonymous protein (TRAP) in Plasmodium
sporozoites (Sultan et al., 1997), circumsprozoite and TRAP-related protein (CTRP) in ookinetes (Dessens et al., 1999; Yuda et al., 1999) and merozoite TRAP (MTRAP) in merozoites (reviewed by Baum et al., 2006). Unifying protein features include von Willebrand factor A (vWA) and thrombospondin type I repeat (TSR) extracellular adhesive modules, a conserved cleavage site within the transmembrane domain, and a cytoplasmic tail domain. The cytoplasmic tail domains are linked to F-actin via the glycolytic enzyme aldolase (Jewett and Sibley, 2003), whilst extracellular adhesive domains bind substrate. Following translocation of the adhesin, the protein is cleaved within the transmembrane domain resulting in its release. This is essential to disengage interactions between the parasite and substrate and is mediated by rhomboid (ROM) serine proteases (Koonin et al., 2003; Urban and Freeman, 2003; Brossier et al., 2005; Buguliskis et al., 2010).

![Diagram of apicomplexan motility](image)

**Figure 1.7. The conserved molecular basis of apicomplexan motility**

Apicomplexan gliding motility is driven by an actin-myosin motor. The gliding associated proteins (GAP) 45 and 50, and the MyoA tail domain-interacting protein (MTIP), connect myosin A (Myo-A) to the inner membrane complex (IMC) of the parasite. Filamentous actin (F-actin) is connected via aldolase to the TRAP-like adhesive protein, which, in turn, interacts with host-cell receptors. Myo-A pushes the transient f-actin scaffolds back onto another MyoA molecule and the parasite glides forward. Taken and modified from (Keeley and Soldati, 2004).
1.5.5. Traversal of the midgut epithelium

The blood meal of a mosquito is usually separated from the midgut epithelium by a peritrophic matrix, comprised predominantly of chitin and glycoproteins produced by midgut secretions following blood feeding (Huber et al., 1991). The peritrophic matrix acts to protect from midgut epithelium immune activation (Kumar et al., 2010) and is considered the first barrier that the ookinete must bypass. Parasite chitinase activity has been shown to be essential for the invasion of *P. gallinaceum* and *P. falciparum* ookinetes, with addition of the chitinase inhibitor allosamidin preventing infections (Shahabuddin et al., 1993). Antibodies against PgCHT1 reduced oocyst formation by 67-96% (Vinetz et al., 1999; Shahabuddin et al., 1993; Vinetz et al., 2000; Dessens et al., 2001) and *P. falciparum* chitinase PfCHT1 is being investigated as a TBV candidate. Unlike *P. gallinaceum* and *P. falciparum* ookinetes, *P. berghei* ookinete infectivity is insensitive to allosamidin (Dessens et al., 2001). Nevertheless, gene disruption of *P. berghei* chitinase (PbCHT1) significantly reduced oocyst formation in *An. stephensi*, suggesting functions beyond aiding digestion of the peritrophic matrix (Dessens et al., 2001).

Interactions between ookinete surface or secreted proteins and glycans present along the microvilli of mosquito midgut epithelial cells appear important in initiating invasion. For example, treating midguts with periodate, which removes exposed carbohydrates, reduces binding of ookinetes to the midgut (Zieler et al., 1999). Furthermore, RNAi-mediated knockdown of *An. gambiae* OXT1, which is required for glycosaminoglycan biosynthesis, markedly reduced parasite development (Dinglasan et al., 2007a). Ookinete attachment can also be blocked by lectins that mask carbohydrate midgut ligands. The lectin-binding mosquito midgut protein *An. gambiae* aminopeptidase N (AgAPN1), which can be blocked by the lectin jacalin, has become the most advanced mosquito-based TBV candidate. Polyclonal antiserum to AgAPN1 has been shown to inhibit both *P. berghei* and *P. falciparum* oocyst formation by up to 80%.

Two other mosquito proteins, calreticulin and annexin B11 – both present on the apical surface of the midgut epithelium – have been identified as potential ligands for invasion. Calreticulin has been identified as a potential ligand using recombinant Pvs25 as a probe (Rodriguez et al., 2007) and annexin B11 was identified by mass spectroscopy in a fraction of midgut proteins capable of binding *P. berghei* ookinetes (Kotsyfakis et al., 2005). $\alpha$-annexin B11 antibodies have been shown to be able to interfere with the
parasite–midgut interaction, reducing oocyst formation by 30-40% (Kotsyfakis et al., 2005).

Ookinete-epithelial interactions can also be blocked by genetic alteration of the mosquito midgut barrier, for example by inducing gene expression from a blood-inducible and gut-specific carboxypeptidase promoter (Jacobs-Lorena, 2003). Identified effector genes include bee venom phospholipase A2 (PLA2), which possibly modifies the properties of the midgut epithelial membrane (Zieler et al., 2001) and salivary gland and midgut binding peptide 1 (SM1), a 12-amino acid peptide which binds to receptors required for ookinete midgut invasion (Ghosh et al., 2001). *P. berghei* oocyst formation is substantially reduced and transmission impaired in transgenic *An. stephensi* expressing either PLA2 (Moreira et al., 2002) or SM1 (Ito et al., 2002).

Key ookinete transmission-blocking antigens include the major ookinete surface proteins Ps25 and Ps28 and several ookinete micronemal proteins. A number of proteins have been identified that are secreted from the micronemes and transported to the surface of the parasite where they may mediate important roles in invasion and/or be targeted by transmission-blocking antibodies. These include:

- Membrane attack ookinete protein (MAOP) – also known as *Plasmodium* perforin-like protein 3 (PPLP3) – and a related protein *Plasmodium* perforin-like protein 5 (PPLP5), both of which contain a membrane-attack complex and perforin (MACPF)-related domain and are thought to facilitate ookinete entry to the lumen of the epithelial cells by disrupting the cell membrane. ∆pplp3 and ∆pplp5 *P. berghei* ookinetes are able to attach to the midgut epithelium but are unable to enter the cytoplasm (Kadota et al., 2004; Ecker et al., 2007).

- Secreted ookinete adhesive protein (SOAP) has been identified as being important to host adhesion (Dessens et al., 2003).

- Circumsporozoite and TRAP-related protein (CTRP) is critical for ookinete motility (Dessens et al., 1999, Yuda et al., 1999).

- Cell traversal protein of *Plasmodium* ookinetes and sporozoites (CelTOS) is critical to host cell traversal (Kariu et al., 2006).

- von Willebrand factor A domain related protein (WARP), which although has a non-essential role in ookinete infectivity is a target of transmission-blocking immunity: anti-WARP antibodies reducing infectivity of both *P. gallinaceum* and *P. falciparum* (Yuda et al., 2001; Li et al., 2004).
With extensive opportunities to elicit transmission blockade the inhibition of midgut invasion is a major focus of transmission-blocking strategies. Antibodies against ookinete surface proteins, antibodies against mosquito midgut ligands present on the apical surface of the midgut epithelium, or genetic modification of the mosquito midgut all represent viable approaches.

1.5.6. Mosquito responses to midgut invasion

Ookinete invasion of the midgut triggers both local reactions in the mosquito epithelium and systemic responses in the mosquito haemolymph, resulting in significant parasite losses occur during and following midgut invasion. A ‘time bomb model’ of ookinete invasion has been developed, depicting invaded midgut cells as ‘bombs’, since the parasite has only a limited time frame before it is killed by cell toxicity or destroyed by apoptotic processes (Han et al., 2000). Ookinete invasion triggers upregulation of nitric oxide synthase (NOS) followed by increased peroxidase activity and causes cellular damage that ultimately leads to apoptosis culminating in an actin–based extrusion of the invaded cell from the midgut epithelia (Han et al., 2000; Zieler and Dvorak, 2000; Gupta et al., 2005). Cell membrane protrusion is mediated by Arp2/3 and Wiskott-Aldrich syndrome protein (WASP) has been identified as an important regulator of actin-based local epithelial responses (Vlachou et al., 2001; Mendes et al., 2008).

Two immune effector mechanisms have been identified that can render mosquitoes completely refractory to *Plasmodium* infection – parasite development can be completely blocked by lysis, as in the *An. gambiae* SUAF2 strain (Vernick et al., 1995) or by melanisation, as in the *An. gambiae* L3-5 strain (Collins et al., 1986). Understanding the molecular basis of these refractory mechanisms is a key focus of investigations into vector-parasite interactions, driven by the possibilities of genetic control of vector populations, and molecular pathways are slowly being deciphered in laboratory models. Lysis is mediated by complement-like killing involving a mosquito thioester-containing protein 1 (TEP1), which is homologous to the mammalian complement protein C3 and directly binds to ookinetes (Blandin et al., 2004). Melanisation is a widespread mechanism of pathogen killing among arthropods, initiated by a serine protease cascade that culminates in the conversion of prophenoloxidase (PPO) to phenoloxidase (PO), which then causes the production of melanin and protein cross-linking around ookinetes in the sub-epithelial space (Lemaître and Hoffmann, 2007; Meister et al., 2004; Christensen et al., 2005). Two leucine-rich repeat-containing molecules; leucine-rich...
repeat immune gene 1 (LRIM1) and *Anopheles Plasmodium*-responsive leucine-rich repeat 1 (APL1; or LRIM2) have been identified as key mediators of both complement-like lysis and melanisation (Osta et al., 2004; Riehle et al., 2006; Povelones et al., 2009). It has been shown that LRIM 1 and 2 exist as a multimeric protein complex in the mosquito haemolymph and directly interact with TEP1, leading to TEP1 activation (Povelones et al., 2009; Fraiture et al., 2009). Several serine proteases have been identified as being (positive or negative) regulators of melanisation, including Clip domain serine proteases (CLIPs) and serine protease inhibitors (SRPNs) (Dimopoulos et al., 2000). Furthermore, two C-type lectins (CTL4 and CTLMA2) have been identified as negative regulators, acting to protect parasites from LRIM1-dependent melanisation (Osta et al., 2004).

The molecular basis of immune effector mechanisms is therefore beginning to be deciphered in laboratory models, providing considerable potential for control via approaches to render mosquitoes refractory to infection. However, many immune-related genes identified in laboratory models, such as LRIM1, CTL4 and SRPN2, have not been confirmed natural *Anopheles/Plasmodium* associations in the field (Cohuet et al., 2006; Michel et al., 2006). It is recognised that considerable variation may exist in different parasite-vector combinations. For example, mosquito immune responses triggered by *P. falciparum* may differ significantly from those induced by *P. berghei* (Dong et al., 2006; Mendes et al., 2008) and silencing of certain immune molecules may cause marked phenotypic changes in one mosquito strain but negligible effects in another (Dong et al., 2006).

**1.5.7. Oocyst formation and maturation**

Oocyst formation occurs in the sub-epithelial space beneath the basal lamina of the midgut epithelium. It is suggested that binding to laminin, a major component of the basal lamina, may promote ookinete-oocyst differentiation and oocyst growth and perhaps protect the parasite against mosquito immune responses (Adini and Warburg, 1999; Vlachou et al., 2001; Nacer et al., 2008). Several studies indicate that ookinete surface proteins interact with mosquito laminin. For example, GST pulldowns and/or yeast-two-hybrid studies demonstrated interactions between *An. gambiae* laminin γ-1 and the ookinete surface proteins Pbs25, Pbs28 (Vlachou et al., 2001), CTRP (Mahairaki et al., 2001) and SOAP (Dessens et al., 2003). The importance of laminin binding is supported
by RNAi-mediated knockdown of *An. gambiae* laminin γ-1, which led to a 60% decrease in *P. berghei* oocyst formation (Arrighi et al., 2005).

Beginning with disassembly of the cytoskeleton, apical complex and IMC, the motile, invasive ookinetes differentiate into a sessile, replicative oocyst, which grows over several days, reaching 50-60 µm in diameter. An oocyst wall, probably synthesised via secretion from golgi-derived vesicles and composed of protein / glycoprotein and an extracellular fibrous coat, surrounds the cell and lies in direct contact with the basal lamina of the mosquito midgut (Sinden et al., 2004). PbCAP380 (oocyst capsule protein) is the first identified oocyst wall protein. It localises to the outer layer of the oocyst wall and gene disruption studies indicate an essential role in oocyst survival (Srinivasan et al., 2008). Repeated rounds of mitosis generates in the region of 2000-8000 haploid nuclei. At 6-8 days, the cytoplasm subdivides by expansion of the ER cisternal space and invagination of the plasmalemma to form sporoblasts; ‘germination centres’ from which sporozoites bud (Terzakis et al., 1967; Sinden and Strong, 1978). MTOCs localising just underneath the sporoblast membrane are involved in the positioning of nuclear lobes at the periphery of sporoblast periphery and initiate formation of the apical complex (Thathy et al., 2002). Sporozoites bud from centres of apical complex formation by the extension of microtubules from polar rings (Bannister and Sinden, 1982). Nuclei move into the developing sporozoites and fully formed, uninucleate sporozoites separate from the residual sporoblast.

The expression of circumsporozoite protein (CSP) begins in the developing oocyst (Hamilton et al., 1988), the protein accumulates at the oocyst plasma membrane, and as the plasma membrane invaginates localises to the developing sporoblast, from where it is incorporated into the membrane of the developing sporozoite (Hamilton et al., 1988; Thathy et al., 2002). Gene disruption shows that CSP plays an essential role in sporoblast/sporozoite formation in the developing oocyst (Menard et al., 1997). It is also implicated in roles in sporozoite egress since targeted gene disruption of region II of CSP prevents sporozoite release (Wang et al., 2005). Sporozoites appear to emerge from mature oocysts via a protease-dependent process, as indicated by the essential role of *P. berghei* egress cysteine protease 1 (ECP1) by gene disruption (Aly and Matuschewski, 2005).
1.5.8. Sporozoite migration and invasion of salivary glands

Sporozoites are released into the haemocoel, within which they are thought to be passively carried with the flow of the haemolymph. Many are destroyed in the environment of the haemocoel; it has been estimated that around 1 in 5 eventually enter the salivary glands (Hillyer et al., 2007). Initial attachment of sporozoites to the salivary glands involves interaction between parasite surface proteins and the basal lamina of salivary gland cells. Following breach of the basal lamina, sporozoites invade salivary gland acinar cells via formation of a tight junction and a transient parasitophorous vacuole and accumulate in the secretory cavity (Pimenta et al., 1994). CSP and TRAP are implicated with essential roles in sporozoite attachment and the SM1 peptide, which inhibits ookinete invasion, also blocks salivary gland invasion (Ghosh et al., 2001). The SM1 receptor has been identified in An. gambiae as saglin and TRAP-saglin binding appears crucial for invasion (Ghosh et al., 2009). TRAP forms a central part of the motility and invasion machinery as demonstrated by gene disruption; ΔPbtrap sporozoites are unable to invade An. stephensi salivary glands and are impaired in gliding motility (Sultan et al., 1997). The inner membrane complex protein IMC1, is also critical for sporozoite motility. Targeted disruption of IMC1 in P. berghei results in mis-shaped sporozoites that are defective in motility.

Sporozoites may undergo significant changes in the salivary glands to prepare for host infection. Sporozoites isolated from salivary glands and inoculated into the haemocoel of uninfected mosquitoes are unable to re-invade salivary glands and whilst salivary gland sporozoites readily establish infections upon injection into hosts, sporozoites isolated from oocysts are regarded as being uninfective to the vertebrate host (Touray et al., 1992). A proportion of sporozoites enter the salivary duct and are injected upon blood feeding along with saliva, the secretion of which is induced during uptake of the blood meal. The salivary glands release a number of components including antihistamines, vasodilators, anticoagulants and immunomodulators, which aid uptake of a blood meal and facilitate sporozoite infection (reviewed by Dhar and Kumar, 2003). Sporozoites residing in the salivary glands are thought to increase their transmission success by modulating mosquito behaviour, for example by decreasing mosquito apyrase activity. Apyrase is a secretory protein which has been shown to inhibit platelet recruitment and aggregation. Reduction in its activity thereby causes difficulties in obtaining a full blood meal and as a result the mosquito probes both more frequently and for a longer duration (Smartt et al., 1995).
1.6. The *Plasmodium* LCCL/lectin adhesive-like protein (LAP) family

1.6.1. Identification of LAPs as potential TBV candidates

The ability to select potential vaccine targets based on predicted properties as encoded by genetic sequence data provides promising opportunities for vaccine development. Putative surface or secreted proteins, which may be accessible to antibodies and thereby logical choices for vaccine candidates, can be identified by *in silico* analysis of sequences encoded by the genome using computer algorithms to predict characteristics common of surface proteins, e.g. presence of signal peptides, transmembrane domains or glycosylphosphatidylinositol (GPI) anchors, and absence of typical intracellular domains or targeting sequences. Such a ‘reverse vaccinology’ approach (selecting candidates based on bioinformatic analysis of a pathogen’s genome, rather than the organism itself) has emerged as a potent approach for the identification of new vaccine candidates – compellingly demonstrated in the development of a vaccine against serogroup B meningococci. Bioinformatic screening for putative surface-associated proteins encoded by the *Neisseria meningitides* genome led to the discovery of meningococcal factor H-binding protein (fHBP) and resulted in the generation of a promising multi-component recombinant vaccine against Meningitis B (reviewed by Rappuoli and Covacci, 2003; Mora et al., 2006; Seib et al., 2009).

Proteomic analyses of distinct stages of the malaria parasite’s life cycle provide key datasets which can be screened by *in silico* approaches in order to select candidates for further study. In the case of TBV development, candidates can be selected from proteome datasets of gametocytes and ookinetes. Among the proteins that stand out as potential TBV candidates are a family of six putatively secreted proteins containing predicted LCCL and/or lectin domains. Termed the LCCL/lectin adhesive-like proteins (LAPs), the proteins were first described as a family after their identification in a proteomic analysis of *P. falciparum* asexual stages and gametocytes (Lasonder et al., 2002). Five proteins, each containing signal peptides and a number of predicted adhesive domains, were recognised as being detected in gametocytes but absent from asexual stages and were grouped as a family (LAP1-5) (Lasonder et al., 2002). A sixth LAP (LAP6) with the predicted presence of an LCCL domain, a lectin domain and a signal peptide was subsequently identified in the *Plasmodium* genome by BLAST analysis (Trueman et al., 2004). Although LAP6 was not detected in the *P. falciparum* sexual stage proteome (Lasonder et al., 2002), all six LAPs have subsequently been detected in a *P. berghei*
ookinete surface-enriched proteome (R. Stanway, PhD thesis 2007), indicating that all are expressed in mosquito stages of the parasite.

The LCCL domain — the primary feature of the LAP family — has been named after the best characterised proteins that were found to contain it, namely Limulus factor C, vertebrate cochlear protein cochlin and mammalian late gestation lung protein (Trexler et al., 2000). The LCCL domain is defined by four conserved cysteine residues and a conserved C-terminal region containing a highly conserved histidine (Trexler et al., 2000). The domain is found predominantly in metazoan multidomain proteins, many of which are extracellular adhesive proteins, containing domains such as complement-type domains, lectin domains and/or von Willebrand type A domains (Bateman and Sandford 1999; Patthy 1999; Trexler et al., 2000; Bikker et al., 2002; Muller 2001; Templeton et al., 2004).

LAPs comprise distinct arrangements of predicted LCCL domains and/or putative adhesive domains, including carbohydrate-binding domains and domains characteristic of cell adhesion proteins, including coagulation factors. LAP1 also contains a lipoxygenase homology (LH) domain, with putative roles in lipid interactions (e.g. membrane attachment; Ponting et al., 1999; Bateman and Sandford, 1999) and furthermore, a tandem array of scavenger receptor cysteine-rich (SRCR) domains. SRCR domains appear highly conserved across a number of metazoan secreted or membrane-bound proteins, within which SRCR domains are thought to mediate protein-protein interactions and ligand binding (Bikker et al., 2002; Muller, 2001). The array of putative adhesive domains found in the protein family (summarised in Table 1.2.) together with the prediction of N-terminal signal peptides strongly suggests that the LAPs have extracellular roles. Predicted domain architectures are reviewed by Dessens et al. (2004) Trueman et al. (2004) and Pradel et al. (2004) and are described in further detail in Chapter 3 of this thesis.
<table>
<thead>
<tr>
<th>Domain</th>
<th>Properties</th>
<th>Protein examples</th>
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<tr>
<td><strong>Limulus factor C, Coch-5b2, late lung gestation (LCCL)</strong></td>
<td>Characterised by four conserved cysteine residues and a conserved C-terminal motif: YxxxSxxCxxAVxGVI containing a highly conserved histidine (Trexler et al., 2000). Roles in lipopolyasaccharide (LPS) binding have been suggested (Wang et al. 2000).</td>
<td>Multi-domain adhesive proteins, e.g. <em>Limulus</em> factor C serine protease; Cochlear protein COCH-5b2; Mammalian late gestation lung protein; Endothelial and smooth muscle cell-derived neuropilin-like protein; and CocoaCrisp proteins (reviewed by Dessens et al., 2004).</td>
</tr>
<tr>
<td><strong>Lipoxygenase homology (LH)</strong></td>
<td>Homologous to a non-catalytic domain of lipoxygenases, with proposed roles in facilitating lipid binding (Ponting et al., 1999). May mediate membrane attachment and may contain a conserved surface lysine or arginine, which possibly represents a ligand-binding site (SMART)</td>
<td>Membrane-bound proteins, e.g. Polycystin-1 which may act as a cell-surface receptor or form part of a large membrane-associated complex (Bateman and Sandford, 1999).</td>
</tr>
<tr>
<td><strong>Scavenger receptor cysteine rich (SRCR)</strong></td>
<td>Can be traditionally subdivided into two groups: group A SRCR domains have six conserved cysteine residues, whilst those of group B are characterised by eight cysteine residues (reviewed by Vilà et al., 2000).</td>
<td>Membrane-bound and secreted proteins with roles in ligand binding, e.g. Macrophage scavenger receptor type 1; Complement factor 1; CD5 T-cell surface glycoprotein; Mac-2 binding protein; Deleted in malignant brain tumors-1; Sea urchin SPERACT egg-peptide receptor (reviewed by Vilà et al., 2000).</td>
</tr>
<tr>
<td><strong>Pentraxin</strong></td>
<td>Comprises a flattened β-jellyroll structure similar to laminin G and concanavalin A-like lectin domains, containing calcium-binding residues (Srinivasan et al., 1994)</td>
<td>Proteins involved in cell recognition and adhesion, including viral and bacterial toxins such as Cholerae neuraminidase; laminins; neurexins; and sialidases; and proteins involved in complement activation, including C-reactive protein and Serum amyloid P component protein (reviewed by Gewurz et al, 1995).</td>
</tr>
<tr>
<td><strong>Neurexin and collagen-like (NEC)</strong></td>
<td>A collagen-binding domain with similarities to a portion of fibrinogen or fibrillar collagen globular domains (identified as a distinct module by Pradel et al., 2004)</td>
<td>Neurexins are neuronal receptors that involved in adhesion and signalling between neurons. Other proteins containing fibrinogen domains include ficolins e.g. <em>Limulus</em> tachylectins), with roles in innate immunity, and intelectins (e.g. <em>Xenopus</em> cortical granule lectin), with roles in fertilisation and embryogenesis.</td>
</tr>
<tr>
<td><strong>Anthrax protective antigen-like</strong></td>
<td>A carbohydrate-binding domain with a beta-barrel structure (reviewed by Rigden et al., 2004).</td>
<td>Cell binding proteins, including anthrax protective antigen (PA) and yeast adhesins (reviewed by Rigden et al., 2004).</td>
</tr>
<tr>
<td><strong>Fibronectin type 2 (FN2)</strong></td>
<td>A collagen-binding domain containing four conserved cysteine residues; related to the Kringles domain family (reviewed by Ozhogina et al., 2001)</td>
<td>Proteins involved in blood coagulation, including fibronectin and blood coagulation factor XII.</td>
</tr>
<tr>
<td><strong>Ricin B lectin</strong></td>
<td>A carbohydrate-binding domain similar to the legume lectin ricin from the seeds of the castor bean plant, <em>Ricinus communis</em>. Characterised by the presence of three QxW repeats (see Hazes, 1996).</td>
<td>Carbohydrate-recognition proteins, including plant and bacterial AB-toxins (see Hazes, 1996).</td>
</tr>
<tr>
<td><strong>Discoidin</strong></td>
<td>Also known as F5/8 type C domain; a ligand binding domain usually containing two conserved cysteines, with diverse ligand recognition mediated by highly variable loops (Fuentes-Prior et al., 2002).</td>
<td>Found in a number of functionally unrelated proteins (Ichikawa et al., 2007) including <em>Dictyostelium discoideum</em> cell adhesion protein and the coagulation factors V and VIII.</td>
</tr>
</tbody>
</table>
The identification of LAPs as multidomain lectin-like proteins immediately led to the suggestion that the protein family may be involved in extracellular functions. It is known that lectin-like properties play important roles in recognition, adhesion and invasion mechanisms of parasites and other pathogens. For example, the negative charge of cell surface glycosaminoglycans or proteoglycans is utilised to enter and infect cells (Rostand and Esko, 1997). In *Plasmodium*, lectin-carbohydrate interaction are expected to be important in mediate cell invasion be ookinetes, sporozoites and merozoites (Siden-Kiamos and Louis, 2004). Differentiation into oocyst is also thought to require specific binding to sugars (of the basal lamina) (Arrighi and Hurd, 2002).

1.6.2. Studies on the LAP family

Genes encoding orthologs of each of the six LAPs are found in all sequenced *Plasmodium* genomes; *P. falciparum*, *P. vivax*, *P. yoelii*, *P. berghei* and *P. chabaudi* (Gardner et al., 2002; Carlton et al., 2002; Gardner et al., 2005; Hall et al., 2005). Furthermore, homologs of LAP1-5 are identified in all sequenced apicomplexan genomes. This includes two species of *Cryptosporidium* (*Cryptosporidium parvum* and *Cryptosporidium hominis*), two species of *Theileria* (*Theileria parva* and *Theileria annulata*) and *Toxoplasma gondii*. Although domains found in LAPs are individually found widely among eukaryotes and other taxa, the domain architectures that make up the LAPs are unique to Apicomplexan parasites.

Studies on the LAP family have been most comprehensively performed in *Plasmodium*, and at the onset of this PhD, a number of studies had been carried out characterising members of the family in both *P. falciparum* (Delrieu et al., 2002; Pradel et al., 2004; Pradel et al., 2006) and *P. berghei* (Claudianos et al., 2002; Trueman et al., 2004; Raine et al., 2007). LAP1 has been named the *Plasmodium* scavenger receptor cysteine rich LCCL adhesive-like protein (PSLAP), and *Plasmodium* scavenging receptor protein (PxSR) (Claudianos et al., 2002; Delrieu et al., 2002). The protein family is now generally known as the LAPs (LCCL/lectin adhesive-like proteins: Lasonder et al., 2002; Trueman et al., 2004; Raine et al., 2007) or also as the CCps (Limulus coagulation factor C domain-containing proteins): the five LCCL domain containing proteins are termed CCP1-5 and the single member of the family lacking an LCCL domain is referred to as FNPA (Pradel et al., 2004; Pradel et al., 2006)
In *P. falciparum*, where the proteins are commonly referred to as the PfCCp protein family, cellular localisation studies have been performed, strengthening the potential of the proteins as vaccine candidates. Through immunofluorescence analyses using antibodies generated against PfCCp recombinant proteins, PfCCp1, 2 and 3 have been shown to be expressed in a stage-specific manner in gametocytes (detectable from stage II onwards). Antibody staining was associated with the plasma membrane, the PV and the PV membrane (Figure 1.7; Pradel et al., 2006). Therefore, following parasite egress from the RBC, it is envisaged that the proteins would be exposed on the surface of the parasite where they may be accessible to immune attack from blood meal components. Studies analysing antibody labelling post-fertilisation indicate that the proteins appear to be secreted from the parasite and thereafter localised extracellularly, with only low level labelling detected post-fertilisation (Delrieu et al., 2002; Pradel et al., 2004).

![Figure 1.8. Immunofluorescent labelling of PfCCp1](image)

Confocal medial section images of mouse polyclonal anti-PfCCp1 antibody in combination with Alexa488 secondary antibody depicting labelling in association with the parasite surface and parasitophorous vacuole. Taken from Pradel et al. (2006).

Antibody labelling studies of select PfCCps therefore suggest that the proteins are specific to gametocytes: expression beginning during gametocytogenesis and ceasing after fertilisation. Accordingly, roles in aiding fertilisation have been suggested. It is proposed that the proteins may act as adhesive molecules on the surface of female gametes: perhaps acting to increase encounters between male and female gametes, or possibly, by promoting cell clustering, aiding gamete survival by surrounding them by cells to shield them from immune attack (Pradel et al., 2006). Yet interestingly, the
outcomes of gene disruption studies, in both *P. falciparum* and *P. berghei*, show that fertilisation is appears to occur normally in *Pblap* or *Pfccp* knockout parasites. Gene disruption of *Pblap1*, *Pblap2*, *Pblap4* and *Pblap6* results in oocysts defective in sporulation (Figure 1.9; Claudianos et al., 2002; Raine et al., 2007). Meanwhile, ∆*Pfccp1*, ∆*Pfccp2*, and ∆*Pfccp3* parasites are capable of forming ooinetes, oocysts and sporozoites in comparable numbers to wild-type parasites, yet sporozoites were unable to invade salivary glands (Pradel et al., 2004). From gene knockout studies it is thereby concluded that the proteins are not essential for asexual blood stage, gametocyte or ooinete development, but are essential for oocyst maturation and sporozoite production or infectivity.

Figure 1.9. Representative morphology of wild-type and ∆*Pblap* oocysts
Images of ∆*Pblap* and wild-type (wt) day 14 oocysts in *An. stephensi* mosquitoes. The ∆*Pblap* oocysts grow, but whereas mature wild-type (wt) oocysts contain thousands of sporozoites, ∆*Pblap* oocysts fail to sporulate.

In cross-fertilisation experiments in which ∆*Pblap* gametocytes are crossed with wild-type parasites, infectious sporozoites are formed (Raine et al., 2007; Trueman et al., 2004) as would be expected for a gene with functions after zygote formation (Ranford-Cartwright et al., 1991). Furthermore, experiments in which ∆*Pblap* gametocytes are crossed with parasite lines deficient in either male or female fertility demonstrate that critical function of *Pblap1, Pblap2, Pblap4* and *Pblap6* depends on molecules inherited from the female gametocyte (Raine et al., 2007). Development is rescued when crossed with ∆*Pbs48/45* gametocytes, specifically deficient in male gamete fertility (van Dijk et al., 2001), whereas crossing with a ∆*Pbs47* parasite line, in which the function of female gametes is disrupted (Khan et al., 2005; Mair et al., 2006) fails to rescue sporulation (Raine et al., 2007). Therefore although the effect of gene disruption is not detected until
sporulation in the oocyst, it is suggested that functions are mediated through the inheritance of transcripts or protein from the female gametocyte (Raine et al., 2007). This is supported by proteomic analyses of separated male and female *P. berghei* gametocytes, though which LAPs are reported to be female specific (Khan et al., 2005). The LAP family have therefore been identified as candidates of interest both in their potential as TBV candidates, and in terms of deciphering aspects of *Plasmodium* developmental biology.

1.7. Thesis aims

General objectives of this thesis were to:

1. Investigate the possibility that anti-LAP antibodies might interfere with parasite development in the mosquito.
2. Gain insights into the biological roles of the LAP family and the molecular processes by which *Plasmodium* completes its development in its mosquito vector.

Using *P. berghei* as a well established system for the study of mosquito stages of the parasite, specific aims were to i) generate LAP recombinant proteins, ii) produce antiserum with anti-LAP activity, iii) evaluate transmission-blocking activity through *in vitro* and *in vivo* assays, and iv) study LAP localisation and other aspects of LAP biology considered relevant.

Results are presented as two chapters: Chapter 3 deals with the generation of antisera against LAPs and evaluates activities in transmission-blocking assays; Chapter 4 addresses cellular localisation and attempts to further investigate LAP biology.
2 MATERIALS AND METHODS

2.1. Maintenance of *Plasmodium berghei*

2.1.1. Parasite strains and clones

*P. berghei* ANKA strain parasites clone 2.34 (wild-type) were used throughout studies. A second clone, 2.33 (defective in the production of mature gametocytes) was used for the purpose of generating gametocyte depleted infections. In addition, the following transgenic parasite lines were used: *PbconGFP*, *Pblap* gene knockout parasites (available for all but *Pblap3*) and *Pb814-myc*. All transgenic parasites were derived from clone 2.34 parental lines. Details are given in Table 2.1.

Table 2.1 *P. berghei* clones

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>clone 2.34</td>
<td>Wild-type (wt)</td>
</tr>
<tr>
<td>clone 2.33</td>
<td>Defective in the production of mature gametocytes</td>
</tr>
<tr>
<td><em>PbconGFP</em></td>
<td>Constitutively expresses GFP (Franke-Fayard et al., 2004)</td>
</tr>
<tr>
<td>Δ<em>Pblap1</em></td>
<td><em>Pblap1</em> knockout (Trueman et al., 2004)</td>
</tr>
<tr>
<td>Δ<em>Pblap2</em></td>
<td><em>Pblap2</em> knockout (Raine et al., 2007)</td>
</tr>
<tr>
<td>Δ<em>Pblap4</em></td>
<td><em>Pblap4</em> knockout (Raine et al., 2007)</td>
</tr>
<tr>
<td>Δ<em>Pblap5</em></td>
<td><em>Pblap5</em> knockout (Ecker et al., 2008)</td>
</tr>
<tr>
<td>Δ<em>Pblap6</em></td>
<td><em>Pblap6</em> knockout (Raine et al., 2007)</td>
</tr>
<tr>
<td><em>Pb814-myc</em></td>
<td><em>Pb814 myc</em> epitope tagged (Dr. M. Delves, unpublished)</td>
</tr>
</tbody>
</table>

2.1.2. Infection of mice

Female Theiler's Original (TO) mice (Harlan, UK) aged 6-10 weeks old were routinely used as hosts for the maintenance of *P. berghei* by cyclical passage. Mice were infected by inoculation with schizonts: approximately $5 \times 10^7$ parasites in a volume of 100-200 µl were injected intraperitoneally. Parasites were obtained either directly from the blood of an infected donor mouse or from a cryopreserved solution (100 µl infected blood in
200 µl 90% (v/v) Alsever’s solution (Sigma) and 10% (v/v) glycerol). To stimulate a quick rise in parasitemia and high gametocytemias, mice were injected 2-3 days prior to *P. berghei* infection with 200 µl phenylhydrazinium chloride (PH; 6 mg/ml in PBS, Sigma). Treatment with PH induces hyper-reticulocytosis and since *P. berghei* preferentially invades reticulocytes, results in an increase of initial cell infection rates and enhanced gametocyte production (Gautret et al., 1996).

### 2.1.3. Assessment of parasitemias

Parasitemias of infected mice were monitored from 3 days post-infection by microscopic examination of Giemsa-stained thin blood smears. A drop of tail blood was spotted and smeared onto a frosted slide. Air-dried blood smears were fixed in methanol and stained with a 1 in 5 Giemsa stain solution (Fluka) for approximately 10 minutes at room temperature. Smears were examined using an Olympus BH2 light microscope at an objective magnification of x100. Parasitemias were quantified by counting the number of infected red blood cells as a percentage of total red blood cells in observations of three random fields.

### 2.1.4. Collection of blood

In order to harvest parasites, mice were anaesthetised by intramuscular injection with a lethal dose (150 µl) of a mixture of xylazine and ketamine; one volume of Rompun® (20 mg/ml xylazine hydrochloride; 5,6Dihydro-2-(2,6-xylidino)-4H-1,3-thiazine-hydrochloride; Bayer) and two volumes of Vetalar® (100 mg/ml ketamine; Fort Dodge Animal Health Ltd.) in three volumes of phosphate buffered saline (PBS). Mice were swabbed with alcohol and a 26 gauge needle and syringe containing approximately 50 µl of heparin (30 units/ml, Sigma) was used to remove blood by cardiac puncture. Typically 0.8-1.0 ml of blood was collected from each mouse.

