The role of the 235 kDA protein multigene family (py235) of the malaria parasite *Plasmodium yoelii* in invasion and host cell selection, virulence and immune evasion

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**THE ROLE OF THE 235 KDA PROTEIN MULTIGENE
FAMILY (PY235) OF THE MALARIA PARASITE
PLASMODIUM YOELII IN
INVASION AND HOST CELL SELECTION,
VIRULENCE AND IMMUNE EVASION.**

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Look up to this day
For it is life, the very life of life
In its brief course
Lie all the verities and realities of your existence
The bliss of growth
The glory of action
The splendour of achievement
Are but experiences of time

For yesterday is but a dream
And tomorrow is only a vision