### 2.1.5. *In vitro* cultivation of ookinetes

Ookinetes were cultured from blood containing mature gametocytes (day 3-4 post-infection). Mice were infected and parasitemias were assessed by observation of Giemsa-stained smears as in 2.1.2. To test for the presence of mature male gametocytes (capable of exflagellation), a drop of tail blood was spotted on a microscope slide, mixed with a drop of ookinete medium (see below), and covered with a Vaseline®-edged coverslip.
Exflagellation typically occurs from 8-12 minutes after removal of blood and can be observed under the microscope as clumps of red blood cells moving in a wobbling motion. Cells were viewed using an Olympus BH2 light microscope under phase contrast at x40 objective magnification.

Parasites were cultured in 20% (v/v) Foetal Bovine Serum (FBS) and 80% (v/v) ookinete culture medium (25 mM HEPES containing 2 mM L-glutamine (Sigma), 0.2% (w/v) Na₂CO₃, 5U/ml penicillin (Gibco), 5 µg/ml streptomycin (Gibco), 50 mg/ml hypoxanthine (Sigma) and 100 µM XA; pH 8.4). 10 ml culture volumes were prepared for the addition of 0.4 ml of blood, 30 ml culture volumes were used for up to 1.2 ml of blood, and 140 ml culture volumes were prepared when larger quantities of ookinetes were required. Blood was collected by cardiac puncture and transferred to the tissue culture flask. Cultures were maintained at 19°C, 70% humidity for 24 hours for ookinete development.

2.1.6. Mosquito infections

Anopheles stephensi SD500 mosquitoes were reared by Ken Baker and Mark Tunnicliff. Adult females (3-6 days post-emergence) were collected using a battery-powered aspirator and potted in cardboard cups (typically 70-80 per pot) covered by mesh netting and secured with a cardboard lid. Mosquitoes were starved overnight to increase the probability of blood feeding. For direct feeds, mice with a parasitemia of 5-10% containing mature gametocytes were anaesthetised with a low dose (40-50 µl) of Rompun®/Vetalar® (mixed as described in 2.1.1.2) and placed onto the mesh netting of mosquito pots. Mosquitoes were allowed to feed for 20-30 min at 19°C, preferably in the dark. Unfed mosquitoes were removed within 24 hours of feeding. Fed mosquitoes can be easily distinguished from unfed mosquitoes since the blood meal is visible in the mosquito abdomen. Mosquitoes were briefly anaesthetised with CO₂ and kept on ice while all unfed mosquitoes were removed. Fed mosquitoes were then maintained on fructose (8% (w/v) fructose, 0.05% (w/v) p-aminobenzoic acid) at 19°C and 70% relative humidity under a 12 h light / 12 h dark cycle.
2.2. Construction of plasmid vectors

DNA plasmids were constructed for the purpose of recombinant protein expression and for tagging endogenous proteins.

2.2.1. Sequence retrieval and bioinformatic analysis

DNA sequences of *P. berghei* laps and corresponding protein sequences were retrieved from PlasmoDB (http://plasmodb.org/plasmo) using the gene ID numbers as follows;

- *Pblap1*: PB000977.02.0
- *Pblap2*: PB000652.01.0
- *Pblap3*: PB001172.01.0/PB000635.02.0
- *Pblap4*: PB000504.02.0
- *Pblap5*: PB001084.00.0
- *Pblap6*: PB000955.03.0

Putative protein domains were predicted using InterproScan (Quevillon et al., 2005) and SMART sequence analysis (Schultz et al., 1998). Nucleotide and protein BLAST searches were conducted using the BLASTN and BLASTP tools (http://www.ncbi.nlm.nih.gov/blast).

2.2.2. Cloning strategy for the generation of vectors for protein expression

Constructs for the heterologous expression of portions of PbLAPS were generated by cloning gene fragments into a pET-46 Ek/LIC vector (Novagen) by ligation independent cloning (LIC). Primers (Table 2.2) were designed with 5’ extensions GACGACGACAAGATX (sense) and GAGGAGAAGCCCGGT (antisense) compatible for annealing into the Ek/LIC vector. Target gene fragments were amplified by PCR from template parasite DNA (2.2.4.1); genomic DNA was used when the target lacked introns, whilst cDNA was used when introns were present in the target sequence. PCR products were purified (2.2.4.4), eluting into TlowE buffer in accordance to the requirements for Ek/LIC cloning. TlowE contains EDTA for sequestration of metal ions and the reducing agent DTT required for cloning steps.

Target gene sequences were inserted into the pET-46 Ek/LIC vector by an annealing reaction following the generation of compatible overhangs by T4 DNA polymerase treatment. The 3’→5’ exonuclease activity of T4 DNA polymerase results in single strand removal of nucleotides from the 3’ end. Since dATP is the only nucleotide available, polymerase activity is only initiated when the first adenine is encountered.
(complementary to the thymidine in the 5’ extensions) thereby generating vector-compatible 13-14 base overhangs. pET-46 Ek/LIC vector and T4 DNA polymerase treated inserts were then annealed by a 10 min incubation at 22°C. Both T4 DNA polymerase treatment and annealing reactions were performed as stated in the Novagen Ek/LIC cloning kit user protocol (#TB163).

Plasmids were initially transformed into E.coli strain NovaBlue Gigasingles (Novagen). Following sequencing, plasmids confirmed to contain an in-frame target of correct sequence were transformed into E. coli expression strains: BL21 (DE3) pMico or Rosetta (DE3) PLysS, allowing for T7 RNA polymerase-mediated expression. An empty vector negative control was generated by transformation of pET-15b. The pET-15b vector is similar to the pET-46 Ek/LIC vector, each containing an N-terminal His•Tag® sequence.

2.2.3. Cloning strategy for generating vectors to tag endogenous genes

All tagging constructs were designed to integrate into the endogenous Pblap locus via single homologous recombination. C-terminal gene portions (minus the stop codon) were PCR-amplified using forward primers equipped with KpnI restriction sites and reverse primers with ApaI restriction sites (see Table 2.2 for primer sequences). Intermediate cloning of purified PCR products was performed using the pGEM® T-Easy vector system (Promega) for ligation into the pGEM® T-Easy vector. Plasmid DNA confirmed by sequencing to contain an in-frame target gene was digested with the restriction enzymes ApaI and KpnI for subsequent ligation into ApaI/KpnI-digested GFP and c-Myc tagging vectors (pDR0007-myc and pDR0008-gfp). Each plasmid encodes the respective tag downstream of the ApaI/KpnI restriction sites and a selection cassette encoding a modified Toxoplasma gondii dihydrofolate reductase / thymidylate synthase gene (Tgdhfr/ts), which confers high levels of resistance to the antimalarial drug pyremethamine.
Figure 2.1. Schematic representation of the strategy for GFP or myc tagging of endogenous genes

The targeting construct corresponds to the sequence of a c-terminal portion of the target gene, lacking the stop codon, in frame with a 3’ eGFP or c-Myc tag and Tgdhlfr-ts. The plasmid is linearised at a restriction site unique to the region of homology (absent in the vector). The linearised vector integrates into the targeting locus by single homologous recombination, resulting in the tagging of the endogenous gene copy (asterisk = stop codon). Modified from Tufet Bayona (2005).
2.2.4. Primers

Primers were synthesised by Invitrogen. Stock solutions of 100 mM and working solutions of 10 mM were prepared and maintained at -20°C.

Table 2.2. Primer sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA11216F</td>
<td>GACGACGACAAATACAAAGTAAAAGTAAATTAGAATCGTGATTC</td>
</tr>
<tr>
<td>RA12121R</td>
<td>GAGGAGAAGCCCGGTTTTAAAAATATATCTCAGAACATTCCTTTCG</td>
</tr>
<tr>
<td>RA12539F</td>
<td>GACGACGACAAATACAAAGTAAAAGTAAATTAGAATCGTGATTC</td>
</tr>
<tr>
<td>RA13157R</td>
<td>GAGGAGAAGCCCGGTTTTAACATGGAATACAAACTCCTGAC</td>
</tr>
<tr>
<td>ABLAP2F</td>
<td>GACGACGACAAATACAAAGTAAAAGTAAATTAGAATCGTGATTC</td>
</tr>
<tr>
<td>ABLAP2R</td>
<td>GAGGAGAAGCCCGGTTTTAACATGGAATACAAACTCCTGAC</td>
</tr>
<tr>
<td>RA2424F</td>
<td>GACGACGACAAATACAAAGTAAAAGTAAATTAGAATCGTGATTC</td>
</tr>
<tr>
<td>RA21323R</td>
<td>GAGGAGAAGCCCGGTTTTAACATGGAATACAAACTCCTGAC</td>
</tr>
<tr>
<td>RA21323R</td>
<td>ABLAP3F</td>
</tr>
<tr>
<td>RA24616R</td>
<td>ABLAP3R</td>
</tr>
<tr>
<td>RA3643F</td>
<td>ABLAP4F</td>
</tr>
<tr>
<td>RA31443R</td>
<td>ABLAP5F</td>
</tr>
<tr>
<td>RA41777F</td>
<td>ABLAP6F</td>
</tr>
<tr>
<td>RA42616R</td>
<td>ABLAP5R</td>
</tr>
<tr>
<td>RA43718F</td>
<td>RALAP6.2F</td>
</tr>
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<td>RA44876R</td>
<td>RALAP6.2R</td>
</tr>
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<td>RA5289F</td>
<td>RALAP6.2F</td>
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<tr>
<td>RA51227R</td>
<td>GAGGAGAAGCCCGGTTTTAACATGGAATACAAACTCCTGAC</td>
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<tr>
<td>RA6_Kpn_3376F</td>
<td>GAGGAGAAGCCCGGTTTTAACATGGAATACAAACTCCTGAC</td>
</tr>
<tr>
<td>RA6_Apa_4416R</td>
<td>GAGGAGAAGCCCGGTTTTAACATGGAATACAAACTCCTGAC</td>
</tr>
</tbody>
</table>

TagRAlap4F         | GGTACCATGTCATGGGAAATATTTATGTAGTAATCGTGATTC                                       |
TagRAlap4R         | GGTACCATGTCATGGGAAATATTTATGTAGTAATCGTGATTC                                       |
TagRAlap5F         | GGTACCATGTCATGGGAAATATTTATGTAGTAATCGTGATTC                                       |
TagRAlap5R         | GGTACCATGTCATGGGAAATATTTATGTAGTAATCGTGATTC                                       |
RA6_Kpn_3376F      | GGTACCATGTCATGGGAAATATTTATGTAGTAATCGTGATTC                                       |
RA6_Apa_4416R      | GGTACCATGTCATGGGAAATATTTATGTAGTAATCGTGATTC                                       |
2.2.5. Preparation of template DNA

In most cases, genomic DNA extracted from wild-type *P. berghei* parasites was used as template for PCR-amplication of target gene fragments; the exception being when the target for protein expression contained introns, in which case DNA was amplified from synthesised cDNA.

2.2.5.1. Extraction of genomic DNA

Genomic DNA was extracted from mixed blood stages or ookinetes. Cells were harvested by centrifugation (500g for 10 min at 4°C) and resuspended in 40 ml ice-cold 0.17 M ammonium chloride and incubated on ice for 30 min to lyse red blood cells. Parasites were then collected by centrifugation (500g for 10 min at 4°C). Genomic DNA was extracted using the Promega Wizard® Genomic DNA Purification Kit. The parasite pellet was mixed in 600 µl Nuclei Lysis Solution and RNA removed by incubation with RNase Solution (15 min at 37°C). Protein and DNA were removed by precipitation (as described in the Promega Wizard® Genomic DNA Purification Kit protocol). The resulting pellet was air-dried for 15 min then the DNA was rehydrated in 100 µl DNA Rehydration Solution for 1 h at 65°C and afterwards stored at 4°C.

2.2.5.2. Synthesis of cDNA

Single-stranded cDNA was produced from extracted RNA using M-MLV Reverse Transcriptase kit (Invitrogen). In order to extract RNA, parasite pellets (prepared as in 2.2.5.1) were homogenised in TRIzol® reagent (Invitrogen) to disrupt and dissolve cellular components. RNA was separated into an upper aqueous layer by the addition of chloroform, and precipitated by isopropanol and ethanol according to the manufacturer’s protocol. The pellet was air-dried and the RNA solubilised in 50 µl Ultrapure™ DNase and RNase-free distilled water (Gibco) by incubation at 65°C. Contaminating DNA was removed by DNase treatment using Ambion’s TURBO DNase. RNA samples were incubated for 3 min at 37°C with 1x TURBO DNase buffer and 1 µl TURBO DNase. The reaction was terminated by the addition of DNase Inactivation Reagent. RNA samples were used immediately or snap-frozen and stored at -80°C. The M-MLV Reverse Transcriptase kit (Invitrogen) was used for cDNA synthesis, using oligo(dT) primers to bind the polyA tails of mRNA and Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) to catalyse the reaction in the presence of RNaseOUT recombinant ribonuclease inhibitor (40 U per µl, Invitrogen). Synthesis was allowed to proceed for 50 minutes at 37°C then the reaction was stopped by heating to 70°C for 15 min. Reverse
transcriptase negative reactions were carried out in parallel, from which it could be
determined if contaminating gDNA was present by PCR amplification.

2.2.6. Polymerase chain reaction (PCR)

To assess the integrity of DNA and/or to check for primer amplification, analytical PCRs
were performed using GoTaq® Green Master Mix (Promega) which contains GoTaq®
DNA polymerase in GoTaq® Reaction Buffer, dNTPs and MgCl\textsubscript{2}. 15 \mu l reactions were
carried out, containing 1x Master Mix, 1 mM of each forward and reverse primers and
1 \mu g of template DNA. For cloning purposes, DNA was amplified using the Advantage®
2 PCR system (Clontech). Typically 50 \mu l reactions were carried out, containing 1 \mu g of
template DNA, 0.2 mM of each dNTP (Promega), 1 mM forward and 1 mM reverse
primers, 1x Advantage PCR buffer, and 1 \mu l Advantage Taq polymerase. Where errors in
sequences were frequently detected in amplified DNA, the Phusion PCR system (NEB)
and/or PWO PCR (Roche) were utilised (reactions were carried out as described in the
respective protocols).

The following standard program was used for all PCRs and adapted if necessary: 94°C
for 5 min (initial denaturing step); 94°C for 45 s (denaturing step), 50°C for 45 s
(annealing step), 62°C for 1.5 min (elongation step), repeated for 30 cycles; 62°C for 5
min (final elongation step) and then held at 4°C. The annealing temperature was
modified as appropriate for each primer pairing, depending on the T\textsubscript{m}. The elongation
time and number of cycles were altered according to the expected product size. PCR
products were visualised by agarose gel electrophoresis (see 2.2.7).

Components of the QIAquick® Gel Extraction Kit were used for direct purification of
PCR products. The supplied binding buffer was added directly to the PCR sample and the
mixture was applied to the spin column. Nucleic acids become adsorbed to the silica-gel
membrane in the high-salt conditions provided by the buffer. Impurities and short DNA
fragments were washed out and purified DNA was eluted in 30 \mu l of miliQ water.

2.2.7. Agarose gel electrophoresis

DNA was resolved using 0.8 % (w/v) agarose gels containing ethidium bromide (BDH
Chemicals Ltd.) at a final concentration of 0.5 \mu g/ml to enable visualisation under UV
light. Tris-acetate-EDTA (TAE; 40 mM Tris-HCl, 1 mM EDTA, 20 mM acetic acid) was
used both in the agarose gel and as running buffer. Samples were loaded in Green
GoTaq™ Reaction Buffer (Promega) and 5 µl HyperLadder™ I (Bioline) was loaded alongside samples to allow estimation of fragment sizes and for approximate quantification. Gels were run at 100 V for 30-60 minutes and photographed using a UV imager box (300-360 nm).

The QIAquick® Gel Extraction Kit (Qiagen) was used to solubilise and purify DNA from agarose gels where required. Excised gels slices were dissolved in Qiagen Solubilisation Buffer QG by incubating at 37°C for 10-20 minutes and QIAquick® spin columns were used to bind DNA according to the manufacturer’s protocol. DNA was eluted in 30 µl of milliQ water or for LIC cloning in TLoweE buffer. Eluates were stored at -20°C if not used immediately.

2.2.8. DNA ligations

PCR products were cloned immediately in the pGEM® T-Easy vector (Promega) which is supplied linearised with single 5’-deoxythymidine (dT) overhangs. Since the terminal transferase activity of Taq polymerase results in the preferential addition of single deoxyadenosine (dA) to the 3’ ends of amplified DNA, PCR products can be directly cloned into the pGEM® vector without the need for digestion with restriction enzymes. Ligations were carried out in 10 µl reaction volumes, comprising 5 µl 2x Ligation Buffer, 1 µl T-Easy vector, 1-3 µl purified PCR product (2.2.4.5) and 1 µl T4 DNA ligase. Reactions were incubated at 16°C for 4 hours and then at 4°C overnight.

Ligations into the tagging vectors pDR0007-myc and pDR0008-gfp were performed using T4 DNA ligase (Roche) or the Roche Rapid DNA Ligation Kit. Concentrations of vector and insert DNA were measured on a NanoDrop® ND-1000 Spectrophotometer. Ligations were typically carried out in 20 µl reaction volumes containing 9-90 fmol of insert and 3-30 fmol vector, at an insert:vector at a ratio of 10:1, 1x ligase buffer and 0.1 U T4 DNA ligase. Reactions were incubated at 16°C for 4 hours and then at 4°C overnight. When using the Rapid DNA Ligation Kit (Roche), reactions comprising 1 µl vector, 3 µl insert, 5 µl buffer and 1µl (0.5 U) T4 DNA ligase were incubated for 5 min at 25°C and then at 4°C overnight.

2.2.9. Transformation of *E. coli* and selection of transformed cells

All transformations were performed using heat shock to facilitate entry of plasmid vectors into bacterial and transformed cells were selected for by growth on Luria-Bertani (LB)
agar containing appropriate antibiotic. All LB agar and media was prepared from premixed powder (Merck) according to manufacturer’s instructions.

Expression vectors were initially transformed into *E. coli* NovaBlue Gigasingles™. 25 µl of competent cells were thawed on ice and added to 1µl of annealed vector/insert. The suspension was incubated on ice for 5 min, then heated for 20 s at 37°C and subsequently placed back on ice for 2 min. 800 µl of salt optimised and carbon (SOC) medium (Invitrogen) was added and cultures were incubated for 1 h at 37°C with agitation at 200 rpm to permit initial bacterial cell growth in absence of selection. Cells were collected by centrifugation (10,000g for 2 min) and a volume of approximately 150 µl was plated onto LB agar plates containing carbenicillin (50 µg/ml) to select for growth of transformants with ampR genotype.

Once the presence of insert had been confirmed, plasmids were subsequently transformed into *E. coli* BL21 (DE3) pMico or Rosetta (DE3) pLysS for protein expression. 25 µl of competent cells were thawed on ice and added to 1µl of annealed vector/insert and incubated on ice for 1 hour. Cells were transformed by heat shock for 30 s at 42°C, and after which were incubated back on ice for 2 min. Initial bacterial cell growth was mediated by culture in 1 ml LB media for 1 h at 37°C with agitation at 200 rpm. Cells were plated as above.

All other transformations were performed using Subcloning Efficiency DH5α competent *E. coli* cells (Invitrogen). 50 µl of competent cells were thawed on ice and added to 10 µl of ligation mixture and incubated on ice for 30 min. Cells were then heat-shocked for 20 s at 37°C and placed on ice for 2 mins. Cells were allowed to recover as above and were plated onto LB agar plates containing ampicillin (50 µg/ml).

2.2.10. Preparation of plasmid DNA

Plasmid DNA was extracted from 5 ml overnight *E. coli* cultures using a QIAprep® spin miniprep kit (Qiagen). Five bacterial colonies were picked under sterile conditions using a P20 pipette tip (Starlab) and the whole pipette tip was placed into a 30 ml Universal tube (Sterilin) containing 5 ml LB broth with ampicillin (150 µg/ml). Cultures were incubated at 37°C with agitation at 200 rpm for 8 h or overnight. Plasmid DNA was isolated from 5 ml overnight cultures using a QIAprep® spin miniprep kit (Qiagen).
Alkaline lysis, followed by neutralisation/DNA renaturation with acetate and purification by binding to the silica gel column was performed as described in the manufacturer’s instructions. DNA was washed with an ethanol-containing buffer and eluted in 30 µl of miliQ water. Glycerol stocks of transformed cells were prepared by mixing 500 µl of bacterial culture (prepared as described above) with 500 µl 50% glycerol. Stocks were maintained at -80ºC.

2.2.11. Restriction digests

Restriction digests were typically carried out using 1 µg plasmid DNA, 2 U of enzyme (NEB or Promega) and 1x compatible buffer (NEB or Promega). Diagnostic restriction digests, to assess for the presence of inserts in plasmid preparations, were carried out in 15 µl reactions and were incubated at 37°C for 2.5 h. Samples (2 µl) were analysed by agarose gel electrophoresis (2.2.6.2.) alongside a sample of uncut plasmid.

ApaI/KpnI digestions for cloning were carried out in 60 µl reactions using 1x multicore buffer. Reactions were incubated overnight at 37°C. For plasmid digests, the possibility of plasmid re-ligation – which is possible when double-digestion does not occur with 100% efficiency – was prevented by dephosphorylation of 5’ ends by incubating for 1 h at 37°C with 1 µl shrimp alkaline phosphatase (Promega). The reaction was terminated by incubation for 15 min at 65°C.

2.2.12. Sequencing

Fully-automated sequencing was carried out by the Molecular Biology Unit at the Natural History Museum. The sequencing of DNA cloned into pET-46 was performed from the T7 promoter and T7 terminator. The sequencing of DNA cloned into pGEM® T-Easy vector was carried out using T7 and SP6 primers. Sequences were analysed manually by alignment of the obtained sequence with that predicted from genomic sequence using ClustalW (http://www.ch.embnet.org/software/ClustalW.html).
2.3. Production of antisera

2.3.1. Expression of recombinant proteins in *E. coli*

*E. coli* expression strains containing recombinant plasmid (or empty plasmid as control) were grown overnight in 5 ml LB containing 50 µg/ml carbenicillin. Each culture was then diluted to the desired volume (between 250-1000 ml) and incubated at 37°C on a shaking platform set at 250 rpm until the bacteria reach mid-late log growth phase (optical density at 600 nm of 0.6 or higher, i.e. ≥ 60 million cells). Overexpression of the target protein was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.2-1.0 mM. Cultures were incubated for 4 h at 37°C or overnight at 28°C on a shaking platform set at 250 rpm. Cells were collected by centrifugation (13,000 rpm for 20 min at 4°C) and either stored at -20°C or lysed immediately.

2.3.2. Extraction of protein

Novagen’s BugBuster® Protein Extraction Reagent was used to release soluble protein by gentle disruption of the bacterial cell wall. Harvested cell pellets were resuspended at 5 ml per gram in BugBuster® HT Protein Extraction Reagent (Novagen) and soluble protein was separated from insoluble protein and cellular debris by centrifugation (13,000 rpm for 20 min at 4°C). To obtain the insoluble protein fraction, the cell pellet was resuspended in 8 M urea buffer (100 mM NaH$_2$PO$_4$, 10 mM tris-HCl, 8 M Urea, pH 8.0) at 2.5 ml per gram and placed on a rotary shaker at room temperature for at least 1 h for cell lysis and protein solubilisation. Benzonase® nuclease (Novagen) was added to a final concentration of 2.5 U/ml to digest nucleic acids and reduce viscosity. The urea-solubilised protein was separated from cellular debris by centrifugation (13,000 rpm for 20 min at 4°C).

2.3.3. Purification of histidine-tagged proteins

Histidine-tagged fusion proteins were purified by nickel affinity using Ni-NTA agarose beads (Qiagen). To purify soluble fractions, 1 ml of Ni-NTA slurry was added to 4 ml of extracted protein in a falcon tube and mixed on a rotary shaker overnight at 4°C. The flow-through was separated by centrifugation (13,000 rpm for 5 min) and removed. Beads were washed four times with 20-50 ml of wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole; pH 8.0) and were transferred to a microcentrifuge tube. Bound protein was eluted by increasing imidazole concentration; eluates were collected by
adding 500 µl of elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole; pH 8.0) followed by centrifugation (13,000 rpm for 2 min). Purification of urea-solubilised fractions (denatured protein) was performed at room temperature. 1 ml of Ni-NTA slurry was added to 4 ml of extracted protein in a falcon tube and mixed on a rotary shaker for 1 h. The flow-through was separated by centrifugation (13,000 rpm for 5 min) and removed. Beads were washed four times with 20-50 ml of wash buffer (100 mM NaH$_2$PO$_4$, 10mM tris-HCl, 8 M urea; pH 6.3) and were transferred to a microcentrifuge. Bound protein was eluted by decreasing pH; eluates were collected by adding 500 µl of elution buffer (100 mM NaH$_2$PO$_4$, 10 mM Tris-HCl, 8 M urea; pH 5.4 for eluates 1-4 and pH 4.5 for eluates 5-8) followed by centrifugation (13,000 rpm for 2 min).

2.3.4. Dialysis and concentration of protein solutions

Proteins were dialysed into PBS in a stepwise manner. Samples were injected into a Pierce Dialysis cassette with a molecular weight cut-off (MWCO) of 20 kDa and capacity of 0.5-3.0 ml. The cassette was placed into a 2 litre beaker, initially containing phosphate buffer with 6 M urea. The solution was stirred at 4°C and the buffer replaced with a 4 M after 2 hours, a 2 M after 4 hours, and with PBS after 6 hours and dialysed in PBS overnight. Protein solutions were concentrated using Centricon® centrifugal filter units (Millipore).

2.3.5. SDS- PAGE

Proteins were separated by Tris-glycine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 12% SDS polyacrylamide gels (80 mm x 73 mm x 1 mm) were cast according to Sambrook et al. (1989) using the Biorad Mini Protean III Electrophoresis System. Protein samples were heated to 98°C for 5 minutes in SDS sample buffer (4x stock solution: 250 mM Tris-HCl; pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.1% (w/v) bromophenol blue) with the addition of 1% (v/v) β-mercaptoethanol (βME) and loaded into the gel. Dual colour pre-stained Precision Plus protein standard (BioRad) was used as a molecular weight marker. Gels were run in 1x Tris-glycine running buffer (10x stock: 250 mM Tris base, 1.92 M glycine, 10% (w/v) SDS) at 100V until the bromophenol blue dye front reached the bottom of the gel. For visualisation of separated proteins, gels were stained in Coomassie blue stain solution (40% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie brilliant blue R250) for at least 30 minutes. Bands were visualised by applying destain solution (40% (v/v)
methanol 7% (v/v) acetic acid) for 20 minutes and 5% (v/v) methanol 7% (v/v) acetic acid overnight.

2.3.6. Extraction of SDS-PAGE-separated protein

Proteins were separated by SDS-PAGE and stained by Coomassie (see 2.3.1.6). The gel was rinsed in PBS and the target band was excised. Attempts were made to acquire a form suitable for immunisations by cutting the gel into small pieces and fragmenting further by passing it through progressively smaller needles, but elution of protein from the gel matrix was considered a preferred approach. To elute protein from the gel, the gel slice was immersed in elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5), crushed using a pestle, and incubated overnight at 30ºC. Supernatant was separated by centrifugation (8,000g for 10 min); a sample was tested by SDS-PAGE and the remainder was used in immunisations.

2.3.7. Detection of histidine-tagged proteins by Western blot

SDS-PAGE separated proteins were transferred onto a nitrocellulose membrane using a semi-dry electroblotter (LKB Bromma, Novablot). A Tris-glycine buffer containing 20% methanol was used as transfer solution (25 mM Tris base, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol). The gel was blotted at 220 mA for 20 minutes per gel. Histagged fusion proteins were detected using His-Probe™-HRP (Pierce); a nickel-activated derivative of horseradish peroxidase (HRP). Nitrocellulose membrane containing transferred proteins were blocked either overnight at 4ºC or for 1 hour at RT in BSA blocking buffer (1% BSA in TBST; 1% (v/v) tween-20 in Tris-buffered saline (50 mM Tris-HCl; pH 7.4, 150 mM NaCl)). Membranes were incubated for 1.5 hours in His-Probe™-HRP at a 1 in 3000 concentration in BSA blocking buffer and then washed 3 times for 10 minutes in TBST. Probed blots were developed using the enhanced chemiluminescence ECL Plus Western Blotting Detection System (GE Healthcare). Membranes were removed from TBST and placed onto transparent film (Niceday), lumigen mixture (prepared according to the manufacturer’s instructions) was pipetted onto the blot and incubated for 5 minutes. Sandwiched between two pieces of transparent films in an X-ray cassette, blots were briefly exposed in the dark to Kodak BioMax MS Scientific Imaging film and developed using an Agfa Curix 60 developing machine.
2.3.8. Immunisation schedule for antibody production

Groups of female BALB/c mice, 6-8 weeks old, were used as hosts for antibody production. Initial immunisations were performed by subcutaneous injection with 40-60 µg of antigen emulsified at a 1:1 ratio in Freund’s complete adjuvant. Boosts were administered with the same dose of antigen emulsified in Freund’s incomplete adjuvant, 14, 28 and 56 days after the primary immunisation. Test bleeds were taken 10 days after each boost immunisation for analysis (see 2.3.9). Mice were placed in a heat box set at 35°C to induce tail vein dilation and approximately 100 µl of tail blood was collected in 1.5 ml microcentrifuge tubes by repeated tail drops. Blood was stored at 4°C overnight to allow for coagulation and sera was subsequently collected following centrifugation (13,000 rpm at 4°C for 20 min). Samples were tested for recognition of recombinant protein by immunoblotting (see 2.3.9). When a specific antibody response was detected sera was collected by a final bleed. Blood was collected by cardiac puncture as in 2.1.2 but without the use of heparin to allow for coagulation of blood and subsequent collection of sera. The blood was allowed to clot at 4°C overnight and sera was collected following centrifugation at 13,000 rpm at 4°C for 20 min. Serum was stored in aliquots at -80°C. Mice whose sera did not display specific antigen binding in immunoblots were re-immunised with a boost injection. A maximum of three boosts were given (14, 28 and 56 days after the primary immunisation) before a final bleed.

2.3.9. Characterisation of antisera by immunoblot

Immunoblots were performed to test serum samples for the presence of antibodies against recombinant LAP (rLAP). 5 ml overnight cultures of bacterial cells transformed with rLAP or empty vector were prepared and split; one to serve as IPTG-induced sample and the second to provide an uninduced sample. Cultures were centrifuged and resuspended in 10 ml fresh LB medium containing carbenicillin and further incubated at 37°C. Protein expression was induced by the addition of 1mM IPTG and incubation for 4 h at 37°C. Cells were collected by (4°C, 8000g for 15 min), resuspended in 200 µl PBS and lysed in reducing sample buffer, heating at 95°C for 10 min. Protein lysates were separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% fat-free milk powder (Waitrose) in TBST for one hour at room temperature or overnight at 4°C. Membranes were cut into 2-lane strips (corresponding to rLAP and empty vector). After washing with TBST, the membranes were incubated with serum samples diluted 1 in 1000 in 1% milk in TBST. Serum collected from a naïve mouse was used as a negative control. Membranes were washed three times for 10 min
each in TBST then a sheep anti-mouse IgG antibody conjugated to horseradish peroxidase (Pierce) (1 in 10,000 dilution in 5% milk in TBST). The membrane was washed again three times for 10 minutes each in TBST, then developed using the ECL Plus Western Blotting Detection System (GE Healthcare) as described in 2.3.7. Western blots for the detection of histidine-tagged protein (see 2.3.7) were performed in parallel to assess and compare the size of the target protein.

2.3.10. Characterisation of antisera by ELISA

Overnight cultures of bacterial cells transformed with rLAP or empty vector were prepared as described in 2.3.10 and whole cell lysates were used as antigen. A 96-well ELISA plate (Nunc) was coated with 100 µl/well antigen or 100 µl/well control at concentration of ~1-10 µg/ml. The plate was covered with clingfilm and incubated overnight at 4°C. Wells of the plate were washed twice with PBS and remaining protein-binding sites were blocked with 100 µl/well 1% BSA. The plate was incubated for 2 h at room temperature 37°C. Wells of the plate were subsequently washed twice with PBS and 50 µl of antibody at a range of dilutions were added. Dilutions were made in 1% BSA, starting at 1:50, with the final well without any sera as a background control for non-specific binding of the secondary antibody. The plate was incubated for 2 h at room temperature. Wells of the plate were then washed twice with PBS and 50 µl of HRP-conjugated anti-mouse IgG secondary antibody (GE Healthcare) was added at a 1:10,000 dilution and incubated for 1 h at room temperature. Freshly prepared o-phenylenediamine (OPD) was used as substrate for the detection of peroxidase activity. Using the SIGMAFAST™ OPD tablet set (Sigma), one OPD tablet and one urea hydrogen peroxide tablet were dissolved in 20 ml of water to form a solution containing 0.4 mg/ml OPD in 0.05 M phosphate-citrate buffer with 0.4 mg/ml urea hydrogen peroxide, pH 5.0. After washing the wells of the plate twice with PBS, the OPD substrate solution was added at 50µl/well. Reactions were allowed to develop for 30 min at room temperature in the dark, after which the reaction was stopped by addition of 50µl/well 3M H₂SO₄. Stopped reactions were read at 492 nm.
2.4. Transmission-blocking assays

2.4.1. Exflagellation assays

A PH-treated mouse injected with *P. berghei* clone 234 (day 4 or 5 post-infection) provided mature gametocytes to assess the affects of antisera on exflagellation. Assays were carried out as follows: 2.5 µl of tail blood containing parasites capable of exflagellation was added to 2.5 µl sera and 5 µl of ookinete medium. The mixture was pipetted onto a microscope slide and covered with a Vaseline®-rimmed coverslip and allowed to stand for 20 minutes. Exflagellation centres were defined as a cluster of cells comprising exflagellating male parasite(s) binding three or more red blood cells (Figure 2.2). The number of exflagellation centres in 20 microscopic fields at 40× objective magnification was counted and experiments were performed in duplicate. Results were expressed as the number of exflagellation centres per field of view and as a percentage compared to the number of exflagellation centres in negative controls (serum from an unimmunised mouse in place of test serum).

![Figure 2.2. *P. berghei* exflagellation centres](image)

Exflagellation centres are observed and counted as clumps of red blood cells. Representative light microscopy image (40x objective). See text for details.

2.4.2. Ookinet development assays

Mice were injected with PH and 2-3 days later infected with wild-type parasites (as in 2.1.2). Blood containing mature gametocytes, day 3 or 4 post-infection, was used to assess the effects of antisera on ookinete development. Ookinete mini-cultures containing 70 µl of ookinete media + 20% FBS and 25 µl test sera (αLAP), control sera (from an unimmunised mouse) or PBS were prepared in duplicate in the wells of a 24-well plate
(Nunc). 5 µl of heparinised blood was added to each well and cultures were incubated at 19°C for 24 h to allow for ookinete development. The numbers of ookinetes per well were subsequently determined using a haemocytometer (Naubauer) using an Olympus BH2 light microscope at x40 objective magnification. For each test sample, the number of ookinetes was calculated as a percentage of the average number of ookinetes in normal mouse sera negative controls.

2.4.3. Membrane feeding assays

Mosquito membrane feeds were performed in order to examine the affects of antisera on parasite development in the mosquito. The PbconGFP transgenic line, which expresses GFP constitutively throughout the life cycle (Franke-Fayard et al., 2004) was used to aid in oocyst counting. Mice without prior PH treatment were infected with PbconGFP and on day 3 were assessed for parasitemia and exflagellation. Enough mice were infected to supply infected blood for the required number of test groups (assuming a maximum of 1 ml of blood is acquired for each mouse and accounting for less). The membrane feeding apparatus was prepared by connecting plastic membrane feeders to each other with silicon tubing. A water bath heated to 39ºC with pump was used to generate a continuous flow of water through each feeder. Thinly stretched Parafilm® (Pechiney Plastic Packaging Company, VWR), through which mosquitoes can probe, was stretched over the underside of each feeder. 125 µl of test sera, pre-warmed to 37°C in a heat block, was used per feed. Blood containing mature gametocytes was removed from mice and, to control for variation in gametocytemia and general health status between different mice, all blood was pooled into a pre-warmed 50 ml nunc tube. Procedures were carried out as quickly as possible to avoid exflagellation before ingestion of blood meal. For each feeder, 375 µl of pooled blood was added to 125 µl of test sera at 37°C. Blood/antisera mixtures were then added to membrane feeders using a blunt-ended syringe and mosquitoes were allowed to feed for 30 min in the dark.
Figure 2.3. Schematic of a mosquito membrane feed to assess the effect of antisera on infectivity to mosquitoes.

The membrane feeding apparatus consists of plastic membrane feeders that can be connected with silicon tubing to a heated water bath to generate a continuous flow of 37°C water through each feeder to attract the mosquitoes. Thinly stretched Parafilm® (Pechiney Plastic Packaging Company, VWR), through which mosquitoes can probe, is stretched over the underside of each feeder. Aliquots of test serum to were prepared in eppendorf tubes and pre-warmed to 37°C in a heat block. Blood containing mature gametocytes is removed from mice and pooled into a pre-warmed 50 ml nunc tube. Gametocyte-containing blood is added to serum aliquots at 37°C such that serum is diluted to the desired concentration. Blood/antisera mixtures are then added to membrane feeders using a blunt-ended syringe. Mosquitoes, within pots covered with a mesh net top, are allowed to feed for around 30 minutes in the dark.

Mosquitoes were maintained as described in 2.1.3. Ten or eleven days following infection, midguts were dissected and oocysts counted. Mosquitoes were anaesthetised using CO₂ and maintained immobilised on ice. The foregut, hindgut and malpighian tubules were removed under a dissecting microscope in a drop of PBS. Dissected midguts were transferred to a 24-well plate containing 4% paraformaldehyde (PFA) prepared (in PBS) from 16% methanol-free formaldehyde (Polysciences Inc.). Midguts were fixed in PFA for 45 min then washed three times in PBS before being mounted onto a slide. GFP-expressing oocysts were counted using a Leica DMR fluorescence microscope under 40x objective magnification.
Prevalence of infection was calculated as the percentage of infected mosquitoes out of those dissected. The number of oocysts per midgut (number of oocysts divided by total number of mosquitoes) and infection intensities (number of oocysts divided by number of infected mosquitoes) were calculated as arithmetic means, as described in (Medley et al., 1993). Mann-Whitney-U tests were carried out to establish if oocyst numbers/intensities in test groups differed significantly from feeds in the presence of sera from an unimmunised mouse. Analyses were performed using GraphPad Prism 5.

2.4.4. Intra-thoracic microinjection of antisera

Antiserum was injected into the thorax of mosquitoes to assess if it could neutralise sporozoites released in the haemocoel. Mosquitoes were fed on gametocyte-containing blood and infections were observed from day 14. Intra-thoracic injections were performed when sporozoites were observed to be released in the haemocoel. Three days post-injection, mosquitoes were anesthetised using CO₂ and salivary glands were removed into PBS by dissection under a light microscope using forceps and a 26 gauge needle. The glands from between 8 and 10 mosquitoes were transferred into a 1 ml glass homogeniser (Jencons) using a Hamilton Microliter syringe. Salivary glands were disrupted to release sporozoites by three turns of the homogeniser and transferred into a 1.5 ml microcentrifuge tube on ice. Sporozoites were counted using a haemocytometer and prevalence of infection was determined by examination of mosquito midguts. Sporozoite numbers were calculated per salivary gland pair per infected mosquito (i.e. total number of sporozoites divided by number of infected mosquitoes).
2.5. Generation of transgenic *P. berghei* parasites

2.5.1. Preparation of DNA for transfection

Plasmids generated as described in 2.3.3 were linearised by restriction digest and purified for transfections. Unique restriction sites were as follows; Lap3: *AarI* (Fermentas); 1x buffer Tango 37°C Lap 1, 4, 6; *BsmI* (NEB) 1x buffer 2 65°C Lap5; *SpeI* (Promega) 1x buffer B 37°C. Digests were performed in 50 µl reaction volumes, containing 40 µl plasmid DNA, 5 µl buffer, 3.5 µl miliQ water, 1.5 restriction enzyme overnight. Following digestion, DNA was precipitated by addition of 0.3 M sodium acetate pH 5.2 and neat ice-cold ethanol and washed once in 70% ethanol. Precipitated DNA was resuspended in 12 µl miliQ water and concentration was estimated by agarose gel electrophoresis; 3-5 µg of DNA in a volume of 5-10 µl was used for each transfection.

2.5.2. Preparation of schizonts for transfection

Purified mature schizonts containing fully developed merozoites provided parasites for transfections. To limit the passage number of potential transgenics, schizont cultures were set up from parasites that had been passaged only once before (P1). A liquid nitrogen stock of P0 passage parasites (i.e. parasites directly after mosquito transmission) were grown up in a donor mouse and then passaged into the required number of mice, with one mouse producing enough schizonts for 2-3 transfections. Blood with a parasitaemia of 3-5% was added to schizont culture medium (RPMI 1640 containing 25 mM HEPES and 2 mM L-glutamine (Sigma) supplemented with 25% (v/v) FBS, 50U/ml penicillin and 50 µg/ml streptomycin (Gibco). The culture was set up in 500 ml Erlenmayer flasks in a total volume of 120 ml. A 3%O₂, 5% CO₂ and 92% N₂ gas mix was blown over the surface of the culture for 5 min, the flask sealed and the parasites were cultured for 16-20 h at 37°C with gentle agitation (50 rpm). Schizonts were harvested by centrifugation at 180g for 15 min, resuspended in around 2-3 ml of culture and layered onto a 5 ml 15.2% Nycodenz gradient (27.6% Nycodenz) in 15 ml Nunc tubes. Centrifugation was performed at 300g for 30 min, with slow brake and acceleration, and the schizonts collected at the interface were removed, washed once with schizont culture medium and pelleted by centrifugation at 300g for 8 min.

2.5.3. Electroporation of parasites

Transfections were performed by electroporation using an AMAXA Human T-Cell Nucleofector system as described by Janse et al. (2006). Briefly, 1-5 µg of digested DNA
prepared for transfection was mixed with 100 µl supplemented T-cell Nucleofector solution, 100 µl of this mixture was transferred into an AMAXA electroporation cuvette, purified schizonts were added to the Nucleofector–DNA solution, and electroporation was performed using the U33 program of an AMAXA Nucleofector. Electroporated parasites were incubated at 37°C in 150-200 µl of naïve blood from a PH-treated mouse to allow parasites to invade reticulocytes. After incubation for 20-30 minutes, blood was injected i.p. into PH-treated mice.

2.5.4. Drug selection of transgenic parasites

Transfected parasites will contain the mutated \textit{Tgdhfr-ts} gene, conferring pyrimethamine resistance. 24-48 h after transfection, mice were treated with pyrimethamine in drinking water prepared by dilution of 100x stock (7 mg/ml pyrimethamine in DMSO) to 1x in tap water (resulting pH 3.5-5). The growth of parasites was monitored by Giemsa-stained smears of tail blood. Stocks of resistant parasites were obtained and parasites were subjected to a second round of drug selection in another mouse.

2.5.5. Diagnostic PCR

Diagnostic PCRs were carried out on genomic DNA extracted from the blood of infected mice. To amplify DNA from any transgenic parasites, forward primers designed to anneal approximately 200 bp upstream of the target site for homologous recombination were used in combination with one of the following reverse primers:

\textit{Tgdhfr-ts} 5'UTR: GATGTGTATGTGATTAATTCATACAC

GFP\textsubscript{R}: GCGGCCGCTTATTTGTATAGTTCATCCATGC

Myc\textsubscript{R}: GGCCGCTTATAAGTCTTCCTCACTTATTAACGTCCAGATCCTC

TTCTGAGATGATTTTGTTCG

Forward primers were as follows:

- Lap1: ATTGATGAAGAAAATGAATTAGTAATAGAACAAAATTGTAATCC
- Lap2: ATCTCAAGATTGTCCAATCGATTTATTTTG
- Lap3: AGATGTAGATTGTGATAATACCTTTAACGACATTCC
- Lap4: ATGTTAACGTTAATATGG
- Lap5: TCTATATCACCAGGGCACCATAATATAAC
- Lap6: ATTGGAGAATGGAAATACACAAGTG

GoTaq Green DNA polymerase Master Mix (Promega) was used to amplify DNA from genomic DNA extracted from the blood mice containing potential transgenics. Reactions were carried out in volumes of 12.5 µl.
2.6. Immunodetection of parasite proteins

2.6.1. Sample preparation for immunoblot analysis

Samples of *P. berghei* asexual blood stages, gametocytes, ookinetes and sporozoites were prepared as described in 2.6.1-2.6.4. Parasite numbers were quantified using a haemocytometer and where necessary purity was determined by Giemsa-stained smears. Samples were suspended in non-reducing sample buffer containing protease inhibitor (prepared from Roche complete, EDTA-free protease inhibitor cocktail tablets) and stored at -20°C if not used immediately.

2.6.1.1. Asexual blood stages

Mice were infected with *P. berghei* ANKA clone 233 (non-gametocyte producing strain). Blood was collected 3-4 days post-infection and white blood cells were removed by passing blood through a CF11 column, comprising glass wool and dry cellulose powder (Whatmann) in a 10 ml syringe. The column was equilibrated using PBS and blood was washed through in 10 ml of PBS. Cells were collected by centrifugation (500g for 5 min at 4°C) and resuspended in 40 ml ice-cold 0.17 M ammonium chloride to lyse red blood cells. After a 30 minute incubation period on ice, parasite pellets were obtained by centrifugation (500g for 5 min at 4°C) and washed three times in PBS.

2.6.1.2. Gametocytes

Gametocyte-enriched samples were prepared by a combination of asexual blood stage depletion and separation of gametocytes by density centrifugation. *P. berghei*-infected mice with parasitemia exceeding 5% (typically 3 days post-infection) were treated with sulphadiazine (Sigma), a schizonticide. Either drinking water was supplemented with 20 mg/L sulphadiazine or mice were i.p. injected each day with 0.24 mg sulphadiazine in PBS. The level of asexual blood-stage parasitemia and gametocytemia were monitored over the following 2-3 days by analysing Giemsa-stained smears. Gametocyte-containing blood was collected when asexual blood-stage parasitemia had sufficiently decreased relative to gametocytemia. The parasites were immediately transferred to 5 ml coelenterazine loading buffer (CLB), containing 20 mM Hepes, 20 mM glucose, 4 mM sodium bicarbonate, 1 mM EDTA and 0.1% BSA in PBS (pH 7.25). Within CLB, gametocytes remain suspended in an unactivated state provided maintenance at 22°C. Cells were passed through a CF11 column equilibrated with CLB and following removal of white blood cells, parasites were collected by centrifugation (600g for 5 min at 22°C)
and resuspended in 2-3 ml of CLB. Gametocytes were further enriched by separation on a
Nycodenz step (5 ml 48% Nycodenz stock solution; 27.6% (w/v) Nycodenz (Axis-
Shield), 3.8 mM Tris-HCl, 300 mM Ethylenediamine tetraacetic acid (EDTA), 3 mM
potassium chloride, pH 7.5). Enrichment was performed by centrifugation (1000g for 20
min at 22°C with slow brake and acceleration). Gametocytes contained at the interface
were collected and transferred to a 1.5 ml microcentrifuge tube, within which they were
washed three times in CLB.

2.6.1.3. Ookinetes
Ookinetes were prepared by the collection of ookinetes produced by in
vitro cultivation. Blood was collected from a P. berghei infected mouse 3-4 days post-
infection, white blood cells were removed by passing blood through a CF11 column at
19°C and ookinetes were cultured as described in 2.1.2. After 24 hours, cells were
harvested by centrifugation (500g for 10 min at 4°C), resuspended in 40 ml ice-cold 0.17
M ammonium chloride and incubated on ice for 30 min to lyse red blood cells. Parasites
were collected by centrifugation (500g for 10 min at 4°C) and resuspended in 2-3 ml of
ookinete culture medium. Ookinetes were enriched by Nycodenz density separation (5 ml
48% Nycodenz stock; as in 2.6.3), performing centrifugation at 500 g for 20 min, with
slow brake and acceleration. Ookinetes contained at the interface were transferred to a 1.5
ml microcentrifuge tube and washed three times in PBS at 4°C.

2.6.1.4. Sporozoites
Mosquitoes harbouring salivary gland sporozoites, typically 21 days following infection,
were anaesthetised and salivary glands were removed into PBS using forceps and a 26
gauge needle. Dissected glands were homogenised in a 1 ml glass homogeniser (Jencons)
and sporozoites were transferred into a 1.5 ml microcentrifuge tube on ice.

2.6.2. Detection of parasite proteins by immunoblot
Parasite preparations were heated for 10 minutes at 95°C in sample buffer (with the
addition of βME for reducing conditions) and were separated by SDS-PAGE on an 8%
polyacrylamide gel. Pre-cast Tris-glycine native 7.5% polyacrylamide gels (Invitrogen)
were used for analysis under native conditions. Samples were loaded and run in buffer
without denaturing SDS (4x stock sample buffer solution: 250 mM Tris-HCl; pH 6.8,
40% (v/v) glycerol, 0.1% (w/v) bromophenol blue; 10x stock running buffer: 250 mM
Tris base, 1.92 M glycine). Separated proteins were transferred onto a nitrocellulose
membrane as in 2.3.7 and membranes were incubated for 1.5 hours at room temperature
or 4°C overnight in blocking solution (5% (w/v) dried fat-free skimmed milk powder (Waitrose, UK) or 1% BSA in TBST). Primary antibodies were used as follows: anti-lapat at 1 in 100; αMyc Rabbit mAb (Cell Signalling) at 1 in 1,500; α-Tubulin Mouse mAb (Prof. Keith Gull) at 1 in 10,000. Incubations were performed for 2 hours at room temperature or overnight at 4°C at the desired dilution in blocking solution. HRP-conjugated anti-mouse IgG (Pierce) or HRP-conjugated anti-rabbit IgG (Sigma), each raised in sheep, were used as secondary antibodies, at dilution 1 in 10,000. Blots were developed using the enhanced chemiluminescence ECL Plus Western Blotting Detection System (GE Healthcare) as described in 2.3.7.

2.6.3. Fixed-cell immunofluorescence assays

2.6.3.1. P. berghei gametocytes, zygotes and ookinetes

Mixed blood stages containing unactivated gametocytes were prepared from blood suspended and washed in CLB. Mixed blood stages post-gametocyte activation were prepared by incubating blood in ookinete media for up to 20 minutes and washed twice in PBS. Zygotes and ookinetes were prepared from ookinete cultures (zygote formation occurs within 1 hour and mature ookinetes are formed within 24 hours) and washed twice in PBS. In each case, parasites were resuspended in PBS / FBS at a 1:1 ratio and 1 µl was spotted and smeared onto frosted glass slides. Following air-drying, a liquid-repellent PapPen (liquid blocker, Daido Sangyo Co. Ltd.) was used to draw sample areas on each slide. Cells were fixed by: i) protein precipitation using either methanol or 50% methanol: 50% acetone (5 minutes at -20°C) or ii) cross-linking using 4% (w/v) PFA in PBS (10 minutes at RT). For permeabilisation of PFA-fixed parasites, cells were washed once with TBS and incubated with 0.2% (v/v) Triton X-100 in PBS for 5 min at RT. All following steps were carried out at RT, and all washes were 5 minutes. Slides were washed three times in TBS, quenched in 0.1% (w/v) sodium borohydride in TBS for 5 min, rinsed in TBS and then blocked for 1 hour in blocking buffer (5% (v/v) goat serum (Jackson Immuno Research), 3% (w/v) BSA in PBS). For antibody incubation, slides were placed in a humidifying chamber and antibody at desired dilution in blocking buffer was pipetted onto sample areas. The slides were incubated overnight at 4°C. The next day, slides were washed 3 times in TBS and secondary antibody incubation (in blocking buffer) was carried out for 1 hour in the dark. Slides were washed three times in TBS and mounted with Mowiol containing DAPI. Slides were kept at 4°C in the dark and were examined using a Leica DMR microscope and Axiovision software.
2.6.3.2. *P. berghei* oocysts

Infected mosquito midguts were dissected in PBS, fixed for 45 seconds in 4% (w/v) PFA, and then opened and blood meal was removed before fixation for 35 minutes in 4% (w/v) PFA at RT. Guts were washed twice in PBS at RT, blocked for 90 min in blocking buffer (1% (w/v) BSA and 0.1% (v/v) Triton X-100 in PBS) and incubated overnight with antibody in blocking buffer at 4°C. Midguts were then washed three times for 20 minutes with PBS and incubated with secondary antibody in blocking buffer for 1 hour at RT. Midguts were then washed as before and mounted in Vectashield/DAPI (Vector Labs).

2.6.3.3. *P. berghei* sporozoites

Sporozoites were dissected into RPMI 1640 and a calculated 3,000 sporozoites in 20µl were incubated on multiwell microscope slides in a wet chamber at 37°C for 30-45 min. The liquid was then taken off and slides dried at RT overnight. Slides were then fixed in 1% (w/v) PFA in PBS for 5 min, blocked in 1% (w/v) BSA in PBS for 20 min and incubated with primary antibody. After three washes with PBS, slides were incubated with secondary antibody for 1 h, washed as before and mounted with Vectashield/DAPI.

2.6.3.4. *P. falciparum* gametocytes

Mature *P. falciparum* gametocytes (strain 3D7) were produced in culture by Dr. A. Blagborough, A. Talman or M. Delves. Parasites were cultured in RPMI 1640, 50 mg/l hypoxanthine, 24 mM NaHCO₃ and 10% (v/v) heat inactivated human AB serum. Cultures were gassed for 20 seconds with 3% O₂, 5% CO₂ and 92% N₂ gas mix and were maintained at 37°C, either manually with daily medium changes or in a semi-automated tipper (Ponnudurai et al., 1982). Day 14 parasites were used for immunofluorescence analyses. 500 µl of gametocyte culture was centrifuged (1 min, 2000g), washed in PBS and 1.5 µl drops were immediately smeared onto a microscope slide and air dried. For activated gametocytes, cells collected by centrifugation were incubated in ookinete medium for 10 min, 15 min or 20 min before being smeared onto microscope slides as above.

2.6.4. Live immunofluorescence assays

Live immunofluorescence assays were performed on parasites collected from gametocyte-containing blood or from ookinete cultures. Samples of 100 µl were washed in PBS and resuspended in 100 µl of PBS. Cells were blocked in 100 µl 2 % BSA in PBS then incubated for one hour in 2 % BSA containing serum diluted at 1 in 50-1 in 500. Cells were then washed three times in PBS, each time collecting the parasites by
centrifugation at 500 g for 2 min. The resulting cell pellet was resuspended in 100 µl 1 % BSA in PBS containing AlexaFluor® 488 goat anti-mouse IgG (Molecular Probes) and cells incubated for one hour in the dark. Cells were then washed in three changes of PBS as above and the final cell pellet resuspended in 50 µl PBS containing Hoechst 33258 (Molecular Probes) at a 1 in 100 dilution. A liquid blocker super PAP pen (Daido Sangyo Co., Ltd) was used to make 15 mm² square wells on glass slides and for each cell sample, 20 µl cell suspension was pipetted into one of the above wells. Cells were then covered with a Vaseline®-edged coverslip and examined by microscopy.

2.6.5. Antibodies

Table 2.3. List of antibodies

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<tr>
<th>Antibody</th>
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<th>Immunofluorescence</th>
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<td>1:50 - 1:500</td>
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<td>-</td>
<td>1:50</td>
</tr>
<tr>
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Secondary

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<th>Immunoblot</th>
<th>Immunofluorescence</th>
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</tr>
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<td>-</td>
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<tr>
<td>Alexa Fluor 546 anti-mouse IgG (Invitrogen)</td>
<td>Goat</td>
<td>-</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Alexa Fluor 546 anti-rabbit IgG (Invitrogen)</td>
<td>Goat</td>
<td>-</td>
<td>1:2,000</td>
</tr>
</tbody>
</table>

2.6.6. Imaging

Parasites were viewed using a Leica fluorescence microscope under 40x, 65x and 100x objective magnification, using the DAPI, FITC and Texas Red filters. Images were captured with a Zeiss AxioCam camera using Zeiss Axiovision40 version 4.6.1.0 software. Post-processing of images was performed using Photoshop version 6.0.
2.7. Phenotypic analysis of ΔPblap parasites

2.7.1. Intra-thoracic microinjection of ookinetes

Ookinetes were cultured and counted using a haemocytometer (Naubauer, Germany). A culture volume containing 11.6x10^5 ookinetes were pelleted by centrifugation (500g for 5 min) and resuspended in 100 µl of ookinete medium. Ookinetes were injected through the mosquito thorax into the haemocoel of mosquitoes using glass capillary needles and the Nanoject II microinjector apparatus (Drummond Scientific Company). Needle tips were created such that the width is large enough to allow enough room for liquid to flow through at a reasonable rate but is narrow enough to inflict a minimal wound during injection. The microinjector was set to deliver a volume of 69 nl per injection and injections were performed with mosquitoes anaesthetised on a CO_2 pad. Mosquitoes were subsequently maintained as described in 2.1.3.

In parallel to haemocoel injections, ookinetes were fed to mosquitoes using a membrane feeding apparatus. To prepare ookinetes for membrane feeds, ookinetes were cultured as in 2.1.2 and counted using a haemocytometer to calculate the number of ookinetes per ml. 4x10^5 ookinetes were pelleted by centrifugation (500g for 5 min) and resuspended in 500 µl of heparinised blood collected from a naïve mouse, providing a final concentration of 800 ookinetes per µl. The membrane feeding apparatus was prepared as described in 2.4.3. The ookinete suspensions were loaded onto membrane feeders and mosquitoes were allowed to feed for 20-30 min at 19°C, preferably in the dark. Unfed mosquitoes were removed the following day and post-feeding maintenance of mosquitoes was carried out as described in 2.1.3.

2.7.2. Transcriptional profiling of ookinetes by microarray

2.7.2.1. Sample preparation

RNA extracted from P. berghei wild-type and Δlap1 ookinetes was used for microarray analysis. Ookinetes were cultured as described in 2.2.6.2 and magnetic beads conjugated to α-Pbs28 monoclonal antibody clone 13.1 were used to purify zygotes and ookinetes, as reported in Siden-Kiamos et al. (2000). 200 µl of sheep anti-mouse Dynabeads® (Dynal Biotech ASA, Invitrogen) were washed four times with PBS; pelleted using a magnetic particle concentrator (Dynal MPC, Invitrogen) and beads were incubated for 30 min on a rotating wheel in 200 µl of α-Pbs28 mAb clone 13.1 (dilution 1:100 in PBS). α-Pbs28-conjugated beads were washed twice further in PBS and resuspended in 200 µl of PBS.
Ookinetes were harvested by centrifugation at 500g for 10 min and resuspended in 8 ml ookinete medium in 15 ml Nunc tubes. Cultures were incubated with 10 µl of 13.1-conjugated beads for 5 min on a rotating wheel. Zygotes and ookinetes expressing Pbs28 were collected using the magnetic particle concentrator through repeated incubations and RNA was extracted from parasite pellets using TRIzol reagent.

### 2.7.2.2. Generation of cRNA and Cy-labelling

All procedures were carried out using reagents from Agilent’s Two-Colour QuickAmp labelling kit according to the manufacturer’s instructions. cDNA was generated from 2 µg of total RNA as template. Reverse transcription was mediated by MMLV-RT and poly dT primer and T7 polymerase promoter sequences were simultaneously incorporated into the 3’ end of the cDNA. Double stranded cDNA was then used as a template for transcription of cRNA using T7 RNA polymerase. Cy3 labelled CTP was incorporated for labelling of ∆lap1 derived cRNA, whilst wt samples were labelled with Cy5. Reactions were performed as described in the QuickAmp labelling protocol (Agilent). Cy3/Cy5 labelled cRNA was purified using Qiagen RNAeasy purification columns and cRNA concentration and dye–incorporation ratios were measured using the Microarray application of a NanoDrop® ND-1000 Spectrophotometer. Labelled probes were stored at -80°C in the dark.

### 2.7.2.3. Hybridisations

2 µg of Cy3-labelled and 2 µg Cy5-labelled cRNA were mixed and were fragmented in Fragmentation buffer at 60°C for 30 min in the dark. The fragmented probes were mixed with Hybridisation solution to a final volume of 80 µl and applied onto the array. All reagents used for hybridisations were supplied in the Agilent in situ Hybridisation Kit. The array was placed into a HybChamber™ and was incubated for 17 hours in the dark in a Hyb oven with rotisserie (65°C, 10 rpm).

### 2.7.2.4. Scanning

Following hybridisation, slides were washed in Gene Expression Wash Buffer containing 0.005% Triton X-102 pre-heated to 37 °C and the HybChamber™ was disassembled whilst immersed in the buffer. Once dry, arrays were scanned using a Gene-Pix 4000B scanner in conjunction with Gene-Pix Pro 4.0 software (Axon instruments). Grid-alignment, recording of spot signal intensity and manual spot inspection was performed using Gene-Pix Pro 4.0.
2.7.2.5. Analysis of microarray data

Processing of microarray data was performed by Dr. D. Vlachou as described in Vlachou et al. (2005). A spot evaluation was carried out whereby the average spot diameter was calculated for each spot and spots with diameter differing by more than three times the standard deviation were removed. Spots were further evaluated by calculating the ratio between spot intensity and local and global background intensity (as defined by negative control spots on the array). Only spots which intensity (foreground) exceeded the local and global background values (spot intensity > average intensity of negative control spots plus one times the standard deviation) were taken forward for further analysis. Following spot evaluation, data was normalised by locally weighted linear regression method (Lowess) method in GeneSpring 6.1 (Axon Instruments). This created a file of valid data, including the expression ratio in fold difference and log transformed expression data for both individual and average spot values. Gene annotations were acquired from PlasmoDB. Clustering was performed using Cluster 3.0 and viewed using Java Treeview.
3 GENERATION OF ANTISERA AGAINST *P. BERGHEI* LAPs & EVALUATION OF ANTIBODY-MEDIATED EFFECTS ON PARASITE TRANSMISSION

3.1. Introduction

Adhesive proteins expressed on the surface of pathogens make prime vaccine targets (reviewed by Dinglasan and Jacobs-Lorena, 2005). In the case of malaria, leading antimalarial vaccine candidates include CSP and TRAP, which contain lectin-like thrombospondin repeats implicated in mediating salivary gland and hepatocyte invasion (Robson et al., 1988), whilst MSP1 and Ps25 contain epidermal growth factor repeats (Blackman et al., 1991; Kaslow et al., 1988). Potentially extracellular adhesive proteins, such as the LCCL/lectin adhesive-like protein (LAP) family, are therefore logical choices for investigation as vaccine candidates.

LAPs are predicted by bioinformatic analyses to encode six putatively secreted proteins with multiple adhesive domains. The presence of N-terminal secretory signals and the absence of predicted transmembrane regions, glycosylphosphatidylinositol (GPI) anchors or ER retention signals indicate a possible surface-exposure and a possible association with the cell surface mediated by adhesion to components of the plasma membrane. Members of the LAP family form four distinct protein arrangements which on current evidence appear unique to apicomplexan parasites (Figure 3.1; reviewed by Dessens et al. (2004); Pradel et al. (2004); Trueman et al. (2004)).

**LAP1 (CCp3)** was first described in *P. falciparum* as the scavenger receptor LCCL adhesive-like protein (PfSLAP) (Delrieu et al., 2002) and in *P. berghei* as the scavenger receptor-like protein (PbSR) (Claudianos et al., 2002). Its predicted multidomain structure comprises four LCCL domains, a lipoxygenase homology 2 (LH2) domain, two tandem scavenger receptor cysteine-rich (SRCR) domains and a pentraxin-like domain. As described in Chapter 1, the LCCL domain (Limulus factor C, Coch-5b2, late lung gestation Lgl1) is defined by four conserved cysteine residues and a conserved motif at the C-terminal end containing a highly conserved histidine, with possible roles in lipopolysaccharide (LPS) binding (Trexler et al., 2000). The LH2 or PLAT (polycystine-
1, lipoygenase, alpha toxin homology) is found among bacteria and eukaryotes, frequently in membrane or lipid associated proteins and may mediate membrane attachment (Bateman and Sandford, 1999). Pentraxin-like domains share similarities with laminin G and Concanavalin A-like lectin/glucanase domains and eukaryotic proteins containing the domains (e.g. sialidases and laminins) are implicated with functions in cell recognition and adhesion (reviewed by Gewurz et al, 1995). The predicted presence of SRCR domains, each containing eight conserved cysteine residues, has been noted as being particularly striking. SRCR motifs are found in surface-associated proteins across a diverse array of metazoans (Muller, 2001). Many are involved in cell-cell adhesion, for example the sperm SPERACT egg peptide receptor of sea urchins (Dangott et al., 1989). Others are involved in regulation of innate and adaptive immune responses, e.g. macrophage SR1 (Freeman et al., 1990) and complement factor 1 (Goldberger et al., 1987). By analogy, this has led to the suggestion that LAP1 may serve a role in protecting the parasite from the mosquito immune response or from complement factors in the blood meal (Claudianos et al., 2002; Delrieu et al., 2002; Lasonder et al., 2002).

**LAP2 (CCp1) and LAP4 (CCp2)** are paralogs, both containing a ricin-B lectin-related carbohydrate binding domain. Similar to the legume lectin ricin from the seeds of the castor bean plant, *Ricinus communis*, the ricin-B lectin-related is found in many plant and bacterial carbohydrate-recognition proteins. Following the Ricin-B domain, the proteins contain a discoidin domain — a domain similar to the coagulation factor 5/8 C-terminal domain — and a neurexin and collagen-like domain (NEC) with similarities to fibrinogen or fibrillar collagen globular domains. The C-terminal regions comprise two tandem levanase-type lectin domains, and two apicomplexan specific cysteine-rich globular domains with homologies to zinc finger binding domains.

**LAP3 (CCp5) and LAP5 (FNPA)** are each predicted to contain a domain related to fibronectin type 2 domain (FN2), a collagen-binding domain, followed by a domain (Anth) similar to anthrax protective antigen (PA14) with potential roles in carbohydrate-recognition. LAP3 also contains a single LCCL domain, whereas LAP5 is the only LAP family member to lack an LCCL domain.

**LAP6 (CCp4)** has a single LCCL domain and two tandem levanase domains. In addition, the C-terminal region of LAP6 displays homologies to a laminin G domain related pentraxin/concanavalin A-like lectin glucanase domain.
Figure 3.1. Domain organisation of the LAP/CCp family

Schematic representations of domain organisations are depicted as in Pradel et al. 2004. All proteins have a signal peptide sequence represented by a black box at the beginning of the architectures. Ric, ricin domain; Disc, discoidin domain; A, ApicA domain; Anth, anthrax toxin NH2-terminal region domain; FN2, fibronectin type II domain; LCCL, Limulus coagulation factor C domain; Lev, levanase domain; LH, lipoxygenase domain; NEC, neurexin and collagen-like domain; SR, scavenger receptor domain. See text for domain descriptions.

Proteomic analyses (summarised in Table 3.1) provide indications that LAPs are expressed — and therefore may be targeted by antibodies — throughout the parasite’s development in the midgut. Studies in both P. falciparum (Lasonder et al., 2002; Florens et al., 2002) and P. berghei (Hall et al., 2005; Khan et al., 2005) detect all but LAP6 in gametocytes. LAP4 and LAP6 are also detected in P. falciparum sporozoites (Florens et al., 2002) suggesting the possibility of expression throughout sporogonic development. This is supported by studies using P. berghei, in which LAP1 and LAP6 have been detected in sporozoites (Hall et al., 2005). Furthermore, through analysing ookinete preparations (Hall et al., 2005; R. Stanway, PhD thesis, 2007; Lal et al., 2009b) studies using P. berghei have identified LAPs as potential surface/secreted protein of ookinetes: LAP1-5 were detected in whole cell proteomic analysis of ookinetes (Hall et al., 2005), whilst in subcellular proteomic analyses, all LAP family members were detected in a putative surface-enriched ookinete proteome (R. Stanway, PhD thesis, 2007) and LAP1-4 were detected in a putative microneme-enriched ookinete proteome (Lal et al., 2009b).

Proteomic expression data relevant to the LAPs is summarised in Table 3.1.
Table 3.1. Overview of LAP proteomic data

<table>
<thead>
<tr>
<th>Species</th>
<th>Description of study</th>
<th>LAP1</th>
<th>LAP2</th>
<th>LAP3</th>
<th>LAP4</th>
<th>LAP5</th>
<th>LAP6</th>
<th>References</th>
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<td>Abs, G, Gm</td>
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<td>OSEP</td>
<td>OSEP</td>
<td>OSEP</td>
<td>OSEP</td>
<td>Stanway, 2007</td>
</tr>
<tr>
<td><em>P. berghei</em></td>
<td>Proteomic analysis of ookinete microneme preparations</td>
<td>MEOP</td>
<td>MEOP</td>
<td>MEOP</td>
<td>MEOP</td>
<td>MEOP</td>
<td>MEOP</td>
<td>Lal et al., 2009</td>
</tr>
</tbody>
</table>

The table indicates the presence (black) or absence (grey) of LAP1-6 in various life cycle stages as detected by proteomic analyses. Abs: Asexual blood stages, Gc: Gametocytes, Gm: Gametes, Mrz: Merozoites, Ocy: Oocysts, Ook: Ookinetes, Spz: Sporozoites, Tpz: Trophozoites. The ookinete surface proteome (OSEP) and the microneme-enriched ookinete proteome (MEOP) were each generated following subcellular fractionation of purified *P. berghei* ookinetes.
Having been identified as potential TBV candidates, this study aimed to determine the feasibility of generating immunogenic recombinant antigens and assess the possibility that anti-LAP antibodies may block the development of *Plasmodium* transmission stages. The *P. berghei* rodent malaria parasite system was utilised since it allows for a robust evaluation of transmission-blocking activity. Both *in vitro* and *in vivo* assays can be reliably performed to assess possible anti-parasitic effects of antisera: the effects of serum on the process of gametocyte activation and exflagellation can be measured by *in vitro* exflagellation assays; *in vitro* ookinete development assays can be performed to assess the number of ookinetes developing after incubation with different antibodies; and above all, *in vivo* effects can be analysed by membrane feeding assays in which mosquitoes are fed on gametocyte-infected blood through an artificial membrane in the presence of test and control sera. Use of a rodent model provides the additional advantages of being able to confirm transmission-blocking activity by testing infectivity of mosquitoes to mice. In contrast, assays with *P. falciparum* relies on the availability of infectious gametocytes produced in culture over 18-20 days, the culture of ookinetes by *in vitro* culture is not well established, and the infection of human malaria parasites in the mosquito vector can be technically challenging. Furthermore, the infection of mosquitoes with human malaria is wrought with considerable safety issues and there is no animal model for sporozoite infectivity. LAP family members are highly conserved between *P. berghei* and human malaria parasites, therefore it is expected that findings in *P. berghei* could reliably correlate with human malaria.
3.2. Protein expression and antibody production

3.2.1. Choice of expression system

Immunisation of suitable hosts with recombinantly produced proteins is an effective and routine method to stimulate the production of antibodies. A number of systems can be utilised for the heterologous expression of *Plasmodium* recombinant proteins. Those commonly used for the purpose of vaccine development include bacterial expression using *Escherichia coli*, and eukaryotic expression systems using yeast (*Pichia pastoris* and *Saccharomyces cerevisiae*) or baculovirus-mediated expression in insect cells.

Difficulties in expressing malarial proteins are well documented. Problems are attributed in particular to the highly AT-rich genome of *Plasmodium*: AT-content is approximately 80% (Gardner et al., 2002). Codon usage in *Plasmodium* is therefore strikingly different to that utilised by heterologous expression systems, with translation requiring tRNA species that are often rare in the expression host (Birkholtz et al., 2008). Furthermore, AT-richness is reflected in proteins by the enrichment of asparagine and numerous low-complexity regions (Aravind et al., 2003), which can trigger mis-translation. *Plasmodium* proteins expressed heterologously are also likely to be processed differently to endogenous proteins due to differential post-translational modification patterns (Chang et al., 2008).

As a result of these difficulties, expression can be problematic in any heterologous expression system. *Escherichia coli* remains the most attractive and most frequently used host for expression, at least for initial analyses. The use of *E.coli* offers the advantages of ease of modification and high protein yields (Flick et al., 2004). Moreover, the availability of codon-enriched *E.coli* strains reduces some of the problems associated with codon biases, to the effect that AT-content has less of an impact on expression (Mehlin et al., 2006). *E. coli* was therefore the selected as the expression system of choice for the generation of recombinant LAPs.

3.2.2. Sequence selection and cloning of *P. berghei* lap gene fragments

Gene fragments for recombinant protein production were selected in a manner aimed at maximising the chance of successful heterologous expression. The Structural Genomics of Pathogenic Protozoa group (SGPP, University of Washington) selected 1,000 *P. falciparum* open reading frames for expression in *E.coli*, of which 30% were expressed.
successfully. They were able to align physical properties of the protein with expression problems and indicated that a relatively low molecular weight is a critical factor in maximising the chance of successful cloning and expression of *Plasmodium* proteins in *E. coli*. 42% of proteins under 20 kDa expressed, while of those over 60 kDa only 20% expresses (Mehlin et al., 2006). Similarly, Vedadi et al. (2007) reported a reduction in recombinant protein solubility with increasing size. Since the LAPs range in size between approximately 90 and 200 kDa, gene fragments predicted to encode protein of size 25-40 kDa were selected for expression. This represented a compromise between expression and immunogenicity: longer protein sequences generally offer a higher probability of recognising epitopes exposed on the native protein and tend to be stronger immunogens but are more difficult to express than smaller molecules.

Bioinformatic prediction of protein domains using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/) was used to generate predicted domain architectures to guide the selection of gene fragments for protein expression. Since individual domains can often be folded and stable independently of the rest of the protein chain, whole predicted protein domains were chosen. Low complexity regions (LCRs) were avoided due to their association with disrupted expression (Vedadi et al., 2007): it has been proposed that *Plasmodium* LCRs may act to regulate protein expression by inducing ribosome pausing (Frugier et al., 2010). A number of sequences, including both unique and conserved regions, were chosen for expression purposes. Antibodies raised against amino acid sequences that are conserved between LAP family members (e.g. the LCCL domain) have the potential to cross-react with multiple LAPs. This could have both advantages and disadvantages: antibody binding to more than one LAP may act synergistically to confer a more potent transmission-blocking activity, but cross-reactivity would also prevent immunodetection of specific LAPs.

For each *Pblap*, two regions of the gene were amplified by PCR. Resulting gene fragments were cloned into a pET-46 vector containing a T7 promoter to drive expression and an N-terminal tag encoding six histidine residues to facilitate purification. Unlike larger tags, such as glutathione S-transferase (GST) and maltose-binding protein (MBP), the small size of the histidine tag is not expected to interfere with specific antibody production.
Recombinant plasmids were generated by ligation independent cloning and transformed into *E. coli* Gigasingles strain. The presence of a correctly sequenced and in-frame gene fragment was confirmed by DNA sequence analysis on plasmid preparations extracted from transformed cells. As shown in Figure 3.2, gene sections encoding the following amino acids of endogenous LAPs were successfully cloned: LAP1SRSR, LAP1PTX, LAP2RG, LAP3LCCL, LAP4LCCL, LAP4C, LAP5PA14, LAP6LCCL. In addition, LAP2AB, LAP3PA14, LAP5AB and LAP6AB were successfully cloned into the same pET-46 expression vector by A. Blagborough and R. Wild and were made available for this study. Plasmids are referred to as plap1SRSR, plap1PTX, plap2RG, plap2AB, plap3LCCL, plap3PA14, plap4LCCL, plap4C, plap5PA14, plap5AB, plap6LCCL and plap5AB.
Figure 3.2. Regions of *P. berghei* LAPs cloned for expression

Domain architecture of *P. berghei* LAP proteins was predicted using SMART software. Amino acid sequences corresponding to gene portions successfully cloned for expression are shown; represented in red for those that were subsequently expressed or grey for non-expressed regions. Positions of introns are indicated I.
3.2.3. Protein expression

_E. coli_ strains carrying the λ DE3 lysogen and mutant lon and ompT proteases were used as hosts for expression. The λ DE3 lysogen expresses T7 RNA polymerase from the IPTG-inducible _lacUV5_ promoter for expression of genes under control of the T7 promoter of the expression vector. Meanwhile, protease deficiency allows for increased yield of heterologous proteins. Two _E. coli_ strains, BL21 (DE3) pMico and Rosetta (DE3) pLysS, were selected for expression on the basis that their use may overcome the problem of _Plasmodium_ codon usage and improve yields of proteins toxic to the bacteria. _E. coli_ BL21 (DE3) pMico contains the plasmid pMico, which helps to improve translation of _Plasmodium_ proteins by encoding the tRNA genes _argU, ileX, and glyT_ – the products of which recognise codons contained in _Plasmodium_ but which are rare in _E. coli_ (Cinquín et al., 2001). pMico also encodes for low level expression of the T7 lysozyme, which binds and inactivates T7 RNA polymerase to reduce basal levels of expression (occurring under uninduced conditions) and thereby allows enhanced bacterial growth and subsequently increased yields of toxic proteins. The Rosetta™ (DE3) pLysS strain provides similar attributes. The BL21 _E. coli_ derivatives carry the pRARE plasmid, which encodes rare tRNAs to provide better codon usage, and the pLysS plasmid, which produces low levels of T7 lysozyme.

The following conditions were tested for expression:

- **E. coli expression strain:** BL21 (DE3) pMico or Rosetta™ (DE3) pLysS
- **Induction temperature/time:** 37°C for 4-6 hours or 28°C overnight
- **IPTG concentration:** 0.2mM, 0.5mM or 1mM

Recombinant protein expression levels were assessed by Coomassie staining and Western blot analysis of SDS-PAGE separated bacterial lysates. Expression of target proteins could not be distinguished by Coomassie staining but the expression of histidine_6-tagged proteins was detected by western blot analysis using an HRP conjugated His-Probe for eight constructs: plap3PA14, plap5AB, plap6AB, plap1SRSR, plap2RG, plap3LCCL, plap4LCCL, and plap6LCCL (Figure 3.3). Proteins generally corresponded with the predicted size of the recombinant histidine_6-tagged proteins (as shown in Table 3.2) exclusively or predominantly expressed in induced preparations were detected. Separation of soluble and insoluble bacterial fractions indicated that in every case, recombinant proteins were found almost entirely in inclusion bodies.
Figure 3.3. Expression of recombinant proteins in *E. coli*

*E. coli* expression strains transformed with recombinant plasmids plap1SRSR, plap2RG, plap3PA14, plap3LCCL, plap4LCCL, plap5AB, plap6AB, plap6LCCL, or an empty pET15b (as a negative control), were cultured and whole cell bacterial lysates of IPTG induced (+) or uninduced (-) cultures were separated under reducing conditions by SDS-PAGE (12% polyacrylamide).Separated proteins were transferred to a nitrocellulose membrane and membranes were incubated with anti-HisProbe-HRP® (Pierce) (1:5000) for the direct detection of recombinant histidine$_6$-tagged proteins. The figure shows representative Western blot analyses of induced (+) and uninduced (-) cultures, indicating the expression of histidine-tagged recombinant proteins predominantly in induced samples. Approximate sizes in kDa, as indicated by Dual Colour PrecisionPlus Protein™ standard, (Bio-Rad) are shown.

Table 3.2. Predicted properties of recombinant proteins and *E. coli* strains for expression

<table>
<thead>
<tr>
<th>Recombinant Protein</th>
<th>Molecular weight</th>
<th>Isoelectric point</th>
<th>Sequence similarity*</th>
<th>Expression strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAP1SRSR aa406-707</td>
<td>37.9 kD</td>
<td>5.6</td>
<td>65%</td>
<td>Ros(DE3)PllysS</td>
</tr>
<tr>
<td>LAP2RG aa161-446</td>
<td>37.9 kD</td>
<td>5.5</td>
<td>81%</td>
<td>Ros(DE3)PllysS</td>
</tr>
<tr>
<td>LAP3PA14 aa3-284</td>
<td>34.6 kD</td>
<td>8.6</td>
<td>51%</td>
<td>BL21 (DE3)pMico</td>
</tr>
<tr>
<td>LAP3LCCL aa215-481</td>
<td>32.3 kD</td>
<td>4.8</td>
<td>56%</td>
<td>Ros(DE3)PllysS</td>
</tr>
<tr>
<td>LAP4LCCL aa596-872</td>
<td>33.2 kD</td>
<td>4.4</td>
<td>70%</td>
<td>Ros(DE3)PllysS</td>
</tr>
<tr>
<td>LAP5AB aa501-756</td>
<td>31.3 kD</td>
<td>8.7</td>
<td>64%</td>
<td>BL21 (DE3)pMico</td>
</tr>
<tr>
<td>LAP6AB aa359-558</td>
<td>24.7 kD</td>
<td>6.0</td>
<td>55%</td>
<td>BL21 (DE3)pMico</td>
</tr>
<tr>
<td>LAP6LCCL aa591-819</td>
<td>27.8kD</td>
<td>8.9</td>
<td>42%</td>
<td>Ros(DE3)PllysS</td>
</tr>
</tbody>
</table>

*% similarity to P. falciparum ortholog.
3.2.4. Protein purification and antigen preparation

The amino terminal polyhistidine-tag of six histidine residues of recombinant proteins facilitated affinity purification of the fusion proteins using nickel coated agarose beads (Ni-NTA). Proteins were expressed as described in 3.2.3, insoluble fractions were extracted and solubilised in urea, and histidine-tagged proteins were purified under denaturing conditions by nickel affinity. Analysis by SDS-PAGE indicated that rLAP1SRSR, rLAP3PA14, rLAP3LCCL, rLAP4LCCL and rLAP6LCCL were all affinity-purified effectively (Figure 3.4). In each case, proteins bound to the nickel-coated agarose beads were eluted using 8 M urea solution, pH 4.5. To prepare antigens for antibody production, eluted fractions of affinity-purified proteins were pooled, and the collected protein solution was dialysed in a stepwise manner from an 8 M urea solution to a 2 M urea solution, and then into PBS overnight. In this way, urea was removed to acquire a non-toxic antigen preparation suitable for immunisations and to allow for protein renaturation. Dialysed proteins were concentrated using a Centricon® column and solutions were assessed by SDS-PAGE (Figure 3.4)

Figure 3.4. Purification of recombinant proteins

Histidine-tagged recombinant proteins were purified from cell lysates by nickel affinity under denaturing conditions. Samples were separated on 12% SDS-PAGE gels and visualised by coomassie blue staining. i) rLAP1SRSR; ii) rLAP3AB; iii) rLAP3LCCL; iv) rLAP4LCCL; v) rLAP6LCCL. For each protein, four eluates (1-4) are shown. Molecular weight markers are displayed in kilo Daltons. Following purification, eluates were pooled and protein solutions were dialysed in a stepwise manner into PBS and then concentrated. Representative examples of resulting protein solutions, separated on 12% SDS-PAGE gels and visualised by coomassie blue staining, are shown in panel vi.
Although Western blot analysis indicated that LAP2RG, LAP5AB and LAP6AB were expressed, the proteins were either undetectable following affinity-purification, or precipitated out of solution following dialysis: proteins failed to be detected by both SDS-PAGE and Western. The loss of protein during dialysis is a frequently encountered problem with the technique, commonly occurring as a result of protein binding to the dialysis membrane or protein precipitation. As an alternative to protein purification and dialysis, rLAP2RG, rLAP5AB and rLAP6AB immunogens were prepared by extraction of affinity purified protein from an excised polyacrylamide gel slice. Proteins were expressed and were enriched from inclusion bodies, suspending the protein pellet in PBS (see methods for details). Protein solutions were separated by electrophoresis, gels were Coomassie-stained to enable visualisation of the protein band and the protein was excised as a gel slice. The gel slice was then destained and washed. Protein was extracted from the gel either by fragmentation or solubilisation (see 2.3.6), thereby generating antigen in a form suitable for immunisations.

3.2.5. Production of antisera
Polyclonal antiserum was produced by immunising groups of female BALB/c mice with 40-60 µg of antigen. Initial immunisations were performed with Freund’s complete adjuvant, which contains mycobacterial cell wall components to potently stimulate humoral responses. Subsequent boost immunisations were administered 14, 28 and 56 days after the primary immunisation with the same amount of antigen in Freund’s incomplete adjuvant. Serum samples were taken 10 days after each boost immunisation, and tested by immunoblot for the presence of antibodies specific to the corresponding recombinant antigen. Recombinant proteins were expressed in E.coli alongside an uninduced control. Whole bacterial lysates were run on a gel under reducing conditions, transferred to a nitrocellulose membrane and probed with sera collected from each immunised mouse. Sera from an unimmunised mouse served as a negative control and non-specific immune reactions were assessed by testing against protein expressed from bacterial cell lysates transformed with an empty plasmid pET-15b. When antibodies specific to the corresponding recombinant antigen were detected, the host mouse was exsanguinated and serum prepared. When no specific reaction was seen, mice were re-immunised. At least two, and up to four independent attempts to generate antibodies against each antigen were made. The serum collected from different mice but containing antibodies reactive against the same antigen was pooled. Antibody reactivity was
confirmed by immunoblot (as above) and ELISA (see 1.3.11) on pooled serum. Representative immunoblots and ELISAs demonstrating the reactivity of antibodies are shown in Figure 3.5.

**Figure 3.5. Immunoreactivity of antibodies against E.coli-expressed antigens**

A) Immunoblot analysis with pooled mouse antiserum: i) αLAP1SRSR, ii) αLAP3AB, iii) αLAP3LCCL, iv) αLAP4LCCL and v) αLAP6LCCL. Whole cell bacterial lysates of IPTG induced (+) or uninduced (-) E.coli expression strains transformed with recombinant plasmids plap1SRSR, plap2RG, plap3AB, plap3LCCL, plap4LCCL, plap5AB, plap6AB, or empty vector p15b as negative control, were separated under reducing conditions by SDS-PAGE (12% gel) and transferred to nitrocellulose membrane. Membranes were probed with anti-LAP serum at a dilution of 1 in 1000 and antibody binding was detected by ECL chemiluminescence following incubation with HRP-conjugated anti-mouse IgG secondary antibody (1:10,000). Immunoreactivity was demonstrating by the presence of a band in lane 1 (cell lysate in which antigen expression was induced), appearing absent from lanes 3-4 (empty vector control). Serum from an
unimmunised mouse (NMS) was used as a negative control. Molecular weight markers are displayed in kilo Daltons.

B) ELISA with pooled mouse antisera. Whole cell bacterial lysates (as above) were used as antigens in ELISAs. Wells of a 96-well ELISA plate were coated with 10 µg/ml of antigen and antibody incubations were performed over a range of dilutions. Antigen-antibody binding was detected by incubation with HRP-conjugated anti-mouse IgG secondary antibody (1:10,000) followed by o-phenylenediamine (OPD) substrate for the detection of peroxidase activity. Reactions were stopped by the addition of 3M H₂SO₄ and were read at 492 nm. Mean absorbance values from duplicate samples (serum at a dilution of 1:500) are indicated. Red = anti-LAP sera; Grey = normal mouse serum (NMS). Error bars represent standard error of the mean.

3.2.6. Summary of recombinant protein expression and production of antisera

- Eight recombinant proteins, corresponding to 25-40 kDa portions of *P. berghei* LAPs, were successfully expressed in *E. coli*: LAP1SRSR (aa406-707), LAP2RG (aa161-446), LAP3PA14 (aa3-284), LAP3LCCL (aa215-481), LAP4LCCL (aa596-872), LAP5AB (aa501-756), LAP6AB (aa359-558) and LAP6LCCL (aa591-819).

- Five of the proteins expressed could be purified effectively by Ni-NTA affinity: LAP1SRSR, LAP3AB, LAP3LCCL, LAP4LCCL, LAP6LCCL, and these were used to immunise mice for antibody production. All were capable of inducing antibodies specific to the heterologously expressed recombinant proteins.

- Remaining antigens were prepared by gel extraction were found to be non-immunogenic in mice, suggesting that the antigens are either poor immunogens or that amounts of antigen were insufficient, resulting in low antibody titre and affinity.
3.3. Immunoreactivity of antibodies against gametocytes

Antisera generated were tested by immunoassays (immunoblot and immunofluorescence) to assess reactivity to *P. berghei* gametocytes.

3.3.1. Detection of gametocyte proteins by immunoblot

To generate gametocyte extracts for immunoblot analysis, PH-treated mice were inoculated with wild-type *P. berghei* clone 2.34 and once parasitemias were established were treated with sulphadiazine to kill asexual blood stages. The *P. berghei* clone 2.33, which is deficient in the production of mature gametocytes, was used for the purpose of generating gametocyte depleted samples. Antisera against the SRCR domain of LAP1, the PA14 domain of LAP3 and the LCCL domains of LAPs 4 and 6 were all found to react with endogenous protein in gametocyte extracts. αLAP1SRCR and αLAP3PA14 were found to detect parasite proteins exclusive to gametocytes: proteins migrating at molecular weights of approximately 150 kDa (αLAP1SRCR) and 120 kDa (αLAP3PA14), corresponding with the predicted sizes of the endogenous proteins, were detected in gametocyte extracts whilst absent from gametocyte-depleted samples. αLAP4LCCL and αLAP6LCCL each recognised multiple protein bands ranging from 80-175 kDa in gametocyte-enriched samples. It is predicted that the immunoreactivity against proteins of different molecular weights may suggest partial protein degradation or processing of the endogenous full-length proteins. Proteins detected by αLAP4LCCL and αLAP6LCCL were predominantly present in gametocytes, although bands were also recognised in gametocyte-depleted samples: at 80 kDa and 85 kDa (αLAP4LCCL) and 175 kDa (αLAP6LCCL). Neither gametocyte nor asexual blood stage proteins were detected by antibodies in the sera from an unimmunised mouse (NMS) or antisera raised against LAP3LCCL. Further analysis indicated that most of the gametocyte proteins detected were present in the soluble fraction following treatment with triton, indicating a possible membrane-bound localisation. Representative immunoblots are shown in Figure 3.6.
Figure 3.6. Detection of gametocyte proteins by immunoblot

Antibodies were tested for immunoreactivity against asexual blood stages (Abs) and gametocytes (Gc). The *P. berghei* clone 2.33, which is deficient in the production of mature gametocytes, was used for the purpose of generating gametocyte depleted (i.e. asexual blood stage enriched) samples. To generate gametocyte extracts, PH-treated mice were inoculated with wild-type *P. berghei* clone 2.34 and once parasitemias were established were treated with sulphadiazine to kill asexual blood stages. Parasite proteins were separated under reducing conditions by SDS-PAGE (8% polyacrylamide) and transferred to nitrocellulose membrane. Membranes were probed with antisera αLAP1, αLAP3, αLAP4, αLAP6, or as a negative control, normal mouse sera, each at a dilution of 1 in100. Membranes were reprobed with monoclonal α-tubulin antibody (1:10,000) to indicate protein loading (lower panel). Antibody binding was detected by use of HRP-conjugated anti-mouse IgG secondary antibody (1:10,000) and ECL chemiluminescence.

3.3.2. Detection of gametocyte proteins by immunofluorescence

Antisera were further analysed by immunofluorescence to visualise the localisation of detected proteins. Cells were fixed by either protein precipitation using either methanol or 50% methanol; 50% acetone or by cross-linking using 4% paraformaldehyde. Fixation using paraformaldehyde followed by sodium borohydride treatment was found to be the most robust and consistent staining methodology between experiments. Fixation with formaldehyde stabilises cells by cross-linking proteins and leads to peroxidation of lipids. Resulting aldehyde formation due to lipid peroxidation is a common cause of background fluorescence (Stoya et al., 2002) but can be effectively masked by sodium borohydride treatment. All antibodies were found to label both unactivated gametocytes (present in fixed blood smears from a *P. berghei* infected mouse) and activated gametocytes undergoing gametogenesis (activated by the suspension of gametocyte-infected blood in
ookinete media containing xanthurenic acid). Since male gametogenesis involves rapid DNA replication, activated males can be easily distinguished from activated females by nuclear staining patterns. Anti-LAP1 (Figure 3.7Bi), anti-LAP3 (Figure 3.7Bii) and anti-LAP6 (Figure 3.7Biv) antisera specifically label female parasites, whilst anti-LAP4 (Figure 3.7Biii) is also immunoreactive against exflagellating males.

**Figure 3.7. Immunofluorescent labelling of gametocytes and gamete**

A) Indirect immunofluorescence assays using mouse sera against LAP1, LAP3, LAP4 and LAP6 revealed labelling of gametocytes. Immunofluorescence analyses were performed on paraformaldehyde-fixed blood smears from a *P. berghei* infected mouse. Antibody binding was detected using Alexa Fluor 488 anti-mouse IgG (green) and cells were counterstained with DAPI nuclear stain (blue). Control serum from an unimmunised mouse (NMS) showed no labelling of gametocytes. Scale bar represents 2 µm.

B) Immunofluorescence analyses on activated gametocytes. Gametocytes were activated by suspending blood collected from a *P. berghei*-infected mouse in ookinete media containing xanthurenic acid and immunofluorescence assays were performed as above on cells 5 minutes post-activation. Representative images of antibody labelling of activated male and female gametocytes are shown.
3.4. Transmission-blocking assays

Having demonstrated immunoreactivity against gametocytes, antisera against the SRCR domain of LAP1, the PA14 domain of LAP3 and the LCCL domains of LAPs 4 and 6 were tested in assays to evaluate antibody-mediated effects on *P. berghei* exflagellation, ookinete development, infectivity to mosquitoes and subsequent transmission.

3.4.1. Effects of anti-LAP sera on exflagellation and ookinete development

Antibody-mediated inhibition of *P. berghei* exflagellation (male gamete release), zygote formation and ookinete development and ookinete formation can be simply detected by *in vitro* assays.

3.4.1.1. Exflagellation assays

Exflagellation can be induced by suspending gametocytes ookinete media containing XA to induce activation. During gametogenesis *in vitro*, emerging microgametes draw in neighbouring erythrocytes, creating a wobbling clump of cells termed ‘exflagellation centres’, which are retained by the residual body of the gametocyte (Eksi et al., 2006). The functional significance of exflagellation centres remains unknown, but their formation can be exploited as a means of measuring male gamete release. To test for antibody-mediated effects on exflagellation, tail blood from a *P. berghei*-infected mouse containing mature gametocytes was suspended in ookinete containing test sera (αLAP1, αLAP3, αLAP4 or αLAP6) or control sera (normal mouse sera (NMS) from an unimmunised mouse). Exflagellation centres were defined as a cluster of cells comprising exflagellating male parasite(s) binding three or more red blood cells and were counted by microscopic examination (x40 objective magnification) after a 20 minute incubation period. The mean number of exflagellation centres per field of view was calculated by counting 20 microscopic fields. Results (Figure 3.8) were collected from three independent experiments, each performed in duplicate (n=6).

Differences between overall means were negligible (αLAP1: 18.37, αLAP3: 15.44, αLAP4: 15.0, and αLAP6: 17.37 compared to NMS: 17.89). Statistical analysis by Wilcoxon signed-rank tests concluded that no significant antibody-mediated effects on exflagellation were detected with any of the antibodies tested (Figure 3.8). The absence of antibody-mediated effects on exflagellation, at least for αLAP1, αLAP3, αLAP4 or αLAP6, was consistent with the lack of labelling of male gametocytes: only anti-LAP4 was found to bind activated male gametocytes (Figure 3.7).
Figure 3.8. Effect of anti-LAP sera on *P. berghei* exflagellation.

Gametocyte-containing tail blood from a *P. berghei*-infected mouse was suspended in ookinete medium containing 25% test serum (αLAP). Sera from an unimmunised mouse (NMS) and PBS were used as negative controls. After a 20 minute incubation period, exflagellation centres were counted under a light microscope (40x objective magnification). The average number of exflagellation centres per field of view was calculated by counting 20 microscopic fields. Assays were performed in duplicate. A) Combined results of three independent experiments (n=6) showing the mean number of exflagellation events for each group. Error bars represent SEM. Values are tabulated in B. P-values were determined by Wilcoxon signed-rank tests performed on the combined results of three independent experiments (n=6).
3.4.1.2. Ookinete development assays

The effects of antisera on ookinete development can be simply detected using *P. berghei* since ookinetes can be readily cultured *in vitro*. Heparinised blood from a *P. berghei*-infected mouse containing mature gametocytes was incubated for 24 hours at 19ºC in ookinete media containing test sera (αLAP1, αLAP3, αLAP4 or αLAP6) or control sera (from an unimmunised mouse). Ookinetes were counted using a haemocytometer to calculate the number of ookinetes per 100 µl culture and results (Figure 3.9) were collected from three independent experiments, each performed in duplicate (n=6).

The antisera tested did not elicit reductions in ookinete numbers, indicating that the antibodies were incapable of inhibiting fertilisation and subsequent ookinete formation. Conversely, an increase in ookinete numbers was observed in the presence of αLAP1, αLAP3 and αLAP6 (αLAP1: +84.0%, αLAP3: +35.1%, and αLAP6: +57.6% relative to NMS control at 100%; Figure 3.9). Statistical analyses by Wilcoxon signed-rank tests demonstrated that the presence of αLAP1 was associated with a significant increase in ookinete numbers (overall mean number of ookinetes 17,942 compared to 9750 for NMS control, *P* = 0.0313). The unexpected antibody-mediated enhancement of parasite development is discussed in 3.5.2.
Figure 3.9. Effect of anti-LAP sera on *P. berghei* in vitro ookinete formation

Gametocytes from a *P. berghei* infected mouse were suspended in 100 µl ookinete cultures containing 25% test serum. Cells were cultured at 19°C for 24 hours and the number of ookinetes culture was calculated by counting ookinetes in a haemocytometer. Assays were performed in duplicate. A) Combined results of three independent experiments (n=6) showing the mean number of ookinetes per 100 µl culture for each group. Error bars represent SEM. Values are tabulated in B. P-values were determined by Wilcoxon signed-rank tests performed on the combined results of three independent experiments (n=6).
3.4.2. Effects of anti-LAP sera on parasite infectivity in vivo

Given that LAPs have been implicated with possible interactions with mosquito molecules (Delrieu et al., 2002; Claudianos et al., 2002; Trueman et al., 2004) it was considered that antibodies raised against the proteins may have effects in vivo even though they do not in vitro. Experimental *P. berghei* mosquito infections have been demonstrated to be highly efficient in *An. stephensi* mosquitoes (Sinden et al., 1996) enabling the reliable detection of potential antibody-mediated effects on parasite development within the mosquito.

3.4.2.1. Membrane feeding assays

The effect of anti-LAP serum on parasite infectivity in vivo was assessed by membrane feeding assays. The use of a membrane feeding system, whereby serum is added to gametocyte-infected blood and mosquitoes are fed through an artificial membrane, enables several different mosquito test groups to be fed on the same pool of gametocyte-infected blood in the presence of different test sera. Counting oocysts (day 10-12 post-infection) is an accurate measure of transmission success. To determine the effect of anti-LAP serum on oocyst numbers, a membrane feeding apparatus was used to feed groups of mosquitoes on *P. berghei* conGFP infected blood mixed with anti-LAP serum (or serum from unimmunised mice (NMS) as a control) at a 1 in 4 concentration. Mosquitoes were dissected 10-11 days post-feed and numbers of oocysts in each midgut were counted by fluorescence microscopy. Values were pooled for analysis: since the intensity of infection varies between experiments, all values were represented as fold change in oocysts compared to NMS treated controls. The combined results of three (for 6 two) independent experiments, each consisting of two duplicate feeds, are shown on Figure 3.10 and the mean values from each experiment are given in Table 3.3.
Figure 3.10. Effect of anti-LAP sera on *P. berghei* development in *An. stephensi* mosquitoes

*P. berghei* conGFP infected blood was mixed with anti-LAP sera or sera from unimmunised mice (NMS) at a 1 in 4 concentration and fed to mosquitoes in using a membrane feeding apparatus. Mosquitoes were dissected 10-11 days post-feed and numbers of oocysts in each midgut were counted by fluorescence microscopy. The graph shows the combined results of three (for αLAP6 two) independent experiments, each consisting of two duplicate feeds. Values are represented as fold change in oocysts compared to NMS treated controls plotted as aligned dotplots on a log2 scale (black=NMS, grey=PBS, red=αLAP). Horizontal lines indicate the mean number of oocysts.
Table 3.3. *P. berghei* infectivity in *An. stephensi* following membrane feeding assays to assess the effects of anti-LAP sera on parasite development in the mosquito

<table>
<thead>
<tr>
<th>Serum</th>
<th>Prevalence (infect/total)</th>
<th>Range</th>
<th>Mean number of oocysts per mosquito (+/SEM)</th>
<th>P</th>
<th>Mean number of oocysts per infected mosquito (+/-SEM)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS</td>
<td>82.8% (24/29)</td>
<td>0-340</td>
<td>55.03 (±17.4)</td>
<td>***</td>
<td>66.5 (±20.3)</td>
<td></td>
</tr>
<tr>
<td>αLAP1</td>
<td>97.6% (41/42)</td>
<td>0-286</td>
<td>83.32 (±18.6)</td>
<td>**</td>
<td>85.17 (±8.8)</td>
<td>**</td>
</tr>
<tr>
<td>NMS</td>
<td>76.9% (20/26)</td>
<td>0-57</td>
<td>15.27 (±3.70)</td>
<td>***</td>
<td>19.85 (±4.3)</td>
<td></td>
</tr>
<tr>
<td>αLAP1</td>
<td>97.4% (38/39)</td>
<td>0-392</td>
<td>71.67 (±14.3)</td>
<td>**</td>
<td>73.55 (±4.5)</td>
<td>**</td>
</tr>
<tr>
<td>NMS</td>
<td>89.4% (42/47)</td>
<td>0-148</td>
<td>24.7 (±7.99)</td>
<td></td>
<td>27.64 (±5.2)</td>
<td></td>
</tr>
<tr>
<td>αLAP1</td>
<td>88.1% (52/59)</td>
<td>0-129</td>
<td>14.25 (±3.09)</td>
<td>ns</td>
<td>16.17 (±3.4)</td>
<td>ns</td>
</tr>
<tr>
<td>NMS</td>
<td>83.0% (±3.60)</td>
<td></td>
<td>31.7 (±12.0)</td>
<td></td>
<td>38.0 (±14.4)</td>
<td></td>
</tr>
<tr>
<td>αLAP1</td>
<td>94.4% (±3.13)</td>
<td></td>
<td>56.4 (±21.4)</td>
<td></td>
<td>58.3 (±21.3)</td>
<td></td>
</tr>
<tr>
<td>NMS</td>
<td>68.0% (17/25)</td>
<td>0-68</td>
<td>8.04 (±3.16)</td>
<td></td>
<td>11.82 (±4.4)</td>
<td></td>
</tr>
<tr>
<td>αLAP3</td>
<td>89.5% (17/19)</td>
<td>0-39</td>
<td>11.21 (±2.78)</td>
<td>*</td>
<td>12.33 (±3.0)</td>
<td>ns</td>
</tr>
<tr>
<td>NMS</td>
<td>82.8% (24/29)</td>
<td>0-340</td>
<td>55.03 (±17.4)</td>
<td></td>
<td>66.5 (±20.3)</td>
<td></td>
</tr>
<tr>
<td>αLAP3</td>
<td>95.3% (41/43)</td>
<td>0-228</td>
<td>92.98 (±8.63)</td>
<td>**</td>
<td>97.51 (±8.4)</td>
<td>***</td>
</tr>
<tr>
<td>NMS</td>
<td>83.7% (36/43)</td>
<td>0-74</td>
<td>18.95 (±2.94)</td>
<td></td>
<td>22.64 (±3.2)</td>
<td></td>
</tr>
<tr>
<td>αLAP3</td>
<td>90.3% (28/31)</td>
<td>0-87</td>
<td>34.1 (±4.67)</td>
<td>**</td>
<td>37.75 (±4.7)</td>
<td>**</td>
</tr>
<tr>
<td>NMS</td>
<td>78.2% (±5.09)</td>
<td></td>
<td>27.3 (±14.2)</td>
<td></td>
<td>33.7 (±16.7)</td>
<td></td>
</tr>
<tr>
<td>αLAP3</td>
<td>91.7% (±1.82)</td>
<td></td>
<td>46.1 (±24.4)</td>
<td></td>
<td>49.3 (±25.2)</td>
<td></td>
</tr>
<tr>
<td>NMS</td>
<td>69.0% (29/42)</td>
<td>0-15</td>
<td>3.31 (±0.60)</td>
<td></td>
<td>4.8 (±0.7)</td>
<td></td>
</tr>
<tr>
<td>αLAP4</td>
<td>69.6% (32/46)</td>
<td>0-54</td>
<td>7.78 (±0.82)</td>
<td>ns</td>
<td>11.2 (±16.5)</td>
<td>ns</td>
</tr>
<tr>
<td>NMS</td>
<td>82.8% (24/29)</td>
<td>0-340</td>
<td>55.03 (±17.4)</td>
<td></td>
<td>66.5 (±20.3)</td>
<td></td>
</tr>
<tr>
<td>αLAP4</td>
<td>100% (25/25)</td>
<td>4-227</td>
<td>68.6 (±12.3)</td>
<td></td>
<td>68.6 (±12.3)</td>
<td></td>
</tr>
<tr>
<td>NMS</td>
<td>83.7% (36/43)</td>
<td>0-74</td>
<td>18.95 (±2.94)</td>
<td></td>
<td>22.64 (±3.2)</td>
<td></td>
</tr>
<tr>
<td>αLAP4</td>
<td>91.2% (31/34)</td>
<td>0-70</td>
<td>23.65 (±3.94)</td>
<td>ns</td>
<td>25.94 (±3.6)</td>
<td>ns</td>
</tr>
<tr>
<td>NMS</td>
<td>78.5% (±4.76)</td>
<td></td>
<td>25.8 (±15.3)</td>
<td></td>
<td>31.3 (±18.3)</td>
<td></td>
</tr>
<tr>
<td>αLAP4</td>
<td>86.9% (±9.03)</td>
<td></td>
<td>33.3 (±18.2)</td>
<td></td>
<td>35.3 (±17.2)</td>
<td></td>
</tr>
<tr>
<td>NMS</td>
<td>82.8% (24/29)</td>
<td>0-340</td>
<td>55.03 (±17.4)</td>
<td></td>
<td>66.5 (±20.3)</td>
<td></td>
</tr>
<tr>
<td>αLAP6</td>
<td>96.0% (24/25)</td>
<td>0-327</td>
<td>78.88 (±15.4)</td>
<td>**</td>
<td>82.17 (±15.7)</td>
<td>*</td>
</tr>
<tr>
<td>NMS</td>
<td>83.3% (35/42)</td>
<td>0-183</td>
<td>16.48 (±5.76)</td>
<td></td>
<td>19.8 (±6.79)</td>
<td></td>
</tr>
<tr>
<td>αLAP6</td>
<td>95.5% (42/44)</td>
<td>0-105</td>
<td>18.55 (±3.28)</td>
<td>*</td>
<td>19.4 (±3.37)</td>
<td>ns</td>
</tr>
<tr>
<td>NMS</td>
<td>83.1% (±0.25)</td>
<td></td>
<td>35.8 (±19.3)</td>
<td></td>
<td>43.2 (±23.3)</td>
<td></td>
</tr>
<tr>
<td>αLAP6</td>
<td>97.8% (±0.25)</td>
<td></td>
<td>48.7 (±30.2)</td>
<td></td>
<td>50.8 (±31.4)</td>
<td></td>
</tr>
<tr>
<td>NMS</td>
<td>69.0% (29/42)</td>
<td>0-15</td>
<td>3.31 (±0.60)</td>
<td></td>
<td>4.79 (±0.7)</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>60.7% (17/28)</td>
<td>0-15</td>
<td>2.54 (±1.97)</td>
<td>ns</td>
<td>4.18 (±1.2)</td>
<td>ns</td>
</tr>
<tr>
<td>NMS</td>
<td>83.4% (36/43)</td>
<td>0-74</td>
<td>18.95 (±2.94)</td>
<td></td>
<td>22.64 (±3.17)</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>89.7% (35/39)</td>
<td>0-86</td>
<td>28.44 (±3.88)</td>
<td>ns</td>
<td>31.69 (±3.97)</td>
<td>ns</td>
</tr>
<tr>
<td>NMS</td>
<td>82.8% (24/29)</td>
<td>0-340</td>
<td>55.03 (±17.4)</td>
<td></td>
<td>66.5 (±20.3)</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>85.7% (12/14)</td>
<td>0-116</td>
<td>41.21 (±10.1)</td>
<td>ns</td>
<td>48.08 (±10.5)</td>
<td>ns</td>
</tr>
<tr>
<td>NMS</td>
<td>78.4% (±4.70)</td>
<td></td>
<td>25.8 (±15.3)</td>
<td></td>
<td>31.3 (±18.3)</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>78.7% (±9.07)</td>
<td></td>
<td>24.1 (±11.4)</td>
<td></td>
<td>28.0 (±12.8)</td>
<td></td>
</tr>
</tbody>
</table>

*P* refers to the statistical significance calculated by Mann-Whitney U tests. Overall mean values are shown in bold.
It was surprising to find that the addition of anti-LAP enhanced oocyst development in *An. stephensi* mosquitoes in comparison with serum from an unimmunised mouse (Figure 3.7 and Table 3.4). Statistical analyses using Mann-Whitney U tests on combined data concluded that the presence of anti-LAP1, anti-LAP3, anti-LAP4 and anti-LAP6 sera led to a significant increase in the number of oocysts per mosquito (P-values 0.0007, <0.0001, 0.0322 and 0.011 respectively). In comparison, feeds in the absence of antiserum (PBS control) did not significantly affect infectivity (P = 0.4297). The increases in infectivity were most notable for anti-LAP1: in the presence of which, more than a two-fold increase was detected (based on the mean of pooled data). The increases in infectivity as indicated by higher oocyst densities were mirrored by increases in both prevalence of infection (αLAP1: +11.3%, αLAP3: +13.5%, αLAP4: +8.1%, and αLAP6: +14.7% compared to NMS control (PBS: +0.3%) and mean differences in intensity of infection αLAP1: +85.7%, αLAP3: +39.8%, αLAP4: +50.4%, and αLAP6: +8.5% compared to NMS control (PBS: -8.1%). Infective sporozoites were formed in each case, as demonstrated by bite back experiments in which mosquitoes from each test group were allowed to feed on anaesthetised mice: in each case leading to blood-stage parasitemia.

The observed antibody-mediated increase in infectivity was unexpected, but transmission-enhancing effects are not uncommon, particularly when antibody concentrations are low. For example, Peiris et al. (1988) report that a monoclonal antibody against *P. vivax* gametes can suppress infectivity of the parasites to the vector at high concentrations, but at lower concentrations, may have the opposite effect and enhance the level of malaria infection in the mosquitoes. Similarly, transmission enhancing effects with low titres of anti-Pfs48/45 and Pfs230 antibodies have been reported (Healer et al., 1999; Van der Kolk et al., 2006; Carter et al., 1990). More recently it was observed that whilst anti-*P. berghei* HAP2 blocks transmission at high concentrations (1 in 5), at low dilutions (1 in 100), its presence appears to promote oocyst development within the mosquito host (Blagborough and Sinden, 2009). Molecular mechanisms underlying transmission enhancing effects are unknown: possibilities are discussed further in the conclusions and discussion section at the end of this chapter (3.5.2).
3.4.2.2. Direct feeds

A caveat of membrane feeding assays using *P. berghei* is that there is no incubation period between the antibody and the parasite. Therefore to determine the in vivo efficacy of antibodies, direct feeds were performed on mice producing anti-PbLAP1 antibodies. Groups of female Balb/c mice were immunised with affinity-purified recombinant LAP1 and tested for production of anti-PbLAP1 antibodies by immunoblot analysis using sera samples acquired from tail bleeds (Figure 3.11A). Following demonstration of antibody production, mice were infected with *P. berghei* and three days post infection, mosquitoes were fed directly on anesthetised mice. In comparison to membrane feeds, much higher oocyst densities were attained, reflecting the higher efficiency of direct skin feeding compared to membrane feeding. Twelve days post-feeding, mosquitoes fed on the blood of mice immunised with LAP1 showed no significant difference in oocyst densities compared to the unimmunised control (LAP1: 75.8 (±10.3), unimmunised: 92.2 (±11.0), P = 0.2511), although when compared to the BSA-immunised control mice a significant reduction in transmission-blocking immunity was detected (P=0.0017). Considering however that mosquitoes fed on the blood of mice immunised with BSA had significantly higher oocyst densities than those fed on unimmunised mice (BSA: 143.4 (±15.4), unimmunised: 92.2 (±11.0), P = 0.0085) it was concluded that differences could be attributable to variation in mouse parasitemias or antibody concentrations, which cannot be standardised in this assay.
Groups of female Balb/c mice were immunised with 50 µg affinity purified recombinant LAP1SRSR or 50 µg BSA emulsified in Freund’s adjuvant. Initial immunisations were performed using complete Freund’s adjuvant and boosts on days 14 and 28 were performed using incomplete Freund’s adjuvant. Mice were tested for antibody responses against corresponding antigens by immunoblot. Two mice with serum containing anti-LAP1 antibodies and two mice producing anti-BSA antibodies were used in direct feeds alongside two unimmunised mice. A) Test sera from two mice immunised with rLAP1SRSR react with LAP1SRSR in whole bacterial lysates expressing the protein (plap1SRSR). The corresponding protein band is absent when tested against whole bacterial lysates transformed with an empty vector (pE). B) Test sera from two mice immunised with BSA are immunoreactive against BSA and negative for PBS. For all immunoblots, proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes and membranes were probed with serum at a 1:1000 dilution. HRP-conjugated anti-mouse IgG antibodies were used for detection by chemiluminescence. Each mouse was infected with \textit{P. berghei conGFP} from the same donor mouse and at day 3 post-infection, groups of mosquitoes were allowed to feed on anaesthetised mice. The graph (C) shows the number of oocysts per mosquito 12 days post-feed. Each data point represents the oocyst density of an individual mosquito. Horizontal lines indicate the mean number of oocysts.
3.5. Conclusions and discussion

3.5.1. Summary of findings

Studies in this chapter represent an initial investigation to evaluate the potential of Plasmodium LAPs as candidate transmission-blocking targets. Experiments were performed using the rodent malaria parasite *P. berghei*, which is far more amenable to the experimental study of transmission than human malaria parasites. Results are summarised as follows:

- Eight recombinant proteins, corresponding to 25-40 kDa portions of the PbLAPs, were successfully expressed in *E. coli*.
- Five of the proteins expressed could be purified effectively by Ni-NTA affinity and these were used to immunise mice for antibody production.
- All affinity-purified antigens were capable of inducing antibodies specific to the heterologously expressed recombinant proteins.
- Immunodetection assays indicated that antibodies against the SRCR domain of LAP1, the PA14 domain of LAP3 and the LCCL domains of LAPs 4 and 6 were all able to bind endogenous *P. berghei* gametocyte proteins.
- Transmission-blocking assays showed that none of the antibodies could block *P. berghei* development and transmission when mixed with gametocyte, and surprisingly an antibody-mediated increase in oocyst numbers was detected.

3.5.2. Antibody-mediated enhancement of infectivity

Antibody-mediated increases in infectivity are not unusual and have been detected with low concentrations of known transmission-blocking antibodies, including anti-Pfs48/45 and Pfs230 antibodies (Healer et al., 1999; Mendis et al., 1987; Van der Kolk et al., 2006; Carter et al., 1990) and anti-*P. berghei* HAP2 sera (Blagborough and Sinden, 2009). It is hypothesised that low amounts of bound specific antibodies may enhance adherence of gametes (to enhance fertilisation) or ookinetes (to enhance midgut invasion), but molecular mechanisms explaining concentration dependent changes in antibody efficacy have not yet been determined. One possible mechanism is by the induction of conformational changes in the antigen by antibody-epitope binding at low concentrations. This may have the potential to elicit increased antigen activity as has been observed with neutralising antibodies against botulinum neurotoxin (Sheridan et al., 2001): When
antibodies are present in excess (antibody:toxin ratio 30:1) internalisation of the toxin is inhibited by binding to epitopes to inhibit antigen-receptor binding; but at low concentrations, antibody binding has the converse effect of making the toxin more active by optimising receptor binding (Sheridan et al., 2001). Another way by which antibody-mediated increases in infectivity can occur is by the presence of cross-reactive antibodies. For example, it is thought that increases in the severity of dengue fever — which is associated with increased exposure to dengue virus — may occur by non-neutralising antibodies sticking to the virus, allowing it to bind to and subsequently invade cells that it would not do normally (Dejnirattijai et al., 2010). Given the polyclonal nature of the anti-LAP antibodies tested in this study, it is possible that enhancing effects may be mediated by antibodies with different specificities against a number of different epitopes. It is hypothesised that increased amounts of immunoglobulins may stick to the parasite to enhance fertilisation or ookinete invasion. Increased amounts of immunoglobulin may also have the potential to inhibit complement binding, thereby increasing infectivity by preventing complement-mediated lysis. The increases in infectivity detected with anti-BSA antibodies (see 3.4.2.2. Figure 3.11) supports the theory that non-specific immunoglobulins may enhance infectivity.

3.5.3. Approaches to improve the biological activity of antibodies

It is possible that the degree of specificity may not have been high enough to elicit transmission-blocking activity. The generation of antibodies with increased specific affinity could perhaps counteract the transmission-enhancing affects. A much greater specificity could be attained by generating monoclonal, rather than polyclonal, antibodies. Monoclonal antibodies are derived from a single clone of antibody-producing cells, producing antibodies specific to a single antigen. Alternatively, the specificity of polyclonal antibodies may be improved by affinity purification of desired antibodies and/or by increasing the affinity of binding to native endogenous antigens by modifying protein expression procedures for the production of antigens more closely resembling the native endogenous protein.

Antibodies generated in this study were found to detect endogenous parasite antigens separated by SDS-PAGE but not by native PAGE, and in immunofluorescence assays antibodies labelled fixed, but not live cells. This is considered to be due to either difficulties encountered with the assays (high levels of background immunofluorescence
and problems in the migration of proteins during native–PAGE) or due to the antibodies predominantly recognising epitopes which are not exposed on the native endogenous proteins. The antibodies may interact with predominantly recognise linear epitopes that are absent from the native protein, or the epitopes recognised by the antibodies may be hidden when the protein is in its native state. Although a number of *E. coli*-derived recombinant proteins have been shown to elicit anti-parasitic antibodies against *Plasmodium*, it is common for antibodies generated from heterologously produced recombinant antigens to interact only weakly with the antigen in its native form (Saul 2007; Williamson 2003). This is largely considered to be because heterologously produced antigens are often conformationally different to the endogenous proteins. Antigen conformation is of crucial importance since antigens and antibodies interact by complimentarity in shape by non-covalent bonds. Yet since expression in *E. coli* occurs in the absence of post-translational modifications, co-factors and modifications which are often required for protein stability and folding are lacking. In addition, the rapidity of bacterial protein expression and the highly reductive environment of the bacterial cytosol often results in unfolded/misfolded proteins.

A number of reagents and techniques have been developed to improve the chance of producing soluble heterologous proteins in *E. coli* by modifying expression procedures. For example, the C-terminal fragment of Pfs48/45 has been generated in its correct conformation in *E. coli* by simultaneous expression with periplasmic folding catalysts (Outchkourou et al., 2008). Alternatively, proteins folding can be improved considerably by using expression systems utilising mammalian, insect or yeast cells as replacements for *E. coli*. This has been demonstrated with the expression of Pb25 in a baculovirus system (Margos et al. 1995), Pf25 in yeast (Barr et al. 1991). Expression of *Plasmodium* proteins in yeast cells (e.g. *P. pastoris*) is often favoured. Importantly, proteins expressed in yeast likely to more closely resemble the conformation of native proteins than if they were expressed in *E. coli*. This is particularly significant for the production of biologically active antibodies since the more similar a recombinant protein is to the native protein, the more likely it is that resulting antibodies will recognise the native protein. Yet as with other heterologous expression systems, expression in yeast has its drawbacks. Most notably, *Plasmodium* genes may contain N-linked glycosylation sites which may be utilised by yeast, but do not appear to be processed by *Plasmodium* since it is believed that malaria performs little N-glycosylation or O-linked glycosylation (reviewed in Gowda and Davidson, 1999 and Kimura et al., 2000). As a result, yeast expressed
proteins could be modified in markedly different way to how they would be in *Plasmodium* and it is possible that glycosylated molecules could mask critical epitopes on an antigen.

Alternatively, protein expression may be avoided altogether by DNA immunisations, whereby plasmid DNA is injected into an animal and is taken up into antigen presenting cells, leading to antibody production and commonly also a cytotoxic T cell response. This approach can induce antibody production against more native-like proteins, but as with eukaryotic protein expression systems, problems may result from differential processing (as described above with glycosylation in yeast) and furthermore antibodies may be difficult to characterise since there are no recombinant antigens to test against.

The biological activity of antibodies may be further affected by the choice of adjuvant used to increase the efficiency of antigen presentation, enhance immunogenicity and prolong antibody responses. For all antisera generated for this thesis, antibody production was induced in mice using Freund’s adjuvant; chosen since it is highly effective in stimulating the proliferation of antibody-producing cells. However, it is possible that antigens may become denatured when emersed in the adjuvant. It has been demonstrated for example that antisera generated after immunisation of mice and rabbits with β-lactamase in Freund’s adjuvant reacted with denatured antigen and only an exposed flexible loop region of the native antigen, whilst after immunisation in PBS, the antisera reacted only weakly with denatured antigen and bound more epitopes on the native antigen (Paus and Winter, 2006).

There are therefore numerous modifications and alternative approaches that could be explored in an effort to improve the chance of generating antibodies with anti-parasitic activity. Investing in such alternatives would be worthwhile if LAPs were to be confirmed as viable TBV candidates.
3.5.4. Are LAPs viable TBV targets?

For a protein to be a viable candidate for inducing antibody-mediated transmission blockade, there are three main criteria:

i) The protein must be able to be produced in a form suitable for immunisation;

ii) The protein must be an effective immunogen in the immunised host to stimulate antibody production;

iii) The endogenous protein must be accessible to antibodies in the mosquito blood meal and antibody-antigen binding must mediate a blockade in parasite development.

The studies presented in this chapter demonstrate that regions of the LAPs can be produced as *E. coli*-expressed recombinant proteins that are immunogenic in mice. Antibodies produced however lack the necessary properties to mediate a blockade in transmission. It is difficult to distinguish whether the anti-LAP antibodies tested fail to block transmission because of properties of the targeted epitopes (e.g. inaccessible) or because of properties of the antisera (e.g. unable to bind native protein with high affinity). During the course of this study, initial assessments of the transmission-blocking potential of anti-PfCCp antibodies were made by *P. falciparum* exflagellation assays, revealing that certain anti-PfCCp antibodies mediated a significant decrease (approximately 30-50%) in the formation of exflagellation centres (Scholz et al., 2008). Antibodies with inhibitory activity were raised against PfCCp1 discoidin-NEC domains, PfCCp2 C-terminal, PfCCp3-SR-LCCL domains, PfCCp4 LCCL domain, and the anthrax like domain of PfFNPA, and inhibition was dependent on the presence of complement: reductions in the formation of exflagellation centres were detected in the presence of active human serum, whilst in the presence of heat-inactivated human serum, there were no significant differences compared to untreated controls (Scholz et al., 2008). The dependency of anti-PfCCp transmission-blocking activity on the presence of complement is shared by the transmission-blocking antigen Pfs230: antibodies against Pfs230 block fertilisation by complement-dependent lysis (Healer et al., 1997; Graves et al., 1998).

It is uncertain as to how antibodies, which are thought to be raised against female-specific proteins, can have an effect on exflagellation. Nevertheless, the results are promising. Following support for their potential as transmission-blocking antibodies in *P. falciparum* exflagellation assays, antibodies raised against PfCCp1 discoidin-NEC domains were tested in membrane feeding assays as a more robust means of assessing transmission-
blocking activity (A. Blagborough and R. Frank, unpublished data). These attempts highlighted the technical difficulties of *P. falciparum* membrane feeding assays, with low prevalence (8-9%) and infection intensities (<2 oocysts) even for negative control groups. Nevertheless, even at these low levels of infection, raised against PfCCp1 discoidin-NEC domains had no inhibitory effect on parasite development (mean oocyst intensity 1.83 +/- 0.44; representing a 1.2 fold increase compared to untreated). Given the advantages of the *P. berghei* system, where realistic repetitions will provide meaningful results, anti-PfCCp antisera were tested for cross-reactivity to *P. berghei*. Immunoblot and immunofluorescence analysis indicated possible cross-reactivity with antibodies against PfCCp1 discoidin-NEC and antibodies against the anthrax-like domain of PfFNPA, yet when tested in a *P. berghei* membrane feeding assay no transmission-blocking activity was observed (A. Blagborough and R. Frank, unpublished data). These results indicate that despite observed reductions in exflagellation assays, infectivity to mosquitoes is not reduced. Support for candidacy of LAPs as transmission-blocking antigens is therefore so far limited to exflagellation assays in *P. falciparum*.

It is possible that transmission-blocking activity of anti-LAP/anti-CCp antibodies may be related to the epitopes targeted. Whilst antisera directed against distinct domains of PfCCp1(LAP3), PfCCp2(LAP4), PfCCp3(LAP1), PfCCp4(LAP6) and PfFNPA(LAP5) up to a 50% reduction in exflagellation, some antibodies, including those against the LCCL domain of PfCCp1(LAP3) and the first SR domain of PfCCp3(LAP1), were not associated with a significant decrease in exflagellation (Scholz et al., 2008). It may be that only certain epitopes are accessible to antibody, due to for example the topology or processing of the native protein, or the formation of protein complexes. Indeed, studies on *P. falciparum* suggest that the PfCCps may interact as a complex; building on the discovery that protein expression of certain PfCCps is co-dependent (Pradel et al., 2004; Pradel et al., 2006), affinity binding studies using recombinant proteins have revealed interactions between the PfCCp proteins, and co-immunoprecipitation studies on parasite lysates have detected binding to Pfs48/45, Pfs230 and Pfs25 (Scholz et al., 2008; Simon et al., 2009). It is also possible that the binding of antibodies to one epitope site may induce formation of the complement membrane attack complex more strongly than binding to other epitopes, or that binding to certain epitopes may act to block complement-mediated lysis. Alternatively it may be unrelated to the epitopes targeted but rather determined by other properties of serum, e.g. immunoglobulin sub-type or competing antibodies.
3.5.5 Concluding remarks

Despite the identification of LAPs as possible transmission-blocking candidates based on a combination of proteomic data and bioinformatic predictions, studies presented in this chapter provided no further evidence to support the candidacy of LAPs as transmission-blocking antigens. Utilising the rodent malaria parasite *P. berghei* as a reliable means of assessing transmission-blocking activity, recombinant LAPs produced in *E. coli* were unable to elicit *P. berghei* transmission-blocking immunity in mice. Given the numerous ways to improve the biological activity of antibodies — most notably by exploiting different protein expression systems — the possibility that different anti-LAP antibodies could elicit transmission-blocking activity cannot be excluded. The study does however highlight the significance of protein expression as a major barrier to vaccine development and raises doubts over the feasibility of generating anti-LAP antibodies with transmission-blocking activity. A better understanding of the biological function of LAPs would help to decipher their suitability as TBV candidates and aid any future attempts to generate anti-LAP sera with transmission-blocking activity.
4 INSIGHTS INTO THE BIOLOGY OF LAPs DURING DEVELOPMENT IN THE MOSQUITO

4.1. Introduction

Members of the LAP family are widely conserved among apicomplexan parasites. At the start of this study, orthologs had been identified in Cryptosporidium (C. parvum and C. hominis), Theileria (T. parva and T. annulata), and Toxoplasma (T. gondii). During this PhD, further genome sequencing projects of apicomplexan parasites were completed, including those of Babesia bovis, Cryptosporidium muris, Neospora caninum and the gregarine Ascogregarina taiwanensis. All these genome sequences are available through the NCBI database and the apicomplexan database ApiDB and LAP orthologs have been identified in each apicomplexan species. The presence of LAPs in the early-branching apicomplexan class of the gregarines (monoxenous parasites of the guts of invertebrates) suggests that the proteins arose prior to the divergence of the apicomplexan clade, whilst their absence in the free-living ciliated protozoa Tetrahymena thermophila suggests that acquisition of the characteristic domain architectures occurred after the apicomplexan lineage split from the ciliates, suggesting that the proteins are unique to the Apicomplexa (Templeton et al., 2007; Templeton et al., 2010). The conservation of LAPs across the entire phylum implies that the proteins may be involved in conserved apicomplexan processes, but this has yet to be confirmed.

Investigations into the LAP family have focused on Plasmodium, but a limited number of studies have been performed on the protein family in Cryptosporidium, Toxoplasma and Babesia. In Cryptosporidium, expression of an ortholog of LAP2, CpCCp1 (also termed Cpa135 or SA35 antigen) has been detected by immunolocalisation studies in the apical region of sporozoites, from where the protein appears to be secreted (Tosini et al., 2004). Accordingly, localisation in micronemes and roles in invasion have been proposed (Tosini et al., 2004). Expression in the sporozoite have similarly been indicated in Toxoplasma, where members of the protein family (TgSR1, TgSR2, TgCCp5A and TgCCp5B) have been shown by immunofluorescent assays to be localised in unknown vesicles of sporozoites (Tosini et al., COST Action Conference, May 2008). Meanwhile,
initial studies on the proteins in Babesia show that, as in Plasmodium, expression of the proteins begins in gametocytes.

A number of studies have examined expression and localisation of LAPs/PfCCps in P. falciparum gametocytes and gametes (Delrieu et al., 2002; Pradel et al., 2004; Pradel et al., 2006; Scholz et al., 2008). These indicate by immunofluorescence that the proteins are expressed during gametocyte maturation, are secreted into the PV surrounding the intra-erythrocytic parasite, and are released or associated with the surface of the extracellular gamete, with little detectable protein in fertilised cells (Delrieu et al., 2002; Pradel et al., 2004; Pradel et al., 2006; Scholz et al., 2008). It is suggested that the proteins form a matrix around macrogametes, possibly acting to enhance fertilisation by aiding adherence or perhaps by forming a protective barrier against factors in the blood meal (Pradel et al., 2006; Scholz et al., 2008). The molecular interactions between the PfCCp proteins have since been investigated by affinity binding studies using recombinant proteins comprising selected adhesion domains (Simon et al., 2009). Immunoblot analysis of eluted protein complexes revealed interactions between the LCCL domains of all PfCCp proteins, the SR domains of PfCCp3 (PbLAP1), and the ApicA, NEC, and discoidin domains of PfCCp1 and PfCCp2 (Simon et al., 2009). Furthermore, interactions between PfCCps and sexual stage TBV candidates Pfs48/45 and Pfs230 have been detected by co-immunoprecipitation assays on gametocyte lysates, and with Pfs25 on activated gametocytes. This has led to the hypothesis that the PfCCps form a large multiprotein complex on the surface of gametes with other sexual stage adhesive proteins (reviewed by Kuehn et al., 2010).

Gene knockout studies have been performed in both P. berghei and P. falciparum, revealing that — despite their predicted function in gametes — parasites in which individual lap gene is knocked out undergo fertilisation and appear function normally until sporulation. In P. berghei, the absence of Pblap results in defects in the developmental processes associated with cell division in the oocysts/sporozoite formation (Claudianos et al., 2002; Raine et al., 2007; Ecker et al., 2008). Meanwhile, in P. falciparum, ΔPfccp disruption leads to deficiency in the transition of sporozoites from oocysts to salivary glands (Pradel et al., 2004; Scholz et al., 2008). Following up on these gene knockout studies, Lavazec et al. (2009) generated double gene knockout parasite lines of Pblap1/Pblap2 and Pblap2/Pblap6, the phenotypes of which appeared indistinguishable from the single gene knockouts (Lavazec et al., 2009). Both individual
and double gene knockout studies thereby clearly indicate roles in sporogonic development. This is supported by proteomic studies in \textit{P. berghei}, by which PbLAPs1-5 have been detected in ookinetes, whilst PbLAP1 and PbLAP6 have also been detected in sporozoites (Hall et al., 2005).

A main aim of the studies in this chapter was to investigate the cellular localisation of LAPs using \textit{P. berghei} to study both the sexual and sporogonic stages of development. While the antibodies described in the previous chapter are useful for immunolocalisation studies, it is realised that their application may be limited due to problems associated with possible fixation artefacts, cross-reactivity and poor specificity. Furthermore, the antibodies generated do not permit the visualisation of live cells. An alternative approach for studying localisation is to ‘tag’ the gene product. This can be achieved through two approaches: an epitope tag, e.g. Myc, will render the tagged protein immunoreactive to an already existing, commercially available and well-characterised antibody (Jarvik, 1998), whilst the use of a fluorescent fluorophore, e.g. GFP, enables the direct visualisation of tagged proteins, without the addition of antibodies, providing the particular benefit of facilitating the visualisation of molecules within living cells (reviewed by Gubbels, 2004). Transfection can be routinely performed in \textit{P. berghei} and the feasibility of integrating gene targeting vectors into \textit{Pblap} coding regions is demonstrated by the successful disruption of \textit{Pblap1,2,4,5} and 6 (Trueman et al., 2004; Raine et al., 2007; Ecker et al., 2008). With this in mind, chief aims of this chapter were to generate GFP and/or Myc-tagged versions of \textit{Pblaps} and to perform cellular localisation studies on sexual and sporogonic stages of \textit{P. berghei}, using both tagged transgenic parasites and antibodies generated in Chapter 3. It was intended that these results, and the outcome of further investigations based upon them, would provide a better understanding of the biological function of LAPs and help to decipher their suitability as TBV candidates.
4.2. Generation of PbLAP-GFP/Myc tagged parasites

Plasmids encoding c-Myc or eGFP and a modified *T. gondii* dihydrofolate reductase/thymidylate synthase gene (*Tgdhfr/ts*) as a selectable marker were used for the generation of constructs aimed at tagging endogenous *Pblap* gene products at their C-terminal end. GFP tagging provides the considerable advantage of fluorescence, which may facilitate a direct method to define localisation, but it is known that the bulky size of GFP can cause mislocalisation through steric hindrance or interruption of critical localisation or retention signals (Huh et al., 2003). Constructs aimed at the integration of c-Myc tagged transgenes were therefore generated in parallel, since the smaller size of c-Myc increases the chance that a protein carrying the tag will retain normal structure and function (Jarvik et al., 1998).

The adopted cloning strategy (see methods) was designed such that following transfection, the linearised vector integrates into the targeted locus by single homologous recombination, resulting in the replacement of the C-terminal gene portion of the endogenous gene with the GFP/myc recombinant. Successful integration thereby results in tagging of the endogenous gene copy, with the full-length gene being expressed with a 3’ eGFP or c-Myc tag. The C-terminal regions of each *Pblap* were cloned into GFP/myc tagging vectors following *Kpn*I/*Apa*I digests. Plasmid preparations were analysed by restriction digest and DNA sequencing to confirm the presence of a correct insert in frame with the GFP/myc tag: constructs encoding *Pblap* C-terminal fragments in frame with a GFP tag were successfully generated for *Pblap4* and *Pblap5* and constructs encoding *Pblap* C-terminal fragments in frame with a c-Myc tag were produced for *Pblap1*, *Pblap3*, *Pblap4*, *Pblap5* and *Pblap6*. Plasmids are referred to as plap4-gfp, plap5-gfp, plap1-myc, plap3-myc, plap4-myc, plap5-myc and plap6-myc.

A restriction site unique to the lap gene fragment (absent from the vector) was utilised to generate linearised plasmid DNA for transfections. Plasmids plap1-myc, plap4-gfp, plap4-myc and plap6-myc were digested with *Bsm*I; plap3-myc with *Aar*I; plap5-gfp and plap5-myc with *Spe*I. Linearised and purified vector constructs were transfected into wild-type *P. berghei* parasites by electroporation of *P. berghei* schizonts: the transfection of purified schizonts, cultured *in vitro*, has been demonstrated to be more successful than transfection of mixed blood stages (van Dijk et al., 1995). Following transfection, parasites were allowed to invade fresh red blood cells by incubation in naïve mouse blood for 20-30 minutes at 37°C, after which blood was injected into donor mice. Transgenic
parasites were selected by treatment of mice with the antimalarial drug pyremethamine. Expression of the *T. gondii* dihydrofolate reductase/thymidylate synthase gene (*Tgdhfr/ts*) renders transgenic parasites resistant to the drug. Parasitemias, which were monitored by Giemsa-staining of mouse tail blood smears, were observed in mice infected with plap4-gfp, plap5-gfp, plap3-myc, plap4-myc and plap5-myc transfected parasites, but not for plap1-myc or pblap6-myc. Drug-resistant parasites were collected by removal of blood from mice, genomic DNA was extracted and integration of the construct DNA was analysed by PCR (Figure 4.1):

- Wild-type (wt) gene fragments were amplified using primers specific for the target *Pblap*.
- A combination of the 5’ primer used for amplification of the 5’ region of homology, and a GFP/Myc or *Tgdhfr/ts* reverse primer was used to amplify the plasmid DNA intended for integration (may be in the form of an episome, existing independently as a plasmid).
- Integration was detected using a combination of a primer binding the upstream of the 5’ region of homology, and a GFP/Myc or *Tgdhfr/ts* reverse primer.

The majority of transfections were unsuccessful as assessed by PCR. This could perhaps be related to the schizont preparations used for transfections, since parasitaemia of blood used at culture set-up, schizont viability, and the number of schizonts used per transfection have been identified as key factors correlated with the successful generation of transgenic parasites (A. Ecker PhD thesis, 2007). Transfection using the Amaxa Nucleofector system has increased the efficiency of transfection, but still several attempts are often required before a transgenic line can be generated.

Integration appeared successful for *Pblap4* and *Pblap5*, although in each case, DNA from the wild-type gene could be amplified more readily (Figure 4.1). PCRs also indicated that a large proportion of the construct DNA may have been present as episomal plasmids. Re-ligation of the linearised plasmid resulting in episomal contamination and generation of resistant parasites without integration is a frequent problem associated with transfections and is one that is not easily overcome. Indeed, it is suggested that the best strategy to deal with episomes is to repeat transfections until a clean population is obtained (A. Ecker PhD thesis, 2007).
Figure 4.1. Diagnostic PCR for the detection of Pblap-tagged transgenic parasites

PCR products were amplified from genomic DNA extracted from parasites surviving pyremethamine–based drug-selection using primer sets represented in panel B. Diagnostic PCRs positive (+) and negative (−) for integration (indicating one successful and one unsuccessful transfection) are shown for both lap4-GFP and lap4-Myc. Fragments present in the wild-type gene were amplified using primers specific for Pblap4 (primer pair a+b). The presence of products from primer pair c+d indicated presence of the introduced vector, but not necessarily integrated into the wild-type gene locus. Integration was determined using primer pair a+d (forward primer binding upstream of the 5′ region of homology and a GFP or Myc reverse primer products (indicated with an asterisk) with which a product was only amplified if integration had been successful. Approximate DNA band size in base pairs is indicated.
Populations of transgenic parasites, in which integrated construct DNA had been detected by PCR, were grown under drug selection in mice and immunoblotting and immunofluorescence analyses were performed on mixed blood stages/gametocytes and cultured ookinetes using commercial anti-GFP or anti-Myc antibodies to determine if the expression of LAP-GFP or LAP-Myc fusion proteins could be detected (Figure 4.2). To detect GFP fusion proteins, immunoblots were performed on lysates from PbLAP4-GFP, PbLAP5-GFP and PbConGFP parasites. ConGFP parasites, which constitutively express GFP throughout their entire life cycle (Franke-Fayard et al., 2004), were used as a positive control: a protein consistent with the expected size for GFP (30 kDa) was detected in immunoblots using anti-GFP antibodies. In contrast no bands were recognised in PbLAP4-GFP and PbLAP5-GFP lysates (Figure 4.2A). Blood-stage parasites and parasites from ookinete cultures were also observed live under a fluorescent microscope to see if GFP fluorescence could be detected, and immunofluorescence analyses were performed on fixed cells using anti-GFP antibodies. Neither however revealed fluorescence above background level for any stages analysed (data not shown). Immunoanalyses were similarly performed using anti-Myc antibodies to see if PbLAP4-Myc and PbLAP5-Myc fusion proteins could be detected. A transgenic parasite line in which *P. berghei* gene number PB000814.03.0 (referred to here as Pb814) is tagged with a C-terminal Myc epitope (Dr. M. Delves, unpublished) was used as a positive control. Immunofluorescence assays on paraformaldehyde-fixed mixed blood stages, zygotes, ookinetes and sporozoites revealed that, whilst Pb814-Myc localisation could be observed in zygotes and ookinetes, no LAP-Myc fusion proteins could be detected in any life cycle stage examined. A representative result, in which Myc labelling is detected in Pb814-Myc zygotes, but is absent from LAP4-Myc and LAP5-Myc parasites, is shown in Figure 4.2. The cellular localisation of Pb814-Myc is described further in 4.5.2.
Figure 4.2. Analysis of lapGFP and lapMyc transgenic parasites by immunodetection
A) lap4GFP, lap5GFP and conGFP gametocytes were extracted by Nycodenz separation of blood collected from infected mice. Parasites were lysed and proteins were separated by SDS-PAGE under reducing conditions. Proteins were transferred to a nitrocellulose membrane and probed with rabbit anti-GFP antibodies (dilution 1:10000). Binding was detected using HRP-conjugated anti-rabbit IgG (dilution 1:100000) and ECL chemiluminescence. Molecular weight marker is indicated in kilo Daltons. A band at approximately 30 kDa was detected in conGFP parasite lysates, whilst GFP was undetectable in lapGFP parasites.
B) Immunofluorescence analyses were performed on lap4Myc, lap5Myc and 814-Myc parasites in paraformaldehyde-fixed blood smears. Cells were incubated with rabbit anti-Myc antibodies (1:200) and antibody binding to Myc epitopes was detected through use of AlexaFluor-488 anti-rabbit IgG secondary antibody (1:1500). Fluorescence was noticeable in 814-Myc zygotes (bottom panel), but not in lap4Myc or lap5Myc gametocytes (upper panels).

Table 4.1. Summary of attempts to generate GFP/Myc-tagged versions of PbLAPs

<table>
<thead>
<tr>
<th>PbLAP</th>
<th>Number of transfections</th>
<th>Drug – resistant parasites</th>
<th>Integration</th>
<th>Immunodetection</th>
</tr>
</thead>
<tbody>
<tr>
<td>lap1 MYC</td>
<td>2</td>
<td>✓</td>
<td>✓</td>
<td>-</td>
</tr>
<tr>
<td>lap3 MYC</td>
<td>2</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>lap4 GFP</td>
<td>3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>lap4 MYC</td>
<td>3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>lap5 GFP</td>
<td>3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>lap5 MYC</td>
<td>3</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>lap6 MYC</td>
<td>2</td>
<td>✓</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Reasons for as to why GFP or Myc fusion proteins could not be detected are unknown. However, since the generation of Pblap knockout parasites demonstrated that it is possible to introduce recombinant DNA into the Pblap gene loci and select for transgenic parasites using pyremethamine, perhaps the most likely explanations are as follows:

- Tagged proteins may be expressed at levels that are too low to enable their detection by immunoblot or immunofluorescence: perhaps due to misfolding of the fusion protein and its subsequent degradation.
- Proteins may be incompletely expressed, and since tags were introduced at the C-terminal end, fusion proteins are not expressed.
- The GFP or myc protein may be unstable in the cellular environment within which it is expressed.
- Incorrect integration of the vector, for example, leading to frameshift mutations resulting in disruption of the protein. Phenotypic examination of transgenic parasites indeed suggested that DNA integration into lap4 resulted in gene disruption: the transgenic lap4 parasites failed to sporulate as is typical of ΔPblap parasites. Other transgenic parasites however appeared phenotypically normal.
4.3 Cross-reactivity/specificity of antibodies

Since it was not possible to determine the subcellular localisation of LAPs from attempts to tag the proteins, cellular localisation studies focused on antibody detection utilising anti-LAP antibodies as described in Chapter 3. Results in Chapter 3 demonstrated that antisera generated against the SRCR domain of LAP1, the PA14 domain of LAP3 and the LCCL domains of LAPs 4 and 6 were all able to detect endogenous *P. berghei* gametocyte proteins in IFAs. To determine their suitability for use in cellular localisation studies, antibody cross-reactivity/specificity was examined using *Pblap* gene knockout parasites (available for all but *Pblap3*). Each gene was disrupted by integration of a gene targeting construct containing the *Tgdhfr* selectable marker by double homologous crossover at the 5’-UTR and 3’-UTR regions of the wild-type gene locus; Δ*Pblap1* (Trueman et al., 2004); Δ*Pblap2*, Δ*Pblap4*, and Δ*Pblap6* (Raine et al., 2007); Δ*Pblap5* (Ecker et al., 2008).

Immunoblots and immunofluorescence assays demonstrated that antibodies directed against LAP1SRSR were specific towards endogenous PbLAP1, whilst antisera that were raised against the LCCL domains of LAP4 and LAP6 cross-reacted with proteins other than the target antigens (Figure 4.3 and 4.4). In immunoblots anti-LAP1 antibodies were readily immunoreactive with wild-type *P. berghei* gametocyte lysates, whilst they did not detect protein in Δ*Pblap*1 gametocyte lysates, clearly indicating that the antibodies specifically react with LAP1 (Figure 4.3A). This specificity was robustly supported by immunofluorescence studies (Figure 4.3B). In contrast, anti-LAP4 and anti-LAP6 antibodies appeared partly non-specific since antibody staining could also be detected on the respective Δ*Pblap*4 and Δ*Pblap*6 parasites (Figure 4.4 and 4.5). In wild-type gametocytes, staining appeared punctate suggesting cytoplasmic labelling. A similar labelling pattern was detected in knockout parasites, all-be-it at a lower intensity of fluorescence (Figure 4.4) in accordance with absence of the target antigen and a degree of cross-reactivity against another, similarly localised, gametocyte protein.
Figure 4.3. Specificity of anti-LAP1 antiserum

For immunoblot analyses (A) wild-type (wt) and Δlap1 P. berghei gametocyte lysates were prepared from gametocyte-enriched samples: infected mice were treated with sulphadiazine to kill asexual blood stages and parasites were subsequently purified by Nycodenz density. Purified parasites were separated by SDS-PAGE (8% gel, reducing conditions). Proteins were transferred to nitrocellulose membrane and membranes were probed with αLAP1 (1:100 dilution) and then re-probed with an anti-tubulin antibody (1:10,000 dilution) as a loading control (lower panel). A band at approximately 150 kDa, present in wt and absent in Δlap1, corresponds to the predicted size of PbLAP1 and indicates αLAP1 specificity. The specificity of αLAP1 was confirmed by immunofluorescence analysis (B). Blood smears from wild-type (wt) and ΔPblap1 infected mice were fixed using 4% paraformaldehyde and cells were incubated with anti-LAP1 serum at a 1 in100 dilution. Antibody labelling was detected with Alexa Fluor 448 anti-mouse IgG, at a dilution of 1 in 2,000 and cells were co-stained with DAPI nuclear stain (blue). Scale bar represents 10 μm.
Figure 4.4. Immunofluorescence analyses indicating cross-reactivity of αLAP4 and αLAP6 antiserum against gametocytes. Immunofluorescence analyses were performed on paraformaldehyde-fixed *P. berghei* blood stages of ∆lap1, ∆lap4, ∆lap6 and wild-type (wt) parasites. Cells were labelled using αLAP antibodies at a dilution of 1 in 100 and Alexa Fluor 448 anti-mouse IgG at a dilution of 1 in 2,000. DAPI (blue) was used to stain nuclei. Whilst antiserum against LAP1 SRSR (αLAP1) was negative for immunoreactivity against the knockout ∆lap1, both αLAP4 and αLAP6 labelled gametocytes of the corresponding knockouts ∆lap4 and ∆lap6, although at a lower intensity than labelling of wild-type (wt) gametocytes. Scale bar represents 2 µm.
Anti-LAP6 antibodies also cross-reacted with asexual blood stages in a non-specific manner (Figure 4.5). Co-labelling experiments in which parasites were labelled with anti-formin antibodies (a gift from J. Baum; Baum et al., 2008b) revealed a localisation at the periphery of merozoites contained in schizonts and at the apical end tip of released merozoites (Figure 4.5). Formin plays an important role in actin polymerisation and localises to the parasite/erythrocyte moving junction during invasion (Baum et al., 2008b). It is possible that the observed labelling with anti-LAP6 antisera at the apical end tip of merozoites could be indicative of cross-reactivity with proteins involved in invasion.

Figure 4.5. Immunofluorescence analyses indicating cross-reactivity of αLAP6 antibodies against asexual blood stages. Immunofluorescence analyses were performed on paraformaldehyde-fixed P. berghei blood stages of Δlap6 and wild-type (wt) parasites. Cells were labelled using αLAP antibodies at a dilution of 1 in 100 and Alexa Fluor 448 anti-mouse IgG at a dilution of 1 in 2,000. DAPI (blue) was used to stain nuclei. A) Whilst antiserum against LAP1SRSR (αLAP1) was negative for immunoreactivity against asexual blood stages, αLAP6 antiserum was found to non-specifically bind asexual blood stages, labelling the tips of both wild-type (wt) and Δlap6 merozoites. Scale bar represents 2 µm. B) Parasites were labelled at the periphery of the ring of developing merozoites and the apical tips of released merozoites, as demonstrated by co-localisation with αFormin. In co-labelling studies αFormin was used at a dilution of 1 in 50 with Alexa Fluor 546 anti-rabbit IgG at a dilution of 1 in 2,000. Scale bar represents 1 µm.
The cross-reactivity of anti-LAP4 and anti-LAP6 antisera could be attributed to antibody recognition of related antigens bearing common epitopes. Both anti-LAP4 and anti-LAP6 antisera were raised against protein regions containing an LCCL domain. Since LCCL domains are characteristic features of all but one LAP, with four in PbLAP1, and a single LCCL domain in each of PbLAP2, 3, 4 and 6, the Average sequence conservation between all PbLAP LCCL domains is 69% and may each maintain a common native structure though the presence of conserved cysteine residues (Figure 4.6). It is also possible for antibodies to react against quite unrelated proteins.

Figure 4.6 Amino acid conservation between PbLAP LCCL domains
Multiple alignment of all predicted LCCL domains of P. berghei LAPs performed using T-COFFEE, version 7.71: http://tcoffee.crg.cat/apps/tcoffee/index.html. Sequences are indicated in rows for each domain: LAP1 LCCL1-4; LAP2 LCCL; LAP3 LCCL; LAP4 LCCL and LAP6 LCCL, with the final row representing the overall consensus (Cons). Columns are aligned with similarities between amino acids indicated by a coloured output, whereby red highlighted residues indicates a high identity, orange corresponds to average and green poor conservation. Asterisks indicate highly conserved residues. The multiple alignment highlights a conserved central core.
4.4. Studies on the expression and localisation of LAP1 during sexual and sporogonic development

4.4.1. Expression in *P. berghei* gametocytes is female-specific and is not dependent on PbLAP2, PbLAP4, PbLAP5 or PbLAP6

Previous studies provide clear indications that LAP expression is specific to female gametocytes. In a proteomic study of separated male and female *P. berghei* gametocytes, PbLAP1, PbLAP2 and PbLAP3 were detected in female but not male gametocytes (Khan et al., 2005). The expression of GFP under the control of *Pblap1* and *Pblap2* promoters confirmed female specificity (Khan et al., 2005). Furthermore, experiments with Δ*Pblap* parasites indicate by genetic crosses that PbLAP1, PbLAP2, PbLAP4 and PbLAP6 are female-inherited (Raine et al., 2007). In contrast, expression of the protein family in *P. falciparum* has been detected by immunofluorescence in both female and male gametocytes (Scholz et al., 2008), although PfCCp labelling has not been observed in exflagellating microgametes. Studies in *P. falciparum* have also demonstrated that in parasites in which *Pfccp3* (*lap1*) is knocked down, the expression of PfCCp1 (LAP2) and PfCCp2 (LAP4) is not detected by immunoblotting — indicating that expression of LAP2 and LAP4 is dependent on LAP1 expression (Pradel et al., 2006) and suggesting that other members of the protein family could perhaps be similarly co-dependently expressed.

In order to conclusively distinguish between male and female gametocytes, co-staining experiments were performed using an antibody against *P. berghei* male development gene-1 (MDV1) as a cellular marker (Lal et al., 2009a). MDV1 (also known as PEG3) is upregulated in developing gametocytes and is associated with the osmiophillic bodies and membranous structures of gametocytes (Silvestrini et al., 2005; Furuya et al., 2005). Recent immunolocalisation analyses demonstrate that antibody staining patterns appear markedly different in male versus female gametocytes. A punctate localisation is detected throughout the cytoplasm of the female gametocyte (Lal et al., 2009a) while staining is confined in one or a few large spots in male gametocytes. The identity of the subcellular structures responsible for the localised staining pattern of the MDV1 antibodies is unknown, but allows for confirmation of the distinction between male and female parasites. Co-staining experiments with rabbit MDV1 antibodies and mouse αLAP1 indeed confirmed LAP1 to be female specific (Figure 4.7A). Further studies were performed to compare LAP1 expression in wild-type (wt), Δ*Pblap1*, Δ*Pblap2*, Δ*Pblap4*, Δ*Pblap5* and Δ*Pblap6* female gametocytes (Figure 4.7B). Whilst previous studies in *P.
*falciparum* (Pradel et al., 2006) have demonstrated that the expression of PfCCp1(LAP2) and PfCCp2(LAP4) is dependent on the presence of PfCCp3(LAP1), the converse does not occur in *P. berghei*: LAP1 expression was found to be expressed in ∆Pblap2, ∆Pblap4, ∆Pblap5 and ∆Pblap6 gametocytes whilst absent from ∆Pblap1 gametocytes.

Figure 4.7. Expression in gametocytes is female-specific and is not dependent on PbLAP2, PbLAP4, PbLAP5 or PbLAP6. Immunofluorescence analyses were performed on paraformaldehyde-fixed blood smears containing wild-type *P. berghei* gametocytes. A) Antibody labelling using αLAP1 demonstrates female specific expression of LAP1 in *P. berghei* gametocytes. Labelling using αLAP1 was detected using Alexa Fluor 448 anti-mouse IgG (green). Cells were co-labelled using antibodies against *P. berghei* MDV1: detected using Alexa Fluor 546 anti-rabbit IgG (red). B) Fixed IFA on wt, ∆lap1, ∆lap2, ∆lap4, ∆lap5 and ∆lap6 female gametocytes. Cells were stained as above with anti-LAP1 (green) and anti-MDV (red) and counterstained with DAPI (blue). Scale bar represents 2 μm.
Interestingly, LAP1 antibody staining patterns frequently (but not exclusively) appeared distinct from the localisation of MDV1, with LAP1 often found predominantly at one side of the gametocyte and MDV1 occupying the other (Figure 4.7B). The apparently separate subcellular compartments/vesicles containing MDV1/LAP1 is consistent with the different functions. Gene disruption of Pbmdv1 indicates a role in the production of fertile female gametes and ookinete formation: Lal et al. (2009) calculated a 27% reduction in the proportion of female gametocytes activated and 83% reduction in the conversion of female gametocytes to ookinetes in ∆Pbmdv1 compared to wt P. berghei parasites. In contrast, the phenotypes of ∆Pblap parasites do not manifest until the late oocyst in which a deficiency in sporulation is observed (Ecker et al., 2008; Raine et al., 2007; Trueman et al., 2004).

4.4.2. Cross reactivity of anti-PbLAP1 antibodies with P. falciparum and antibody labelling patterns during gametogenesis

Considering the sequence similarities between PbLAP1 and its P. falciparum ortholog PfCCp3, immunofluorescence experiments were performed to assess the potential for cross-reactivity of anti-PbLAP1 antisera on P. falciparum parasites. Cultured P. falciparum gametocytes (provided by A. Blagborough, A. Talman or M. Delves), were smeared onto a slide, fixed in 4% formaldehyde and immunoreactivity of anti-PbLAP1 antisera was tested. The antisera labelled cytoplasmic regions of P. falciparum female gametocytes, but not males (Figure 4.5A). Serum from an unimmunised mouse was used as a negative control and showed no labeling (data not shown). The demonstration of cross-reactivity enabled comparative analyses to be made. Firstly a comparison of anti-PbLAP1 staining patterns on P. berghei versus P. falciparum, to assess if there a difference between P. falciparum and P. berghei LAP localisation. Secondly a comparison of anti-PbLAP1 versus anti-PfCCp staining patterns on P. falciparum, to assess if anti-PfCCp antibodies label gametocytes differently.

Antibody staining appeared markedly reduced in activated gametocytes compared to pre-activation in both species (Figure 4.7B and 4.7C). Antisera against PfCCp3 (LAP1), PfCCp2 (LAP4) and PfCCp4 (LAP6), generated and provided by Pradel et al., were used in parallel IFA experiments. These demonstrated that antisera against PfCCp3 (LAP1) and PfCCp2 (LAP4) label parasites in a manner indistinguishable from that observed with anti-PbLAP1 antisera (Figure 4.7D and 4.7E). In contrast, antiserum against PfCCp4 (LAP6) demonstrated an association with the parasite plasma membrane as previously
reported by Scholtz et al. (2008) and were the only to label activated male parasites (Figure 4.8F).

Figure 4.8. Cross reactivity of anti-PbLAP1 antiserum with *P. falciparum* and antibody labelling patterns in gametocytes/gametes

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Immunofluorescence analyses were performed on paraformaldehyde-fixed cells from a *P. falciparum* gametocyte culture (A, B, D, E and F) and on paraformaldehyde-fixed cells collected from the blood of a *P. berghei*-infected mouse (C). For each assay in A-C, αLAP1 antisera was used at a 1:100 dilution and labelling was detected with AlexaFluor-448 anti-mouse IgG secondary antibody at a dilution of 1:2,000. In D-F, mouse antisera raised against the *P. falciparum* proteins PfCCp3, PfCCp2 and PfCCp4 (orthologs of PbLAP1, PbLAP4 and PbLAP6 respectively) were used to stain *P. falciparum* gametocytes and gametes. αPfCCp2 and αPfCCp3 antisera were used at a dilution of 1:100 and αPfCCp4 antiserum was used at a dilution of 1:50. In each case, antibody labelling was detected using AlexaFluor-448 anti-mouse IgG at a 1:2,000 dilution. A) Immunofluorescence analysis using anti-PbLAP1 antisera on *P. falciparum* demonstrated immunoreactivity against female *P. falciparum* gametocytes. B) LAP1 labelling patterns in *P. falciparum* gametocytes undergoing gametogenesis were observed following gametocyte activation by suspension in ookinete medium. The top panel is representative of staining patterns on unactivated gametocytes and the three panels underneath this are of ‘rounding up’ gametes at various stages post-activation. C) LAP1 labelling patterns in unactivated *P. berghei* gametocytes and gametes, following activation by suspension in ookinete medium. D) Labelling of PfCCp3 (LAP1 ortholog) in unactivated *P. falciparum* gametocytes and gametes showing localisation at the poles of gametocytes and reduced levels of expression in gametes. E) Localisation of PfCCp2 (LAP4 ortholog) appears similar to that of anti-PfCCp3; labelling is detected at the poles of gametocytes whilst expression seems reduced in gametes. F) PfCCp4 localisation appears markedly different; labelling appears associated with the periphery of the parasite in both gametocytes and gametes, with expression also detected in activated male gametocytes. Scale bar represents 2 µm.

Gametocytes of *P. berghei* and *P. falciparum* differ markedly in their morphology: *P. falciparum* gametocytes are crescentic shaped possess a subpellicular complex and reside in an extensive PV. In contrast, *P. berghei* gametocytes are round in shape, lack a subpellicular complex and fill almost the entirety of the iRBC. Considering these differences, the observed PbLAP1 staining patterns on both *P. berghei* gametocytes (punctuate across the entire cytoplasm) and *P. falciparum* gametocytes (towards the poles of the cell) appears consistent with an association with the ER or a vesicular network of the endomembrane system, in accordance with entry into the secretory pathway. In each species, antibody staining appeared markedly reduced in female gametes (Figure 4.8B and 4.8C), complying with the observation that expression of the proteins ceases after fertilisation and the suggestion that the proteins are secreted from the parasite (Pradel et al., 2004; Pradel et al., 2006).

In parallel assays, immunofluorescent labelling of PfCCp3 (LAP1 ortholog), PfCCp2 (LAP4 ortholog) and PfCCp4 (LAP6 ortholog) demonstrated that antisera against PfCCp3 (LAP1) and PfCCp2 (LAP4) label parasites in a manner indistinguishable from that observed with anti-PbLAP1 antisera (Figure 4.8D and 4.8E). In contrast, antiserum against PfCCp4 (LAP6) demonstrated an association with the parasite plasma membrane.
as previously reported by Scholtz et al. (2008). Interestingly only anti-PfCCp4 serum was found to label activated male parasites (Figure 4.8F). The possible association of PfCCp4 (LAP6) with the plasma membrane could have considerable implications for TBV development since based on antibody staining patterns it would appear to suggest that PfCCp4 (LAP6) as the most favourable target, although no transmission-blocking effects were detected when anti-PbLAP6 sera was tested in transmission-blocking assays (Chapter 3).

4.4.3. Immunodetection of LAP1 in *P. berghei* zygotes

Despite the observed reduction in PbLAP1 antibody staining of female gametes in both *P. berghei* and *P. falciparum* follows, further experiments revealed that LAP1 is expressed post-fertilisation in the developing zygote. *P. berghei* zygotes were cultured in vitro from gametocyte-infected blood and immunofluorescence assays were performed on samples containing zygotes (from 1 hour post activation). Co-staining with antibodies against MDV1 allowed developing zygotes to be distinguished from unfertilised gametocytes. Four hours after activation, the antibody staining pattern of MDV1 localisation changes dramatically from a punctuate distribution, to a ‘patch’ on the surface of the zygote (Lal et al., 2009). In parasites identified with this distinct anti-MDV1 antibody-labelling pattern, LAP1 antibody staining appeared punctuate across the parasite (Figure 4.9).

Whilst it has been suggested that the apparent reduction in LAP/CCp represents secretion of the proteins from the cell, it could alternatively be explained by i) inhibition of expression or protein degradation, thereby reducing the amount of protein; or ii) a block in epitope access/conformational change in epitope(s), preventing antibody binding. The immunodetection of the protein again in the developing zygote could be explained by these effects being temporary in nature. Renewed levels of expression post-fertilisation could also represent the translation of a sub-population of previously translationally repressed *lap* mRNAs. In this way the female gametocyte may provide *lap* mRNA which is required for further zygote development, providing a possible explanation for the essential contribution of the female in *lap* function. Indeed there is evidence to suggest that *Pblap4*, *Pblap5*, and *Pblap6* are regulated by DOZI (Development of Zygote Inhibited) RNA helicase, which is responsible for repression of translation of certain genes until after fertilisation. In ΔPbdozi parasites, mRNAs that in wild-type parasites are
transtranslationally repressed become degraded. The group of identified down-regulated genes includes *Pblap4*, *Pblap5*, and *Pblap6*, but not *Pblap1* (Mair et al., 2006).

**Figure 4.9. Immunofluorescent labelling of PbLAP1 during zygote development**

Mice were infected with wild-type *P. berghei* parasites. Gametocyte-containing blood was removed and added to ookinete culture medium. Cultures were incubated at 19°C and samples removed between 4 and 6 hours post activation and fixed using 4% paraformaldehyde. Immunofluorescence assays were performed using anti-PbLAP1 antisera (αLAP1, 1:100; Alexa 448, 1:2,000) and anti-PbMDV-1 antibodies (αMDV, 1:1,000; Alexa 546, 1:2,000).
4.4.4. PbLAP1 is expressed in *P. berghei* ookinetes, localising to the crystalloids

Intriguingly, imaging of LAP1 localisation within ookinetes revealed that fluorescence was concentrated intracellularly in one or two large circular spots, with overlay images of fluorescence and light microscopic images demonstrating that the antiserum stained pigmented areas (Figure 4.9A), corresponding to intracellular compartments known as the crystalloids. Immunoblot analysis confirmed the specificity of anti-LAP1 antisera on ookinet lysates and demonstrated the expression of LAP1 in ΔPblap2, ΔPblap4, ΔPblap5 and ΔPblap6 ookinetes (Figure 4.10B). At the ultrastructural level, crystalloids appear as a cluster of electron-dense particles with no visible membrane (Figure 4.10C). Crystalloids have been observed by electron microscopy in the sporogonic stages of several species of *Plasmodium* and other haemosporidia (including *Haemoproteus* and Leucocytozoon species) since the 1960s. Among the first descriptions noted the resemblance to viral aggregation, comprising virus-like particles (Garnham et al., 1962; Das Gupta, 1968; Terzakis, 1969; Terzakis et al., 1976). The possibility of a viral nature was doubted however due to the transient nature of the crystalloid, appearing only in certain life cycle stages, and was confirmed by the absence of DNA or RNA in the crystalloid inclusions of *L. simondi* (Desser et al., 1971). Various speculations have been made regarding their nature (discussed later in this thesis) but functions remain elusive.
Figure 4.10. PbLAP1 expression and localisation in *P. berghei* ookinetes

A) Immunofluorescence analyses were performed on paraformaldehyde fixed ookinetes, grown in culture from gametocyte-containing mouse blood: mice were infected with wild-type (wt) or Δlap1 *P. berghei* parasites, gametocyte-containing blood was removed, added to ookinete culture medium, and cultures were incubated at 19°C for 24 hours. Immunofluorescence assays were performed using anti-PbLAP1 antisera (αLAP1, 1:100; Alexa Fluor 448 anti-mouse IgG, 1:2,000). Scale bar represents 2 µm. B) Western blot analysis of wt and Δlap gametocyte lysates probed with αLAP1 (1:100). Antibody binding was detected by use of a secondary antibody anti-mouse IgG (1:10,000) and ECL chemiluminescence. C) Electron micrograph (x45,000) of *P. gallinaceum* ookinete crystalloid (CR) taken from Mehlhorn et al (1980).
4.4.5 Immunoreactivity of anti-LAP1 antibodies against *P. berghei* sporozoites

Immunofluorescent staining of isolated salivary gland sporozoites with anti-LAP1 antibodies indicated an apparent labelling of the surface of the parasite, with no substantial staining seen within the cell (Figure 4.11A). Immunoblot analysis of sporozoite lysates supported the detection of LAP1, demonstrating reactivity against a 150 kDa protein band corresponding to the predicted size of LAP1. A protein of approximately 40 kDa was also detected alongside potential full-length protein (Figure 4.11B).

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**Figure 4.11. Immunoreactivity of anti-LAP1 antiserum against *P. berghei* sporozoites**

An *stephensi* mosquitoes were allowed to feed on anaesthetised mice infected with mature *P. berghei* gametocytes (day 3 post-infection). Salivary gland sporozoites (day 21 post mosquito feeding) were collected by dissection of salivary glands and immunoanalyses were performed to examine anti-LAP1 reactivity against sporozoite proteins.

A) Immunofluorescence assays were performed on paraformaldehyde-fixed sporozoites. Antibody incubations (1:100) were performed with αLAP1, or as a control normal mouse serum (NMS) from an unimmunised mouse, using AlexaFluor-488 anti-mouse IgG (1:1500) for detection. αLAP1 appeared to label the periphery of the sporozoite; NMS showed no cross-reactivity with the parasites.

B) Immunoblots were performed on sporozoite proteins separated by SDS-PAGE in 8% polyacrylamide under reducing conditions. Proteins were transferred to a nitrocellulose membrane and antibody binding was detected by incubation with anti-mouse IgG secondary antibody (1:10,000) and ECL chemiluminescence. αLAP1 bound proteins migrating at approximately 150 kDa and 40 kDa; No protein bands were detected following incubation with normal mouse serum (NMS) from an unimmunised mouse.
Immunoreactivity against sporozoites is in agreement with previous studies: the expression of LAP1 in *P. berghei* sporozoites has been detected by both proteomic analysis (Hall et al., 2005) and by immunodetection (Claudianos et al., 2002; Trueman et al., 2004). In immunofluorescence assays, Claudianos et al. (2002) showed that an anti-peptide antibody against two peptides specific to the SR domains of LAP1 stained the apical portion of sporozoites, whilst immunoblots demonstrated reactivity against two sporozoite proteins of approximately 40 and 60 kDa (Claudianos et al., 2002). Using antibodies against *P. falciparum* LAPs (Delrieu et al., 2002), which were found to cross-react against *P. berghei*, Trueman et al. (2004) observed surface labelling of *P. berghei* sporozoites by immunofluorescence and detected a 60 kDa band by immunoblotting. The consistent demonstration of immunoreactivity against proteins with molecular weights considerably lower than the predicted size of the full-length protein (Claudianos et al., 2002; Trueman et al., 2004; and this study) is indicative of a proteolytic processing, as previously suggested by Claudianos et al. (2002).

Although the possibility of cross-reactivity with other parasite proteins was not excluded, the indication of anti-PbLAP1 immunoreactivity against the sporozoite surface raised the question: is anti-LAP antiserum able to prevent sporozoite invasion of salivary glands? In the mosquito, sporozoites emerge from oocysts into the haemocoel within which they migrate to and invade salivary glands. The ability to deliver set volumes of a solution into the haemocoel of *P. berghei*-infected mosquitoes by microinjection enabled in vivo assays to be performed to determine if anti-LAP1 antiserum could inhibit sporozoite invasion of salivary glands. For each experiment, six pots of 70-80 mosquitoes were allowed to feed on three anaesthetised mice infected with *P. berghei* (gametocytemia approximately 5%). Mice/mosquitoes were rotated every 5 minutes to compensate for variation in host parasitemia and immune factors. All unfed mosquitoes were removed the following day and those remaining were maintained in new pots at 19°C. From day 14 post-infection, mosquitoes were taken as samples for dissection to assess for the presence of sporulating oocysts (usually) and haemocoel injections were performed once the first ruptured oocysts had been observed. For each experiment, two pots of approximately 30 mosquitoes were injected with anti-LAP1 antiserum and the same number with normal mouse serum (NMS) from an unimmunised mouse. Antiserum was injected into the haemocoel of mosquitoes using a microinjector and glass needle to deliver a volume of 138 nl by double injection of 69 nl. Mosquitoes were subsequently maintained at 19°C. Mosquito post-injection mortality appeared similar for each group: ranging from
approximately 43-67% over a period of three days. Surviving mosquitoes were dissected and salivary gland sporozoites counted on day 3 post-injection. Mean values for the number of sporozoites per mosquito were attained by analysis of the infection of groups of between 5 and 10 mosquitoes. The glands were homogenised together in a 1 ml glass homogeniser to release sporozoites and sporozoites were counted using a haemocytometer to calculate the number of sporozoites per ml. Sporozoite numbers per mosquito were estimated by dividing the total number of sporozoites divided by the number of infected mosquitoes. Overall data revealed that sporozoite salivary gland invasion appeared reduced by approximately 50% in mosquitoes injected with anti-PbLAP1 sera in comparison to mosquitoes injected with normal mouse serum from an unimmunised mouse (Table 4.2).

Table 4.2. Antiserum raised against LAP1 appears to partially block sporozoite invasion of mosquito salivary glands

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Mortality</th>
<th>Mosquitoes dissected</th>
<th>Prevalence of infection</th>
<th>Mean number of sporozoites</th>
<th>Sporozoite invasion (% of control)</th>
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<td></td>
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<tr>
<td>i</td>
<td>NMS</td>
<td>67%</td>
<td>20 (two groups of 10)</td>
<td>65% (13/20)</td>
<td>4233</td>
<td>100%</td>
<td></td>
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<tr>
<td></td>
<td>αLAP1</td>
<td>53%</td>
<td>28 (two groups of 10 and one group of 8)</td>
<td>71% (20/28)</td>
<td>1791</td>
<td>42.3%</td>
<td>*</td>
</tr>
<tr>
<td>ii</td>
<td>NMS</td>
<td>50%</td>
<td>30 (three groups of 10)</td>
<td>67% (20/30)</td>
<td>4717</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>αLAP1</td>
<td>53%</td>
<td>28 (two groups of 10 and one group of 8)</td>
<td>79% (22/28)</td>
<td>1823</td>
<td>38.7%</td>
<td>***</td>
</tr>
<tr>
<td>iii</td>
<td>NMS</td>
<td>53%</td>
<td>28 (four groups of 5 and one group of 8)</td>
<td>86% (24/28)</td>
<td>3005</td>
<td>100%</td>
<td></td>
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<tr>
<td></td>
<td>αLAP1</td>
<td>43%</td>
<td>37 (two groups of 5, 2x6, 1x7 and 1x8)</td>
<td>84% (31/37)</td>
<td>2056</td>
<td>68.4%</td>
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|                | NMS      | 56.7%     |                      |                         |                           |                                    |       |
|                | αLAP1    | 49.7%     |                      |                         |                           |                                    |       |

NMS 72.7% 3985 100%
αLAP1 78.0% 1890 49.8%

138 ng of anti-LAP1 antiserum or normal mouse serum (NMS) was injected into the thorax of *P. berghei*-infected mosquitoes harbouring sporulating oocysts. Groups i and ii represent results obtained from the same initial feed, with serum injections performed on day 16 (i) and day 17 (ii) post-feed. In iii, serum injections were performed on day 15 post-feed. Mortality indicates the proportion of mosquitoes that died in the 3-day period post-injections; an approximate value based on the injection of 60 mosquitoes per group. Mosquitoes were dissected three days post-injection; the glands from between 5 and 10 mosquitoes (as indicated) were homogenised together in a 1 ml glass homogeniser to release sporozoites and sporozoites were counted using a haemocytometer to calculate the number of sporozoites per ml. Prevalence of infection was determined following examination of mosquito midguts. Mean numbers of sporozoites (per infected mosquito) were estimated by dividing the total number of sporozoites divided by the number of infected mosquitoes; this is
represented as a percentage compared to NMS. P indicates the significance in differences between anti-LAP1 and NMS injection based on P-values determined by Mann-Whitney tests on estimated means; i)* (P=0.0375), ii)***(P<0.0001), iii)** (P=0.0201). Overall mean values of the three experiments are shown in bold.

The possibility that natural variation in parasite loads could account for the differences observed is not ruled out, therefore results are considered with caution. Given that values for the mean number of sporozoites per mosquito represent an estimate based on total number of sporozoites for a group of up to ten mosquitoes, taking into account prevalence, it is noteworthy that a few very heavily infected mosquitoes and/or levels of prevalence could have a considerable impact. Nevertheless, the apparent reductions in sporozoite salivary gland infection following the injection of anti-LAP1 antiserum into haemocoel indicate that the serum antibodies may bind to sporozoites in the haemolymph and inhibit invasion of salivary glands. Possible mechanisms for antibody-mediated blockade in sporozoite invasion may be by stearic interference or by inducing lysis of sporozoites. It is also possible that the antibodies may initiate an inhibitory effect by binding mosquito molecules. As a starting point for further investigations, it would be interesting to establish whether or not the antibodies are capable of neutralising sporozoites in vitro. In P. berghei, this could be performed by incubation of sporozoites obtained from An. stephensi followed by in vitro incubation in culture media containing antisera. The effect on infectivity can be assessed by following liver-stage development in cultured mouse hepatocytes: after inoculation of a known number of sporozoites, the number of sporozoites capable of invasion and transformation into trophozoites can be determined.
4.4.6. Summary of PbLAP1 localisation

- PbLAP1 expression was shown to be specific to female gametocytes, as revealed by double-labelling experiments with anti-MDV1 serum, which is known to differentially label male and female gametocytes.

- The absence of PbLAP2, PbLAP4, PbLAP5, or PbLAP6, does not affect the expression of PbLAP1, as determined by the immunodetection of LAP1 in ΔPblap2, ΔPblap4, ΔPblap5, and ΔPblap6 parasites.

- Labelling of PbLAP1 appears in distinct areas of the cytoplasm of female gametocytes.

- Antisera against the SR domains of *P. berghei* LAP1 cross-react with female *P. falciparum* gametocytes, with antibody staining patterns in each species appearing similar.

- Antibody labeling of LAP1 appears reduced following gametogenesis, yet despite this, LAP1 expression is detected by immunofluorescence in developing zygotes.

- In *P. berghei* ookinetes, LAP1 was found to localise to the crystalloids.

- Labelling of LAP1 was undetectable in oocysts, but antibodies were found to label the periphery of salivary gland sporozoites.
4.5. PbLAPs localise in ookinete crystalloids and appear necessary for crystalloid formation

IFAs using anti-LAP3, anti-LAP4 and anti-LAP6 antisera on *P. berghei* ookinetes revealed that all antibodies labelled the ookinete crystalloids (Figure 4.12). Immunofluorescent labelling with anti-LAP4 revealed an antibody staining pattern indistinguishable from that observed with anti-LAP1 antisera (Figure 4.12B) whilst anti-LAP3 and anti-LAP6 antisera distinctly labelled both the crystalloids and also a small apical component at the tip of the ookinetes (Figure 4.12A and C). Interestingly, despite the cross-reactivity of anti-LAP4 and anti-LAP6 in gametocytes (as demonstrated in Figure 4.4), neither antibody labelled the ookinete crystalloid in corresponding knockout parasites. Using anti-LAP6 antisera, both crystalloids and apical tips were labelled in wild-type ookinetes, whilst in ΔPblap6 ookinetes labelling of crystalloids was abolished but the apical tip staining remained present. Through these observations it became noticeable that the *Pblap* mutants were somewhat deficient in crystalloid formation.

![Figure 4.12. Antisera against PbLAP3, 4 and 6 label ookinete crystalloids](image)

Immunofluorescence analyses were performed on paraformaldehyde fixed cells collected from a 24 h *P. berghei* ookinete culture. Wild-type (wt) or Δlap *P. berghei* parasites were stained using αLAP, 1:100 (secondary Alexa 448, 1:2,000) and αFormin 1:50 (secondary Alexa 546, 1:2,000) and counterstained with DAPI nuclear stain (blue). Scale bar represents 2 µm.
Given that crystalloids have previously been demonstrated to be positive for lipids (Desser et al., 1971), the lipophilic dye Nile Red was chosen as a stain in order to facilitate their visualisation and quantification. Nile Red fluoresces in lipid-rich or highly hydrophobic environments and indeed was found to label ookinete crystalloids (Figure 4.13B). The seeming impermeability of a large proportion of ookinetes to the stain and the high amount of background fluorescence however made analysis problematic. Nevertheless, crystalloids could be clearly distinguished by Giemsa-staining — in wild-type ookinetes, appearing as a vacuole surrounded by pigment (Figure 4.13A). In contrast, in ΔPblap ookinetes, pigment often appeared more dispersed and mutant ookinetes appeared to lack an obvious pigmented surrounded vacuole, suggesting a lack of cellular organisation. Assessment of the number of crystalloids in cultured wt and ΔPblap ookinetes revealed that the proportion of ookinetes with crystalloids was constantly lower in ΔPblap ookinetes compared to wild-type ookinetes (Figure 4.13C).

Figure 4.13. ΔPblap ookinetes appear deficient in crystalloid formation
A) Giemsa stained ookinetes; wt and ΔPblap ookinetes were grown in culture from gametocyte-infected mice. Parasites were collected by centrifugation from 24 h cultures, smeared onto a slide and stained with Giemsa solution. Crystalloids were clearly visible as vacuoles surrounded by pigment in wild-type ookinetes (upper panel) whereas ΔPblap ookinetes lacked an obvious pigment surrounded vacuole. Images shown are representative of at least three independent ookinete cultures. B) Ookinete stained with Nile Red; cultured ookinetes were suspended in PBS containing Nile Red and after a five minute incubation were observed by light and fluorescent microscopy.
Although During the course of this study, transgenic parasite lines of PbSR (LAP1) tagged at C-terminal by GFP or the N-terminal by mCherry were successfully generated by Carter et al. (2008) and GFP-tagged versions of PbLAP2 and PbLAP3 were subsequently generated by Saeed et al. (2010). Reassuringly, examination of the tagged parasites revealed cellular localisation very similar to the results presented in this thesis. Labelling was detected throughout the cytoplasm of female gametocytes, macrogametes, zygotes and early retorts and in mature ookinetes and young oocysts, fluorescence was confined to the crystalloids (Carter et al., 2008). Furthermore, the disruption of PbLAP1 was shown to be associated with a deficiency in crystalloid formation by both fluorescent microscopy and electron microscopy (Carter et al., 2008).

Several studies based on electron microscopy indicate that crystalloids derive from electron-dense particles interspersed in the female gametocyte (Desser et al., 1971; Canning and Sinden, 1973; Davies et al., 1974; Gallucci et al., 1974; Trefiak et al., 1973; Mehlhorn et al., 1980), an observation which is consistent with the involvement of LAPs in their formation given that PbLAPs have been shown to be inherited from the female gametocyte: it has been demonstrated through genetic crossover experiments with \textit{Pb}lap gene knockout parasites that LAPs are inherited from the female gametocyte (Raine et al., 2007). It may thereby be envisaged that LAPs may be among the molecules derived from the female gametocytes that are required for crystalloid formation, perhaps playing important roles in binding, sorting or packaging components to the crystalloid, their knockout therefore leading to disruption of cellular organisation.
4.6. Features and predicted roles of *Plasmodium* crystalloids

4.6.1. Crystalloids have hypothesised roles as cellular storage compartments which may be essential for oocyst development

Through electron microscopy, crystalloids have been recognised in several species of *Plasmodium* over the past 50 years. Although their functional significance remains unknown, a number of suggestions have been proposed regarding possible roles. Principal hypotheses include an involvement as a metabolic resource, as initially proposed by Garnham (1969) and Desser et al. (1970). It is considered that the crystalloid may act as a cellular storage compartment which is essential for oocyst development; for example by aiding in the proliferation of ER, Golgi apparatus, secretory vesicles and mitochondria (Sinden et al., 1984) or by providing a source of oocyst wall components (Garnham, 1969; Terzakis et al., 1976).

*Plasmodium* is assumed to depend on its host for the acquisition of amino acids. During blood stages, the parasite acquires nutrients from both the RBC cytoplasm and the surrounding plasma. Proteins are converted to amino acids in the parasite’s digestive vacuole and amino acids are transported to the rest of the cell. In contrast, no known nutrient uptake occurs once the parasite is in the mosquito blood meal. It is thereby speculated that the crystalloid may act as a protein store, containing surplus protein generated from nutrient acquisition in blood stages to be released as amino acids for oocyst development. Cell storage bodies can take a variety of different forms. For example, protein storage organelles can release amino acids used for further synthesis; lipid bodies comprise an energy reserve of fatty acids; polyphosphate bodies/volutin granules comprise polymers of phosphates joined by pyrophosphate bonds which may be broken down to supply inorganic phosphate (e.g. for ATP); glycogen or amylopectin granules form a carbohydrate reserve in many cell types, providing a source of glucose.

It is understood that crystalloid bodies may be analogous to refractile bodies (RBs), which among apicomplexan parasites are particularly prominent in *Eimeria* sporozoites (Roberts et al., 1970). Yet rather than a homogeneous electron dense cellular structure, as is typical of *Eimeria* RBs, crystalloids appear as electron-dense particles, which may be arranged either in a random orientation or in an ordered honeycomb-like pattern, (Figure 4.15). Also termed paranuclear bodies, usually two RBs are present, one either side of the nucleus — with an anterior RB localised between the nucleus and the apical complex often smaller than a large posterior RB. Associations with glycogen/amyllopectin granules
have been detected in several *Eimeria* species (Roberts and Hammond, 1970). The RB has proposed roles as a reservoir for proteins involved in invasion, perhaps eliciting the relocalisation of proteins such as the aspartyl protease Eimepsin to the apical end of sporozoites just before invasion (Jean et al., 2000). This is supported by the detection of a number of invasive proteins in an *Eimeria* RB-enriched proteome (Venevelles et al., 2006) and is consistent with the disappearance of the RB after sporozoite invasion: the anterior *Eimeria* RB has been described to break up and disappear within 10 hours post-invasion, although in a few species, RBs may be found in merozoites (Roberts et al., 1970). Proteomic analysis of *Eimeria* RB-enriched preparations also detected several proteins implicated with energetic and metabolic functions (Venevelles et al., 2006). Proteins detected included a lactate dehydrogenase, involved in metabolism, and a group of proteins involved in redox mechanisms, namely a carbonyl reductase, a haloacid dehalogenase type hydrolase (implicated in detoxification in bacteria) and a 2-cys peroxiredoxin. A protein of the ubiquitin family was also detected (Venevelles et al., 2006). Potential for involvement in a form of energy metabolism has also been indicated in *Cryptosporidium* by the finding that a unique fusion protein pyruvate:NADP+ oxidoreductase (CpPNO) has been shown though immunofluorescent and transmission electron microscopy to localise to the *C. parvum* crystalloid bodies (Crtnacta et al., 2006). Therefore, although literature on the nature of crystalloids and potentially similar organelles in apicomplexan parasites is limited, there are indications to support roles as a store for the distribution of proteins, and/or an involvement in energy metabolism.
Figure 4.14. Examples of crystalloid and refractile bodies of apicomplexan parasites

A) Drawing of a *P. gallinaceum* ookinete showing five crystalloids (CR). Also shown: MN, micronemes; PG, protein granules; S, spindle apparatus; PE, pellicle; Taken from Mehlhorn et al. (1980).

B) Electron micrographs of haemosporidian crystalloids from:
- i) *Leukocytozoan simondi* ookinete (x47,600);
- ii) *L. simondi* early oocyst (x17,000);
- iii) *L. simondi* sporozoite (x42,500);
- iv) *P. gallinaceum* ookinete (x40,000);
- v) *Parahaemoproteus fringillae* ookinete (x40,000);
- vi) *P. velans* ookinete (x40,000);
- vii) *Haemoproteus columbiar*
- viii) *Isospora ohioensis* day 7 (x23,800);
- ix) *I. ohioensis* day 14 (x23,800). Taken from Trefiak and Desser (1973); Gallucci (1974); Lindsay et al. (1997).

C) Drawing of an *Eimeria* sporozoite showing two prominent refractile bodies (RB), one on either side of the nucleus. Also shown: C, conoid; MN, micronemes; RH, rhoptries; MP, micro pore; MI, mitochondria; ER, endoplasmic reticulum; DB, dark bodies; L, lysosome; N, nucleus; NU, nucleolus; V, vacuole; IM, inner membrane; OM, outer membrane; Taken from Scholtyseck (1979).

D) Drawing of an *Isospora* sporozoite showing a large central refractile body (RB). Also shown: P, polar ring; C, conoid; RH, rhoptries; N, nucleus; MI, mitochondrion; PE, pellicle; A, amylopectin granules;
4.6.2. The crystalloid is a transient cellular compartment of *P. berghei* ookinetes and early oocysts to which Pb814, a potentially micronemal protein, localises

Recent efforts to characterise putative ookinete micronemal proteins (identified by Lal et al., 2009b) have led to the identification of a further crystalloid protein — the first to be identified outside of the LAP family. PB000814.03.0 (referred to here as Pb814) is a small (30 kDa) hypothetical protein shares no sequence homologies to other known proteins with no known putative protein domains. In an initial attempt to characterise the protein, a transgenic parasite line expressing a c-Myc-tagged version of Pb814 has been generated (Dr. M. Delves, unpublished). These parasites (a gift from Dr. M. Delves) provided a positive control for attempts to tag PbLAPs (section 4.2). Interestingly, cellular localisation studies using an anti-Myc antibody revealed the protein to be localised to *P. berghei* crystalloids and thereby provided a means of tracing the progression of crystalloid development.

Unlike LAPs, Pb814 expression was absent from gametocytes, with the tagged protein first detectable in zygotes, approximately 4 hours post-activation (Figure 4.14), consistent with the indication that mRNA transcripts of Pb814 may be held under translational repression in the female gametocyte (Mair et al., 2006). Fluorescent staining of Pb814-Myc depicts a cytoplasmic localisation during the early stages of ookinete development and as ookinete development proceeds via protrusion from the apical end, becomes noticeably associated with pigmented areas of the parasite, clearly concentrating in a single focal spot in ookinetes 12 hours post-activation. Over the following 12 hours, the nucleus migrates from the posterior end to the middle of the cell and mature ookinetes are characterised by an enlarged anterior end, a central nucleus, and pigment representing the crystalloid at one or both sides of the nucleus. In mature ookinetes, Pb814 appears localised in the crystalloids, and interestingly, a putative association with ookinete micronemes is apparent — indicating a potential link between the crystalloid and the apical organelles.

In *An. stephensi* midguts with early stage infections with transgenic Pb814-Myc parasites (24-48 hours post-blood-feed) labelling of Pb814 is observed in the crystalloid of
transforming ookinetes (tooks), representing recently invaded ookinetes. Labelling of the crystalloid remains prominent in young oocysts (day 2 post-blood-feed) and appears diminished in 4-day old oocysts. The described pattern of Pb814-Myc labelling of crystalloids is shown in Figure 4.14. Labelling was absent in mature oocysts and sporozoites (data not shown).

Figure 4.14. Subcellular localisation of Pb814 during zygote/ookinete development and in early oocysts

Upper panel shows representative images of ookinete development on parasites cultured in vitro. The ookinete develops by protrusion of the apical end into a mature 24 h ookinete with dense pigment representing crystalloids at one or both sides of the nucleus. Lower panel shows representative images during the first five days of development in the mosquito. The crystalloid is present in young oocysts, but is lost during oocyst development and is absent in mature oocysts.
Concentration of Pb814 in the early stages of development in the oocyst and its apparent dispersion and later disappearance by day 5 oocysts is consistent with previous electron microscopic observations of *Plasmodium* crystalloids, which describe their presence only during the early stages of oocyst development and not in mature oocysts or sporozoites (Mehlhorn et al., 1980). Regression of the crystalloid appears to follow one of two patterns, relating to oocyst growth:

- In *Plasmodium* and *Haemoproteus*, oocysts undergo dramatic cell growth and produce thousands of sporozoites from multiple sporoblasts. Crystalloids typically disperse in young oocysts, prior to the formation of sporoblasts (Mehlhorn et al., 1980).

- In *Leucocytozoon* species, oocysts remain small and a relatively small number of sporozoites (less than 100) are formed from a single sporoblast. The crystalloid fragments and a portion of the crystalloid progresses into budding sporozoites (Valkiunas, 2005).
4.7. Can bypassing the midgut restore \( \Delta Pblap \) sporulation?

A comparison of the albeit limited protein expression and localisation data available for the LAP family in apicomplexan parasites reveals an interesting association with life cycle stages involved in infection of the host gut;

- In *Plasmodium*, the proteins are expressed in stages within the mosquito midgut.
- In *Babesia divergens* are expressed in phases of parasite development in the mosquito midgut (Becker et al., 2010).
- In *Cryptosporidium*, The immunolocalisation of *C. parvum* CCp1 (an ortholog of LAP2) shows possible associations with the micronemes.
- In *Toxoplasma*, members of the protein family have been shown to be localised in sporozoites, immunolocalisation studies on *T. gondii* CCps (TgSR1, TgSR2, TgCCp5A and TgCCp5B) indicate their localisation in dense granules of sporozoites (Tosini et al., COST Action Conference, May 2008).

This raises the interesting possibility that the proteins may play essential roles in the environment of the gut.

Interestingly, Carter et al. (2008) demonstrated that when \( \Delta Pblap1 \) oocysts were cultured *in vitro*, sporulation appeared to occur normally. The ability of \( \Delta Pblap1 \) to form sporozoites *in vitro* but not in the environment of the midgut indicates that *Pblap1* may not be essential for sporulation *per se*, and raises the possibility that functions might involve mosquito factors (Carter et al., 2008). It is possible to bypass midgut invasion and study the development of *Plasmodium* in the absence of midgut interactions by the microinjection of ookinetes into the haemocoel of mosquitoes. This technique can be used to investigate if the phenotype of \( \Delta Pblap \) parasites alters if ookinetes are allowed to develop in the absence of mosquito factors and the midgut epithelial barrier bypassed. A rescue of phenotype would indicate an essential role during ookinete invasion of the midgut epithelium, as is demonstrated for example for the ookinete micronemal protein *PbCTRP*; injection of \( \Delta Pbctrp \) ookinetes into the haemocoel restores infectivity (Nacer et al., 2008).

To assess if interaction with the midgut plays a role in \( \Delta Pblap \) phenotypes, haemocoel injections were performed and subsequent sporozoite development was assessed by enumeration of salivary gland sporozoites. For each experiment, ookinete haemocoel injections were performed in parallel to ookinete membrane feeds. Wild-type ookinetes
were able to establish an infection in the mosquito independent of the mode of administration and \( \Delta Pblap \) phenotypes appeared indistinguishable whether ookinetes were fed or injected (Table 5.2). Although some salivary gland sporozoites were detected in \( \Delta Pblap1-5 \), numbers were extremely low compared to wild-type. No sporozoites were detected in \( \Delta Pblap6 \) infections.

The inability to rescue phenotypes when the midgut epithelium barrier is bypassed by ookinete haemocoel injections indicates that LAPs function independently from interactions with the mosquito epithelia and lends support to the theory that the crystalloid may act as a storage compartment during ookinete development, with subsequent functions in the oocyst. In seeking possible reasons for the observed \( Pblap1 \) redundancy \textit{in vitro}, it is perhaps possible that growth factors present within the \textit{in vitro} oocyst culture medium could provide signals for proliferation that are otherwise absent during development in the mosquito. This would imply that \( \Delta Pblap \) oocysts may be able to respond to growth signals if provided, resulting in sporulation, but in the absence of such additional cues in the mosquito oocysts remain defective. Alternatively, the observed \textit{in vitro} \( \Delta Pblap1 \) phenotype may be similar to that of \( \Delta Pfccp3 \) parasites, which are capable of forming sporozoites, but that these are defective and unable to invade salivary glands.
Table 4.3. Injection of \( \Delta Pblap \) ookinetes into the haemocoel of mosquitoes does not restore sporulation.

<table>
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<tr>
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</tr>
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</tr>
<tr>
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<td>10</td>
</tr>
<tr>
<td>wt</td>
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</tr>
<tr>
<td></td>
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<td>( \Delta Pblap2 )</td>
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</tr>
<tr>
<td>( \Delta Pblap4 )</td>
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</tr>
<tr>
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<td>20</td>
</tr>
<tr>
<td>( \Delta Pblap6 )</td>
<td>20</td>
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</tbody>
</table>

Ookinetes were cultured in vitro and the same culture was delivered to An. stephensi mosquitoes either by membrane feeding or by haemocoel injection. Salivary gland sporozoites were counted 21 days post-feed/injection. N refers to the number of mosquitoes dissected. Salivary glands were dissected, homogenised in groups of 5-10, and sporozoites were counted using a haemocytometer. Sporozoite numbers are shown as the mean number of salivary gland sporozoites per mosquito.
4.8. Differential gene expression profiles of wild-type and \( \Delta Pblap1 \) parasites

LAP expression appears to cease prior to sporulation, yet essential effects of \( Pblap \) are not apparent until sporozoite formation, as revealed by gene knockout studies. Given the new finding that \( \Delta Pblap \) parasites appear deficient in crystalloid formation in zygotes/ookinetes, it is hypothesised that the loss of LAP may have molecular effects in the ookinete, leading to a defect in sporulation through widespread knock-on effects. To investigate this, microarray-based transcriptional profiling was performed to analyse how the loss of \( Pblap \) and consequent absence of crystalloid formation impacts gene expression.

Microarray-based transcriptional profiling provides an effective means of examining patterns of gene expression in a cell population on a genome-wide scale. Based on the hybridisation between immobilised DNA probes and labelled cDNA / RNA targets derived from RNA samples, DNA microarrays can simultaneously measure mRNA activity for each gene within a particular mRNA sample. Therefore through the application of microarray experiments, it can be distinguished how \( Plasmodium \) responds to the disruption of \( lap \) in terms of gene expression across the entire genome. In doing so there exists the possibility of identifying how defects in sporulation are related to the loss of \( lap \) and the signalling pathways required for sporozoite formation.

Since phenotypic differences between wild-type and \( \Delta Pblap \) parasites first appear noticeable in the ookinete, it was predicted that at this stage of development, differences in gene expression may be detectable. Therefore an analysis of the transcriptional activity of \( in \text{ vitro} \) cultured \( \Delta Pblap1 \) ookinetes was made as a comparison to \( wt \) ookinetes cultured in parallel, with the aim of measuring the transcriptional profile of \( \Delta Pblap1 \) ookinetes relative to \( wt \). mRNA was reversed-transcribed into cDNA, labelled with a fluorescent dye (\( \Delta lap1 \) derived cRNA with Cy3 labelled CTP and \( wt \) derived cRNA with Cy5 labelled CTP) and hybridised on the array. Expression levels at each spot of the array (corresponding to a specific gene) were quantified by fluorescent imaging. Intensity of fluorescence correlates to the amount of mRNA in a sample complementary to each gene, providing a means of comparing expression levels among wild-type and knockout samples. Four independent samples were analysed in a single microarray experiment, identifying 274 genes showing at least fold average change >1.5 (log2 normalised ratios >0.6) in regulation in the absence of LAP1.
4.8.1. Genes downregulated in \(\Delta Pblap\) ookinetes

Genes most strongly downregulated (Table 4.4) included two members of the PyST-B-2TM family. This is a gene family found in the rodent malaria parasites \(P. yoelii\), \(P. chabaudi\) and \(P. berghei\) with no direct orthologs in \(P. falciparum\), but have a structure similar to that of the \(P. falciparum\) PfMC-2TM and PfST-2TM family (Carlton et al., 2002). These gene families are among those located in the variable subtelomeric regions of the chromosomes. The gene products are predicted exported transmembrane proteins of the asexual blood stage parasites involved in protein transport and trafficking (Smyth et al., 2008). Despite this, PB103795.00.0 was found to be significantly (3.7 fold) down-regulated in \(\Delta Pbdozi\) parasites compared to wild-type (Mair et al., 2006).

Table 4.3 lists the 34 most down-regulated genes. Of these approximately a third are specific to \(Plasmodium\), and encode hypothetical proteins with no predicted homologies to known protein domains. Around half however are widely found across eukaryotic phyla, representing genes that encode proteins essential to key signalling pathways, many of which could be potentially be important for \(Plasmodium\) sporogony. Those with a potential involvement in cell cycle regulation most notably included cyclin 4 and a HORMA domain-containing protein, both of which are known in other eukaryotes to be key regulators of mitosis (Aravind and Koonin, 1998). A number of translational machinery components were also down-regulated including a tRNA synthetase, a tRNA ligase and an RNA polymerase. Furthermore, proteins with potential biosynthetic, metabolic and trafficking activities were also down-regulated. These included a putative patatin-like phosphatase, with predicted roles in lysophospholipid lipolytic activity. Known patatin domain containing proteins include the yeast ER integral membrane protein is lysophospholipase neuropathy target esterase-1 (NTE1), which has an identified role in lipid membrane homeostasis and is regulated by components of the yeast secretory machinery which are involved in lipid metabolism and vesicular trafficking (Fernandez-Murray and McMaster, 2007). In addition, a putative member of the mitochondrial carrier protein family, with associated roles in the transfer of molecules across the membranes of organelles for energy transfer (Walker, 1992).
Table 4.4. Genes most strongly downregulated in \( \Delta Pblap1 \) ookinetes

<table>
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<th>Gene ID</th>
<th>Gene annotation</th>
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4.8.2. Genes upregulated in ΔPblap1 ookinetes

Genes upregulated in ΔPblap1 ookinetes (Table 4.5) included a number of cytoskeletal associated proteins. These included MTIP, an essential component of the apicomplexan invasion machinery and a second putatively inner membrane complex (IMC) related skeletal protein. MTIP localises to the IMC and interacts with MyoA tail domain, which is essential for gliding (Meissner et al., 2002; Bergman et al., 2003), whilst the putatively IMC related membrane skeletal protein may have important roles in cell development, shape, and structural integrity (Tremp et al., 2008). The IMC for example forms a scaffold for the assembly of daughter parasites therefore is essential for cytokinesis and is likely to be required to maintain the shape of invasive stages. In addition, a 2-fold upregulation in circumsporozoite protein (CSP), which plays essential roles in organising the formation of the cytoskeleton of budding sporozoites (Thathy et al., 2002) was also upregulated. It is possible that the upregulation of genes may represent a compensatory mechanism for the absence of Pblap1. Both CSP and PbLAPs are implicated with roles in sporozoite development, as indicated by gene disruption studies, lending support to the idea that csp transcripts may be upregulated to compensate for the loss of lap1. Alternatively, mis-regulation of events in the ookinete as a result of lap1 gene disruption could perhaps trigger premature csp expression. A number of conserved enzymes including kinases, ribonucleotide reductase, phosphatidylserine decarboxylase, which may initiate various interactions and reactions involved in cellular development were also detected as being upregulated.
<table>
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4.8.3. Interpretation of transcriptional profiles

Transcriptional analysis demonstrated that the absence of *Pblap1* in ookinetes is associated with differential expression of a number of genes that could potentially account for defects in cytokinesis. A total of 274 genes were identified showing at least >1.5 fold average change in regulation in the absence of LAP1. This included the upregulation of genes involved in cell growth and the downregulation of genes required for cellular division, which could potentially lead to the characteristically large unsporulated Δ*Pblap1* oocysts, typified by growth despite the absence of division.

The microarray analysis was designed to be an initial single-point analysis, comparing gene expression in Δ*Pblap1* ookinetes with wt *P. berghei* ookinetes. Clearly it would be of interest to extend this to a time-series analysis, incorporating comparisons through from gametocytes to oocysts. However, although microarrays have great potential in offering insights through transcriptional profiling, they also have a number of imitations. Most notably, they are expensive, require a large amount of high-quality mRNA, which can be difficult to acquire, and artefacts are common. It is predicted that a proportion of the genes in an array may be found to be differentially expressed in different by chance alone. In order to verify results it is desirable to confirm differentially regulated genes by RT-PCR: it would be wise to test the genes that appear to be most strongly up-/down-regulated in Δ*Pblap1* ookinetes by RT-PCR analysis. Furthermore, the transcription of a gene does not necessarily indicate its translation. Considerable changes in protein levels may act at the level of protein expression rather than the level of transcript, perhaps through alterations in protein stability or targeting. This is clearly demonstrated by the finding that the disruption of *Pfccp3* (*Pblap1*) causes loss of LAP2 and LAP4 protein, but not of the transcript (Pradel et al., 2006).
4.9. Conclusions and discussion

4.9.1. Summary of findings

Studies presented in this chapter aimed to provide a better understanding of the biological roles of LAPs and their suitability as TBV candidates. Main findings were as follows:

- The absence of surface labelling of gametes, zygotes and ookinetes with anti-LAP sera correlates with the lack of transmission-blocking immunity conferred by the antibodies.
- Anti-LAP1 sera labelled the periphery of *P. berghei* salivary gland sporozoites and may act to inhibit sporozoite migration/invasion of salivary glands.
- Antisera raised against the SR domains of LAP1, the PA14 domain of LAP3 and the LCCL domains of LAPs 4 and 6 were all found to label the crystalloids of *P. berghei* ookinetes. ∆Pblap1, ∆Pblap2, ∆Pblap4, ∆Pblap5, and ∆Pblap6 parasites were observed to be defective in crystalloid formation by light microscopy, indicating that LAPs are in some way involved in crystalloid formation and/or its maintenance. The association of PbLAPs with the crystalloids provides an immediate indication of a potential link to the observed phenotypes of the ∆Pblap parasites, on the basis that the crystalloid may be associated with cellular events in the oocyst.
- Immunofluorescent labelling of Myc-tagged Pb814 protein identified Pb814 as a protein localised to crystalloids demonstrated that crystalloids appear as transient compartments of *P. berghei* ookinetes and early oocysts, fragmenting thereafter, consistent with observations through electron microscopy.
- The injection of ∆Pblap parasites into the haemocoel of mosquitoes (thereby bypassing the midgut) did not rescue sporulation, indicating that LAPs function independently from interactions with the mosquito epithelia.
- The outcome of the microarray analysis comparing the transcriptional profiles of ∆Pblap1 and wild-type *P. berghei* ookinetes highlights the potential ‘knock-on’ effects of lap gene disruption and provides insight into how the loss of LAP1 and associated defect in crystalloid formation may result in disrupted development.

4.9.2. PbLAP cellular localisation studies

Main aims were to study the cellular localisation of PbLAPs through a parallel assessment of localisation by antibody detection methods and of GFP and/or Myc-tagged versions of the PbLAPs. However, transfection attempts were unsuccessful in generating
transgenic parasites in which GFP fluorophores or Myc epitope tags could be detected. The inability to detect GFP or Myc fusion proteins may be related to low expression levels of tagged proteins, incomplete expression of the fusion proteins, mis-folding and/or degradation of the GFP fluorophore or Myc epitope, or loss of protein function caused by integration of the tagging construct (as described in 4.2). During the course of this study, transgenic parasite lines of PbSR (LAP1) tagged at C-terminal by GFP or the N-terminal by mCherry were successfully generated by Carter et al. (2008) and GFP-tagged versions of PbLAP2 and PbLAP3 were subsequently generated by Saeed et al. (2010). These studies confirmed that LAPs can be tagged to study cellular localisation and comparing methods/results reveals possible reasons as to why attempts described as part of this thesis may have failed to yield parasites expressing LAPs where the tag could be detected. Carter et al. (2008) interestingly indicated by immunoblot analysis that whilst the N-terminal tagged PbSR/mCherry remained intact (detecting full-length PbSR product), the C-terminal GFP was cleaved (giving rise to a band of 27 kDa corresponding to monomeric GFP). Although the GFP appeared to remain associated with the target protein, cleavage of the tag represented a problem in tagging at the C-terminal end. A similar scenario could potentially explain why C-terminal GFP/Myc tagging attempts as part of this thesis were unsuccessful. Indeed the subsequent successful generation of GFP-tagged PbLAP2 (Saeed et al., 2010) was produced by single crossover homologous recombination at the 3’end. Meanwhile, a double crossover homologous recombination strategy targeting the entire gene was used in the generation of GFP-tagged PbLAP3 (Saeed et al., 2010).

Although for this thesis the localisation of LAPs could not be traced by tagging the proteins, antisera generated against the SR domains of LAP1, the PA14 domain of LAP3 and the LCCL domains of LAPs 4 and 6 as described in Chapter 3 proved to be effective tools for the study of LAP localisation in fixed cells. Studies focused on the localisation of LAP1 given that only anti-LAP1 sera, raised against the two unique SR domains, was confirmed to react specifically against the endogenous target protein. The outcome of studies (summarised in 4.4.6) revealed interesting insights into LAP expression and localisation. The protein was notably expressed specifically in cytoplasmic regions of female gametocytes, with no labelling of male gametocytes. Detection of the protein appeared markedly reduced in female gametes, yet — whilst previous studies in *P. falciparum* (Pradel et al., 2004; Pradel et al., 2006; Scholtz et al., 2008) suggested that expression ceases following fertilisation— anti-LAP labelling in this study indicated the
continuation of expression beyond fertilisation. Intriguingly, anti-LAP sera were found to label the crystalloids of *P. berghei* ookinetes, becoming the first antibodies known to do so. Reassuringly, examination of PbSR-GFP and mCherry-PbSR parasites (Carter et al., 2008) and PbLAP2-GFP and PbLAP3-GFP (Saeed et al., 2010) revealed cellular localisation very similar to the antibody based detection methods. Localisation was detected throughout the cytoplasm of female gametocytes, macrogametes, zygotes and early retorts; whilst in mature ookinetes and young oocysts, labelling was confined to the crystalloids (Carter et al., 2008; Saeed et al., 2010).

4.9.2. How does the observed cellular localisation compare to bioinformatic predictions and proteomic data?

Bioinformatic predictions, analysing LAP sequences from each *Plasmodium* species for which genome sequence data is available, indicate that LAPs contain an N-terminal signal peptide (as predicted using SignalP: http://www.cbs.dtu.dk/services/SignalP/) and implies that LAPs are extracellular proteins (as predicted using WoLF PSORT: http://wolfpsort.org/). The presence of an N-terminal signal sequence, composed of 5-10 hydrophobic amino acids followed by a peptidase cleavage site, mediates transfer to the ER. It is predicted that the proteins are not retained in the ER or any other subcellular compartments, since further typical eukaryotic sorting signals (which may denote retention in the ER or specify transport to another organelle) appear absent. It is therefore predicted that the proteins progress through the parasite’s secretory pathway and are distributed to the plasma membrane. The proteins lack transmembrane regions or GPI anchors, but may it is thought that they may be retained on the parasite’s surface by protein-protein interactions through their multitude of putative adhesive domains.

The absence of sorting signals however does not necessarily preclude transport to intracellular compartments. Apicomplexan parasites have intricate cellular organisations, containing several specialised cytoplasmic structures and organelles, and mechanisms for intracellular segregation are poorly understood. For example, no protein sorting sequences are known for directing transport to the micronemes, rhoptries or dense granules. Proteins can be transported without sorting signals, most commonly through the bulk flow pathway, according to which proteins remain in a donor compartment until reaching a bulk concentration, at which point they are trafficked to an acceptor compartment. It is thought that proteins may be delivered to the dense granules by the
bulk flow pathway (Coppens et al., 1999). Apicomplexan parasites also have sorting signals that are not found in typical eukaryotic cells, for example, proteins targeted to the apicoplast possess a two-part N-terminal signal comprising a signal peptide to enter the ER and a transit peptide to cross the two inner membranes of the plastid (Waller et al., 2000). It is interesting that, using software to predict apicoplast-targeted sequences (PATS: http://gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php; Zuegge et al., 2001), certain LAP sequences are predicted to be targeted to the apicoplast. These include *P. berghei* LAP 2, 5 and 6; *P. falciparum* LAP 2, 3 and 6; and *P. yoelii* LAP 1, 2, 4, 5 and 6. Cellular localisation studies do not confirm apicoplast targeting, nevertheless, the predicted presence of a transit peptide following the signal peptide may be important in directing LAP cellular transport. To investigate this further, tagged versions of mutant parasites could be generated in which the putative transit peptide is disrupted and the signal peptide retained.

The finding that LAPs localise intracellularly within *P. berghei* ookinetes contrasts with subcellular proteomic studies, which have detected PbLAP1-6 in an ookinete surface protein preparation (Stanway, 2007) and PbLAP1-4 in a putative ookinete micronemal preparation (Lal et al., 2009). It could be reasoned that LAPs represent contaminating proteins of the preparations rather than true surface or micronemal proteins. During preparation of the putative ookinete surface proteome, which was generated by biotin-labelling of surface proteins followed by affinity purification, it is possible that parasites may rupture or become permeabilised during or prior to biotinylation. As such, the biotinylation reagent may be able to enter the cell and label intracellular proteins, and/or proteins that are released from the ookinete may adhere to the biotin-labelled ookinete surface. A number of intracellular proteins such as histone h2A, RNA helicase and components of the cytoskeleton, including myosin and tubulin appear enriched in the surface proteome (Stanway, 2007). Given the high protein-binding capabilities of LAPs, it is plausible that they may have associated with the ookinete surface and thereby be among the contaminating proteins detected as being ookinete surface-enriched. The presence of contaminants is similarly recognised in the ookinete microneme preparation, which was generated by separation of the supernatant of lysed nycodenz-purified ookinetes on a sucrose gradient (Lal et al., 2009b). The 1.1 M sucrose fraction was identified as being enriched in known microneme proteins such as CTRP and SOAP and was shown by electron microscopy to be predominantly comprised of dense granules characteristic of micronemes (~85% of total structures in the fraction were assessed to be
micronemes). However, 68 ribosomal proteins were detected in the preparation (Lal et al., 2009b) and is quite possible that LAPs may be similarly detected as contaminants, particularly since they are found associated with the electron dense regions of the crystalloids, which could conceivably co-segregate with the dense granules of the micronemes. It is possible of course that the crystalloids represent secretory granules, within which LAPs are stored prior to their secretion and that the antibodies generated, whilst able to bind the intracellular proteins, may fail to recognise secreted forms.

4.9.3. Possibilities for LAP1 roles in sporozoites

Although LAPs have not been localised in sporozoites by tagging approaches (Carter et al., 2008; Saeed et al., 2010), the detected antibody labelling at the periphery of salivary gland sporozoites with anti-LAP1 antiserum (4.4.5) alludes to putative roles of the protein beyond sporulation, with possibilities for involvements in functions relating to sporozoite survival, development and infectivity. Several surface/secreted proteins of *Plasmodium* sporozoites are for example known to mediate roles in invasion. The sporozoite is the only developmental stage of the parasite’s life cycle to invade multiple cell types: sporozoites must first invade the salivary glands of the mosquito (described in 1.5.8), and once in the vertebrate host, actively migrate through skin cells (Mota et al., 2001; Vanderberg and Frevert, 2004) before entering the bloodstream and subsequently invading hepatocytes. Those sporozoites that travel through the portal venule or hepatic arteriole stop their migration by abruptly adhering to extracellular matrix proteoglycans protruding through endothelial fenestrations on the sinusoidal cell layer (Coppi et al., 2007). They glide across the sinusoid until encountering and entering a Kupffer cell (macrophage of the liver), in which the sporozoite becomes enclosed in PV, and safely passes through to traverse the sinusoidal cell layer and reach hepatocytes (Baer et al., 2007; Prudencio et al., 2006). Sporozoite surface proteins (e.g. CSP and TRAP) are known to interact with carbohydrate receptors on both the salivary glands and the extracellular matrix of the sinusoidal layer to initiate invasion. Given their adhesive properties, it is certainly plausible that any of the LAPs may be involved in mediating invasion by binding to host receptors. Such a role would be consistent with the phenotypes of ΔPfccp parasites — individual gene knockouts of all but PfCCp6 resulted in parasites that were capable of forming sporozoites, but these were unable to invade the salivary glands, leading to the suggestion that the proteins may be involved in sporozoite binding and invasion of the salivary glands (Pradel et al., 2004).
Less well characterised potential roles of proteins secreted from the sporozoite include roles in mediating immune protection/modulation of the parasite’s surrounding environment. Among the possible functions that may be attributed to LAP1 may be a role in mediating protection against such responses for example by interfering with immune signalling cascades. Immunomodulatory roles have indeed been suggested as putative functions of the protein in light of the indication that predicted domains of the protein are found in many immune-related proteins. For example, SRCR domains are found in macrophage SR1 (Freeman et al., 1990) and complement factor 1 (Goldberger et al., 1987). This has led to the suggestion that LAP1 may serve a role in protecting the parasite from complement in the blood meal (Claudianos et al., 2002; Delrieu et al., 2002; Lasonder et al., 2002), although it could equally act in the haemocoel where parasites are exposed to mosquito immune response mechanisms such as phagocytosis and melanisation (described in 1.5.6), or indeed perhaps in the vertebrate host. Among the speculated mechanisms through which the protein may be involved in immune evasion is by competitive binding. For example, it has been suggested by Trueeman et al. (2004) that LAP1 may compete for ligand binding with *An. gambiae* serine protease Sp22D, which like LAP1, contains two SRCR domains (Gorman et al., 2000). The protein could also play roles in modulation whilst parasite’s residing in salivary glands or hepatocytes. An example of parasite-mediated modulation of mosquito physiology and manipulation of feeding behaviour is the reduction of mosquito apyrase activity, resulting in difficulties in obtaining a full blood meal and therefore increased feeding attempts (Smartt et al., 1995).

Meanwhile, in infected hepatocytes, it has been revealed that CSP is exported across the PV mediated by a pexel/VTS motif and is detected in the cytoplasm of the host cell and been found to localise around and within the hepatocyte nucleus where it is implicated with binding to importins and thereby inhibits nuclear translocation of NFkB to promote parasite growth by influencing the expression of host genes (Singh et al, 2007). As with CSP — for which roles in sporozoite formation, sporozoite invasion, and host-cell modification have been determined — it is speculated that LAP1 and other LAP family members may have multiple roles in parasite development.

### 4.9.4. Are LAPs proteolytically processed?

Throughout this study, immunoblotting analysis frequently detected proteins of molecular masses lower than that expected for the full-length LAPs, possibly indicating the detection of proteolytic processed products of the protein. Indeed, the possibility of LAP proteolytic processing has been previously suggested by Claudianos et al. (2002)
following the finding that antibodies raised against LAP1 appeared to be immunoreactive against two sporozoite proteins of approximately 40 and 60 kDa (Claudianos et al., 2002). A sporozoite protein of 60 kDa was similarly detected by Trueman et al. (2004). Furthermore, antibodies against PfCCp2 and PfNPA were found to recognise differentially sized proteins in gametocytes: in immunoblots, αPfCCp2 detected bands of 180 kDa and 60 kDa, whilst αPfNPA detected proteins migrating at molecular weights corresponding to 100 kDa and 60 kDa (Scholz et al., 2008).

Proteins are often expressed in the form of a precursor, which is then cleaved by specific proteolytic enzymes to yield mature protein. A well-characterised example of proteolytic processing of *Plasmodium* proteins is the cleavage of MSP1. Synthesised as a high molecular weight precursor of around 200 kDa during early schizogony (Holder, 1988), MSP1 undergoes proteolytic processing at the end of schizogony to form a complex consisting of four fragments of approximately 83, 30, 38 and 42 kDa, which is bound to the merozoites surface by the C-terminal GPI-anchor of the 42 kDa fragment (MSP142) (Blackman et al., 1991; Blackman and Holder, 1992; Gerold et al., 1996; Kauth et al., 2006). During invasion, MSP142 is cleaved by proteolysis mediated by a subtilisin-like serine protease (SUB2) (Blackman et al., 1990; Blackman and Holder, 1992; Barale et al., 1999). A 33 kDa fragment is shed with the rest of the complex, whilst a 19kDa C-terminal fragment (MSP119) remains attached to the merozoite surface via the GPI-anchor and is carried into the erythrocyte upon invasion (Blackman et al., 1990; Blackman et al., 1991; Blackman and Holder 1992). The micronemal merozoite protein AMA1 is also processed (Howell et al., 2003; Howell et al., 2005; Narum and Thomas, 1994) as is Pfs230: mature Pfs230 (310 kDa) is present on the surface of gametes following processing during gametogenesis of a 360 kDa precursor protein (Pfs260) which exists on the gametocyte surface (Vermeulen et al., 1985).

It is predicted that LAPs are expressed with an N-terminal signal peptide, the synthesis of which mediates entry into the ER via interaction with a signal recognition particle, enabling the ribosomal complex to enter the ER by binding to a SRP receptor on the ER membrane. The N-terminal signal peptide may be cleaved from the rest of the protein by a specific signal peptidase of the ER membrane. Disulphide bonds — each linking two cysteine residues — are known to form between various conserved cysteines in several of the LAP domains. These form as newly synthesized proteins enter the ER, after which the proteins may undergo any number of additional enzymatically catalysed post-translational
modifications, including cleavage by proteases. Processed forms may then be transferred to a final destination — for example, secreted into the extracellular environment or transported to a specific organelle.

To study potential proteolytic processing, antibodies against various regions of the protein would be raised. For such purposes, peptide antibodies would be suitable, since antibodies generated against short, unique amino acid sequences are likely to be highly specific. The occurrence of proteolytic cleavage can be examined by immunoblot analyses, in which changes in molecular mass detected by different antibodies would be indicative of proteolytic cleavage. It would be possible to identify protein fragments by excision of a gel slice containing the protein of interest followed by proteolytic digestion and mass spectrometric analysis of the resulting peptides, based on the output of which the identity of the protein can be determined by software to match peptide sequences to a protein in the sequence database. For the purpose of vaccine development, it would be important to determine the subcellular localisation of the processed products. Subcellular fractionation experiments followed by further immunoblot analyses could be performed to decipher if differently processed protein forms are detected in different cellular compartments. More informative would be immunolocalisation studies through which the antibody staining patterns of specific anti-peptide antibodies corresponding to different regions of the protein (e.g. C-terminal or N-terminal) can be compared.

Regarding the nature of proteolytic processing, the use bioinformatic prediction software (for example PeptideCutter; http://expasy.org/tools/peptidecutter/) can be used to reveal potential protease cleavage sites based on protein amino acid sequences. Based on this, the effects of specific protease inhibitors can be examined to see if they interfere with processing. It is interesting to note that gene knockout of a P. berghei aspartyl protease, Pbasp, results in mutant parasites with phenotypes similar to those of ∆Pblaps: high numbers of large unsporulated oocysts and substantial reductions in number of salivary gland sporozoites observed (Ecker, 2007; Ecker et al., 2008). Since a common mutant phenotype is often indicative of involvement in the same molecular pathway, this raises the question as to whether LAPs and ASP may interact during the parasite development — as such it could be envisaged that ASP could potentially be involved in processing of LAPs. The mRNA transcript of PbASP appears under translational repression in the female gametocyte (Mair et al., 2006) and the protein is identified in the P. berghei ookinete proteome (Hall et al., 2005). Attempts to study the cellular localisation of the
protein in *P. berghei* by c-Myc epitope tagging were not successful (Ecker, 2007), yet interestingly, a homolog in *Eimeria*, Eimepsin, has been localised to the RB of sporozoites (Jean et al., 2000). Given the similarities between the phenotypes of ∆Pbasp and ∆Pblap oocysts, ∆Pbasp ookinetes were examined for the presence of crystalloids during this PhD study; revealing that indeed ∆Pbasp ookinetes may indeed have possible defects in crystalloid formation: in experiments described in section 4.5, only 14% of ∆Pbasp ookinetes were deemed to contain a compartment resembling a crystalloid as assessed by light microscopy of Giemsa-stained ookinetes (data not shown). Further studies investigating the immunodetection of LAPs in ∆Pbasp ookinetes were attempted but unfortunately were inconclusive since in these studies the antibodies tested failed to recognise wild-type LAP. It would be interesting to investigate this with a new set of antibodies.

4.9.5. Thoughts on crystalloid formation and the involvement of LAPs

*Plasmodium* crystalloids have been identified as transient cellular compartments, forming in the developing zygote/ookinete, and fragmenting during early stages of oocyst development. Previous observations by electron microscopy indicate that the crystalloids derive from electron-dense particles interspersed in the female gametocyte (Desser et al., 1971; Canning and Sinden, 1973; Davies et al., 1974; Gallucci et al., 1974; Trefiak et al., 1973; Mehlhorn et al., 1980), providing a notable parallel to the manner by which PbLAPs have been shown to be female inherited (Raine et al., 2007). It is thereby hypothesised that LAPs are among the molecules derived from the female gametocytes that are required for crystalloid formation. Two main possibilities are considered regarding crystalloid formation: i) it may be derived directly from the female-inherited ER; or ii) it may be formed from molecules inherited independently from the ER.

As is typical of metazoan species, the *Plasmodium* zygote acquires membrane-bound organelles such as the ER from the female cell through cytoplasmic inheritance. There exists the potential that the crystalloid may correspond to a compartment of the endomembrane system. The possibility that the crystalloid represents an ER-derived organelle is supported by electron microscopy observations, which in *P. gallinaceum* ookinetes have revealed a close association between the crystalloid and budding sheets of smooth ER and Golgi-like vesicles (Mehlhorn et al., 1980). The ER is classically divided into three subcompartments — the rough ER, smooth ER, and nuclear envelope — but is a highly complex and dynamic structure, capable of undergoing dramatic morphological
changes (reviewed by Federovitch et al., 2005). Among the most widely found forms of differentiation is proliferation of the smooth ER to form a regularly arranged crystalloid structure (reviewed by Federovitch et al., 2005). Meanwhile, plant cells display the capacity to assemble large protein bodies, which may be either retained in the ER or transported via release into the cytoplasm to protein storage vacuoles (reviewed by Chrispeels, 1991; Okita and Rogers, 1996; Galili, 2004). The protein bodies/protein storage vacuoles provide the cell with a store of proteins that are degraded by proteases during germination known to provide plant cells with resources for rapid growth upon germination or after periods of dormancy (reviewed by Hermin and Larkins, 1999). *Plasmodium* crystalloids could perhaps represent similar forms of differentiated ER compartments. Although typical ER retention signals are absent from LAPs, they may perhaps be retained in the ER by other protein-protein interactions, as is suggested for several protein body components of plant cells (reviewed by Okita and Rogers, 1996).

Another possible hypothesis regarding the nature of crystalloids is that they may be analogous to transient Golgi complexes. The Golgi apparatus is known to undergo reorganisation at various stages of the cell cycle in many eukaryotic cells and parallels can be made between crystalloid formation and Golgi inheritance. Notably, in mammalian cells, the Golgi disassembles during mitosis by fragmentation and redistribution of its components, and subsequently reassembles (possible processes involved in generation and regulation of the Golgi are discussed by Jesch et al., 2001 and reviewed by Jesch, 2002). Patterns of Golgi biogenesis are generally poorly understood in protozoan parasites and the extent to which the Golgi is reorganised is uncertain. However, there are studies to support the existence of transient Golgi-like structures in the simple protozoa *Giardia* (Stefanic et al., 2009). *Giardia lamblia* lacks a distinct Golgi complex, but compartments termed encystation specific vesicles are known to occur in encysting trophozoites. The vesicles appear important for cyst wall formation and evidence for a Golgi-like nature is provided by dependence of their assembly on the GTPases Sar1 and Rab1 (Stefanic et al., 2009). Similar studies in *Plasmodium*, for example using specific inhibitors blocking trafficking out of ER and examining the effects of select gene knockouts on crystalloid formation, would help to determine the degree to which crystalloid formation is dependent on the ER.

It is hypothesised LAPs are in some way required for incorporating molecules to form the crystalloid and/or for retaining the structure of the crystalloid. In this regard, it could be
envisaged that LAPs may be involved in trafficking processes — for example, by acting as chaperones to direct molecules to the crystalloid; and/or act as a type of scaffolding matrix involved in guiding crystalloid formation and maintaining its structure. Given that LAPs are predicted to form a complex, it is considered that interactions between one or more LAP partners may be required for crystalloid formation. Complex formation is known to be essential in the transport of a number of proteins. For example, families of chaperones are known to act as complexes (Pavithra et al., 2007), whilst formation of nuclear pore complexes is generally required to direct the selective transport of specific molecules between the cytosol and nucleus. A specific example in *Plasmodium* includes the formation of a complex between the rhoptry proteins PfRhopH1/H2/H3 and the rhoptry-associated proteins PfRAP1/2/3 (Topalska et al., 2004).

Further studies examining the nature of crystalloid formation would be of interest not only regarding the biology of LAPs, crystalloids and *Plasmodium* sporogony, but also because there may be the possibility of inhibiting crystalloid formation by chemotherapeutic attack of vertebrate blood stages (as suggested by Dessens 2011): according to the understanding that crystalloid formation appears to depend on inheritance of molecules from gametocytes.

### 4.9.6. Concluding remarks

Cellular localisation studies were unable to confirm a surface labelling of gametocyte, gametes, zygotes and ookinetes, correlating with the lack of transmission-blocking immunity conferred by the antibodies. However, a possible surface localisation on sporozoites was identified using anti-LAP antiserum and *in vivo* assays indicated that the antibodies may be capable of inhibiting sporozoite invasion of salivary glands — suggesting that perhaps the lack of transmission-blocking activity at earlier stages of development in the mosquito may be associated with absence of surface localisation or inaccessibility of epitopes in these stages.

Based on the results of immunolocalisation studies, it was intriguingly revealed that LAPs may be key intracellular regulatory proteins involved in formation of crystalloid compartments in ookinetes. It is hypothesised that the crystalloids may be required for sporulation and that LAPs are among the components inherited from the female gametocyte required for crystalloid formation. The functional significance of the crystalloids remains unestablished; however, it is hypothesised that it may represent a
cellular compartment within which molecules are captured and stored/inhibited — the predicted tight packaging of proteins inhibiting their degradation, activation, or involvement in further signalling by preventing accessibility to enzymes and binding partners. The cellular events that lead to the formation of sporozoites in the oocyst are poorly understood, but it could be envisaged that it depends of the timely release of crystalloid contents early in oocyst development. In accordance, the defects in sporulation observed in \( Pblap \) gene knockouts could be attributed to indirect effects of the loss of LAP — the defect in sporulation being linked to a defect in crystalloid formation — rather than a more direct involvement of the proteins in sporulation. Microarray-based transcriptional analysis of \( \Delta Pblap1 \) ookinetes compared to wild-type ookinetes has provided an initial indication of differential expression of a number of genes in \( \Delta Pblap1 \) parasites that could potentially account for defects in cytokinesis: identifying for example upregulation of genes involved in cell growth and the downregulation of genes required for cellular division. Future studies into the role of the crystalloid, for example its association with the endomembrane system, cytoskeletal components and events during cytokinesis could reveal new insights into the processes of \( Plasmodium \) sporogony and potentially reveal new targets for malaria transmission blockade.
Genome sequencing has revolutionised biological research, providing masses of genetic information, and the potential of identifying new therapeutic targets. With the availability of a wealth of data stemming from the sequencing of *Plasmodium* genomes it is necessary to characterise targets of interest and determine which should be pursued for the development of control strategies. Incentivised by the requirement for new targets to block transmission, this thesis aimed to examine whether a group of mosquito-stage malaria parasite proteins referred to as LCCL/lectin adhesive–like proteins (LAPs) could be feasible candidates for the development of antimalarial transmission-blocking vaccines (TBVs).

### 5.1. Viability of LAPs as TBV candidates

The idea that LAPs could represent potential TBV candidates followed the proteomic and bioinformatic analyses, whereby six LAPs have been identified as putatively secreted mosquito-stage proteins with predicted extracellular adhesive domains — properties which are desirable for TBV candidates. Having been identified as prospective TBV candidates, this study aimed to use *P. berghei* to evaluate the potential of LAPs as transmission-blocking antigens.

Codon-enriched *E.coli* strains were utilised for expression of LAP recombinant proteins as means of reducing protein expression problems associated with extreme AT codon bias of *Plasmodium* (Mehlin et al., 2006; Flick et al., 2004). Due to the large size of the proteins (up to 200 kDa) *Pblap* gene fragments predicted to encode protein of size 25-40 kDa were selected for expression rather than attempting to express whole proteins; since low molecular weight proteins are more likely to be expressed successfully (Mehlin et al. 2006; Vedadi et al., 2008). Proteins corresponding to specific regions of the LAPs (LAP1SRSR, LAP2RG, LAP3PA14, LAP3LCCL, LAP4LCCL, LAP5AB, LAP6AB, and LAP6LCCL) were successfully expressed in *E. coli*, although expression was almost exclusively in an insoluble form. Antibodies were effectively raised against the SR domains of LAP1, the PA14 domain of LAP3 and the LCCL domains of LAPs 3, 4 and 6, of which αLAP1SRSR, αLAP3PA14, αLAP3LCCL, αLAP4LCCL, and αLAP6LCCL
were reactive against endogenous proteins. Antibodies however, did not elicit transmission-blocking activity, as determined by a combination of in vitro and in vivo parasite development assays, and did not show an obvious localisation with the surface of early sexual and sporogonic stages of the parasite, as assessed by immunofluorescence assays. Therefore although feasible to generate in a heterologous expression system and immunogenic in mice, antibodies generated were perhaps reactive against epitopes not exposed on the surface of the parasite or were only weakly active against antigens in their native forms.

Difficulties in expressing recombinant antigens in a form that allow the generation of high titres of biologically active antibodies is a frequently encountered problem, posing renowned barriers to malaria vaccine development (Saul, 2007; Williamson, 2003). Antibody activity generally depends on the recognition of antigens in their native conformation, yet the generation of recombinant antigens with tertiary structures mirroring that of the native antigen is often difficult to achieve, particularly when antigens are expressed in E. coli. Consequently, recombinant proteins produced in E. coli often fail to induce antibodies with anti-parasitic activity generally due to the low concentration of properly folded epitopes within the recombinant protein preparation (Milek et al., 1998; Anders et al., 1998; Hodder et al., 2001). Antibody properties can alter considerably depending on antigen expression system, host species for antibody production, and adjuvant. For example, expression of Pb25 in a baculovirus system (Margos et al., 1995), Pf25 expression in yeast (Barr et al., 1991) and Pfs48/45 in a modified E. coli expression system (Outchkourou et al., 2008) considerably improved the production of properly folded proteins.

It remains uncertain whether anti-LAP antibodies generated in a different manner may yield antibodies that might disrupt parasite development. Further investment in the generation of anti-LAP antibodies was not pursued due to the uncertainty over whether LAPs are accessible to antibodies on the surface of the parasite. In their native, endogenous states, the proteins, or at least critical residues, may be inaccessible to antibody. They may, for example, be absent from the parasite surface, or perhaps hidden within protein complexes. Absence of an obvious surface localisation of PbLAP1, PbLAP2 and PbLAP3 has been reaffirmed by tagging the proteins. It is possible that LAPs may be secreted from the parasite in a soluble form and are not retained on the surface of the parasite, or are perhaps retained in intracellular compartments of the cell.
Immunofluorescence studies using anti-PfCCp4 (LAP6) sera indicate that the localisation of LAP6 may be distinct from that of the other family members (noticeably associated with the plasma membrane rather than cytoplasmic). This should perhaps distinguish LAP6 as the focus of any LAP-based TBV studies and it would be interesting to examine the cellular localisation of a tagged version of LAP6.

There are indications that LAPs may undergo both processing and complex formation: the detection of differentially sized protein bands by immunoblotting may be an indicator of processing, whilst co-immunoprecipitation studies indicate possible complex formation with other sexual stage proteins. This complicates antibody design since both proteolytic processing and complex formation affect which portions of a protein are accessible to antibody. For example, Pfs230, exists as a 360 kDa precursor protein (Pfs260) on the gametocyte surface (Vermeulen et al., 1985) and during gametogenesis is processed to a mature 310 kDa protein (Pfs230), therefore antibodies must be raised against the mature processed protein to elicit transmission-blocking activity. The merozoite micronemal protein AMA1 is among the antimalarial vaccine candidates known to be involved in complex formation. AMA1 is transported to the parasite surface where it forms a complex with rhoptry neck proteins (RONs) as has been demonstrated in *T. gondii* (Alexander et al., 2006; Cao et al., 2009). Initial studies of LAP complex formation have been performed by affinity binding studies with PfCCp recombinant proteins and by co-immunoprecipitation assays on *P. falciparum* gametocyte lysates. Studies could be extended to investigate molecular interactions in *P. berghei* gametocytes and ookinetes.

5.2. Insights into LAP biology

Prior to this study it had been demonstrated that LAP expression appears to begin in gametocytes, where it has been suggested that the proteins may play critical roles in extracellular interactions during gametogenesis and fertilisation, possibly acting to enhance fertilisation by aiding adherence or perhaps by forming a protective barrier against factors in the blood meal (Pradel et al., 2004; Pradel et al., 2006). This work has since been extended by studies utilising recombinant proteins to investigate the molecular interactions between the PfCCp proteins in affinity binding studies (Simon et al., 2009). Immunoblot analysis of eluted protein complexes revealed interactions between numerous domain combinations (Simon et al., 2009). Furthermore, interactions between PfCCps and sexual stage TBV candidates Pfs48/45 and Pfs230 have been detected by co-immunoprecipitation assays on gametocyte lysates, and with Pfs25 on activated
gametocytes, leading to the suggestion that the PfCCps form a large multiprotein complex on the surface of gametes with other sexual stage adhesive proteins (reviewed by Kuehn et al., 2010). Nevertheless, the outcome of gene knockout studies in both *P. falciparum* and *P. berghei* indicate that the proteins may be involved in further roles later in parasite development, namely in oocyst maturation and sporozoite production or sporozoites infectivity: gene disruption of *Pblap1*, *Pblap2*, *Pblap4*, *Pblap5* and *Pblap6* results in oocysts defective in sporulation (Claudianos et al., 2002; Raine et al., 2007; Ecker et al., 2008), whilst in ∆*Pfccp1-3*, ∆*Pfccp6* and ∆*Pffnpa* parasites, sporozoites are produced but are incapable of invading salivary glands (Pradel et al., 2004; Scholz et al., 2008).

Using *P. berghei* as an amenable system to study *Plasmodium* development beyond fertilisation, studies presented in this thesis were able to use antibody detection methods to examine the cellular localisation of LAPs and potential functions in later stages of sporogony. Antisera against the SR domains of LAP1, the PA14 domain of LAP3 and the LCCL domains of LAPs 4 and 6 were all found to label the crystalloids of *P. berghei* ookinetes. Despite being identified by electron microscopy in the 1960s, the functional significance of *Plasmodium* crystalloids remains unknown, although among one of its most early suggested roles is that of a storage organelle, perhaps supplying molecules required for oocyst development (Garnham (1969) and Desser et al. (1970). ∆*Pblap1*, ∆*Pblap2*, ∆*Pblap4*, ∆*Pblap5*, and ∆*Pblap6* parasites were observed to be at least partially defective in crystalloid formation by light microscopy, supporting the localisation of LAPs in crystalloids and indicating that LAPs may play important roles in intracellular regulation to maintain the cellular organisation of *P. berghei* ookinetes (possibilities are described in 4.9.5)

LAPs are predicted to be extracellular proteins in light of their identification as potentially secreted multidomain lectin-like proteins, with similarities to many known extracellular adhesive proteins (outlined in 1.6.1). Lectins however are an extremely diverse protein family — with many playing central roles in intracellular regulation (reviewed by Dodd and Drickamer, 2001). The specificity of lectin binding is for example commonly utilised to direct protein entry into a certain cellular pathway, (e.g. secretory or lysosomal) and promote the correct folding of proteins:

- L-type lectins recognise correctly folded proteins and assist their exit from the ER and progression along the secretory pathway;
Calnexin and homologues are ER-resident lectins that bind incompletely processed glycoproteins bearing terminal glucose residues prevent misfolded glycoproteins from exiting the ER and mediate their presentation to proteins which assist in folding.

Members of the glycoside hydrolase protein family (M-type lectins) recognise permanently misfolded proteins and assist their exit from the ER into the cytoplasm for degradation.

F-box lectins recognise misfolded proteins that have been removed from the ER and promote their proteasomal degradation.

P-type lectins recognise mannose 6-phosphate residues, for example on hydrolases, and direct proteins to lysosomal compartments.

R-type lectins play roles in directing enzyme targeting and glycoprotein turnover. (reviewed by Dodd and Drickamer, 2001).

There are a number of notable similarities between LAPs and lectins which may be involved in intracellular regulation. For example, pentraxin/ConA-like domains are found in calnexins and L-type lectins; levanase-type lectin domains and PA14 domains are widely found in enzymes involved in processing of N-linked glycoproteins; and R-type lectins contain a ricin B domain. Therefore, whilst LAPs were distinguished as being putative extracellular proteins in light of their lectin-like properties, analogies can equally be made to intracellular lectins. The need for lectins in directing protein trafficking and protein quality control processes in Plasmodium is debatable due to evidence suggesting that N-glycosylation of proteins is absent in the parasite (Dieckmann et al., 1992; Gowda et al., 1997; Gowda et al., 1999). In agreement with the predicted lack of N-glycosylation, calnexin family proteins, conserved members of which are involved in glycoprotein processing across eukaryotes, including plants, fungi, and animals, are not identified in the Plasmodium genome (Pavithra et al., 2007). Thereby, studies into the possible intracellular roles of LAPs in Plasmodium have the potential to reveal novel insights into the parasite’s biology.
5.3. Future directions

The possibility of generating transmission-blocking antibodies against LAPs is not excluded and different approaches to produce antibodies with improved biological activities (as described in 3.5.3) could prove fruitful. The major aim would be to produce recombinant proteins closely resembling the native structure of the corresponding endogenous proteins. This would not only be important for antibody production but also for any structural analyses. Perhaps as an alternative strategy to expressing the entire full-length proteins, it may be possible to effectively generate antibodies against epitopes along the full length of the proteins by the expression of protein fragments and subsequent immunisation of hosts for antibody production with a mixture of fragments constituting the full protein. This method could be useful as a more conclusive screen to establish whether a protein may be a viable TBV target. However, the inclusion of certain epitopes may have a counterproductive effect, masking potential transmission-blocking activity induced by other epitopes.

Aside from the further generation of antigens and antibodies for testing in transmission-blocking assays, there are countless further experiments that could be performed regarding the biology of the proteins. Of particular interest would be elucidating possible roles in intracellular trafficking. The use of small molecule inhibitors would be an interesting start to study the effects of specific trafficking steps and to determine the possible contribution of components of the endomembrane and cytoskeletal systems on LAP function and crystalloid formation. For example, the fungal toxin Brefeldin A or acyl-CoA:cholesterol acyltransferase inhibitors can effect multiple membrane trafficking pathways in eukaryotic cells including inhibition of vesicle budding from the ER and inhibition of trafficking of material to the ER (Brown and Schimidt, 2005). The ability to culture *P. berghei* ookinetes, oocysts and sporozoites provides a system for the application of such studies.

Expression in heterologous systems could be used to infer the functional attributes of *Plasmodium* proteins (reviewed by Birkholtz et al., 2008), for example by studying their interactions with endogenous proteins or by studying the adhesive properties of proteins by expression of the proteins via endogenous signal sequences and GPI-anchoring motifs to elicit expression on the plasma membrane. The single-celled amoeba *Dictyostelium discoideum*, which can be readily cultured and genetically manipulated, has become an important model organism for studying numerous aspects of eukaryotic cell biology,
including cell division, cell motility, intracellular signalling and trafficking pathways. It offers considerable advantages for the heterologous expression of *Plasmodium* proteins since it shares an AT codon bias (77.4%) (Szafranski et al., 2005). The system has been used to reveal insights into a number of *Plasmodium* proteins. For example, expression of *P. falciparum* chloroquine resistance transporter (PfCRT) demonstrated targeting to the digestive compartments of *D. discoideum* and provided evidence that mutations in PfCRT mediate resistance to chloroquine by reducing chloroquine accumulation (Naudé et al., 2005). Expression in *Xenopus* oocytes has become a valuable tool for the functional characterisation of *Plasmodium* transporters, for example *P. falciparum* hexose transporter PfHT1 (Woodrow et al., 1999) and the aquaglyceroporins PfAQP and PbAQP (Hansen et al., 2002; Promeneur et al., 2007). The *Xenopus* oocyte system has been used to study how PfCRT affects CQ susceptibility by modulation of food vacuole transporters (Nessler et al., 2004) and to identify a SERCA-type Ca2+ ATPase PfATP6 as a target of artemisinins (Eckstein-Ludwig et al., 2003). Furthermore, roles of a *P. falciparum* leucine-rich repeat antigen (PfLRR1) in regulating protein phosphatase activity and cell cycle progression were implied from expression in oocytes, where the protein was found to regulate the G2/M cell cycle transition by binding *Xenopus* protein phosphatase PP1 (Daher et al., 2006).

As potentially multifunctional proteins, it is certainly possible that LAPs may have both intracellular and extracellular functions. For example, it is recognised that the proteins may be secreted from the parasite as soluble molecules that impact mosquito physiology or innate immune responses. Investigation of the potential roles of LAPs in interactions with the mosquito host was beyond the scope of this thesis, but the predicted structural properties, of LAP1 in particular, have initiated the hypothesis of immunomodulatory roles. For example, it is suggested that PbLAP1 could scavenge or compete with the ligands of its molecular homologues such as *Anopheles* Sp22D. Sp22D is the ortholog of *Drosophila* GRAAL and appears to be involved in immune recognition in a manner analogous to that of the horseshoe crab clotting factor C (Danielli et al., 2000; Gorman and Paskewitz, 2001). In order to investigate possible interactions between PbLAPs and mosquito immune factors, it may be possible to study potential co-localisation of PbLAPs and known mosquito immunity proteins, provided reliable markers can be generated. It is also possible to study the effects of the presence and/or absence of PbLAPs on the expression of mosquito genes, at a genome-wide level by microarray analysis, or on select genes by RT-PCR. Differences in gene expression could be examined following the
administration of recombinant LAPs compared to control protein in membrane feeds, or by comparing the expression of mosquito genes in following feeds with $\Delta Pblap$ compared to wild-type parasites.

The intriguing finding that gene disruption of $Pblap$ can lead to an increase in ookinete and oocyst numbers when compared to wild-type infections (Raine et al., 2007; A. Ecker PhD thesis, 2007) could possibly be explained by affects on mosquito physiology (for example, higher oocyst loads could be related to slower digestion of the mosquito blood meal, as is the case in younger mosquitoes) or by a deficiency in regulatory mechanisms, such as inefficient clearance of cells in the absence of LAPs. Regarding the latter, it could be hypothesised that LAPs may act as signal molecules for phagocytosis or apoptosis, perhaps via a mechanism similar to the mode of action of mannose-binding lectin. Mannose-binding lectin does not bind viable cells since sialic acid moieties present on the cell surface inhibits binding of the protein, but the absence of sialic acid residues on apoptotic cells facilitates the binding of mannose-binding lectin, resulting in activation of complement-mediated lysis to promote the clearance of apoptotic cells (Tsutsumi et al., 2005). A number of apoptotic markers could be utilised, such as acridine orange or annexin (see Ali et al., 2010), to observe apoptosis: for example the compare the number of apoptotic cells in $Pblap$ knockout parasites with wild-type and to perform co-localisation studies with markers of apoptosis to determine if LAPs are present on cells undergoing apoptosis.

Future studies such as these mentioned would not only help to decipher more conclusive roles of the proteins, but could also yield insights into processes $Plasmodium$ development in the mosquito perhaps aid in the further development of transmission-blocking strategies.

5.4. Value of the $P. berghei$ experimental system

Studies presented in this thesis utilised the rodent malaria parasite species $P. berghei$, which provides an amenable system for the study of $Plasmodium$ transmission stages, offering exceptional opportunities for understanding molecular mechanisms underlying the developmental processes of $Plasmodium$ in the mosquito, for identifying and characterising putative targets for transmission blockade, and for performing transmission-blocking assays. Genomes of $P. berghei$ and human malaria parasites contain a core of conserved genes (Hall et al., 2005), reflecting conserved biological features (reviewed by Kooij et al., 2006) and the high level of sequence similarities
between *P. berghei* LAPs and orthologs in *P. falciparum* strongly implies that the proteins may perform analogous roles in each species. There are however notable differences between human and rodent malaria parasites, in particular at the level of gametocyte biology. Gametocytes of *P. falciparum* are unusual in morphology (crescent-shaped rather than round) and have a prolonged development (9-12 days compared to 26-30 hours). These discrepancies are associated with marked differences in biological processes such as sequestration and nutrient acquisition, and even conserved proteins may be observed to elicit different functions in the two species. Indeed differences in gene knockout phenotypes of highly conserved members of the 6-cys protein family are apparent. For example, *P. berghei* Pbs47 is essential for female fertility (Mair et al., 2006), whereas although *P. falciparum* Pfs47 is similarly expressed on the surface of female gametes, but does not appear crucial for fertilisation (van Schaijk et al., 2006).

Differences are apparent between the gene disruption of phenotypes of *Pblaps* and their orthologs in *P. falciparum*, *Pfccps*: ∆*Pblap1*, ∆*Pblap2*, ∆*Pblap4*, ∆*Pblap5* and ∆*Pblap6* are defective in sporulation, whilst ∆*Pfccp1*(*Pblap2*) ∆*Pfccp2*(*Pblap4*) and ∆*Pfccp3*(*Pblap1*) results in a defect in the transition from midgut to salivary gland sporozoites (Pradel et al., 2004) and interestingly ∆*Pfccp4*(*Pblap6*) shows no essential function (Scholtz et al., 2008). It is difficult to distinguish whether these discrepancies relate to true differences in the biological functions of the genes in each species, or whether distinct phenotypes are observed as a consequence of variation in experimental procedures. For example, *P. falciparum* is grown in culture and *P. berghei* in mice, and different mosquito species were utilised to examine phenotypes in the mosquito. In a similar manner, variable results due to species and experimental differences between *P. berghei* and *P. falciparum* transmission-blocking assays cannot be excluded. Nevertheless the technical advantages of studying *Plasmodium* transmission in *P. berghei* overwhelmingly justify the examination of transmission-blocking candidates using *P. berghei*. Furthermore, investigating LAPs in *P. berghei* has highlighted their nature as potentially multifunctional proteins with possible roles throughout sexual and sporogonic development of the parasite in the mosquito — revealing possible functions that have not yet been identified through studies in *P. falciparum*. 
5.5. Perspectives

Efforts to control and eliminate malaria have to date been largely limited to the application of drugs and insecticides, with only limited success in areas of high endemicity. Therefore, although understanding the molecular mechanisms underlying a pathogens development is not essential to control a disease, attempts to understand the molecular basis of Plasmodium infection have been initiated to improve the chances of successful long-term control, in particular by identifying new targets for intervention. The development and distribution of drugs, vaccines, or novel vector control interventions intended to interrupt the transmission of malaria are expected to be key to the success of future malaria control and elimination programmes. A transmission-blocking vaccine (TBV) is particularly appealing, especially given the recent advances in vaccine development against pre-erythrocytic stages of the parasite. Knowledge of the biology of malaria has been transformed by molecular biology and post-genomic technologies and continued research on parasite development at a molecular level is certain to yield further targets for intervention and reveal insights into the intriguing biology of the malaria parasite. Meanwhile, re-consideration of the feasibility of antimalarial vaccines based on whole parasites could offer an alternative approach to sub-unit vaccines.


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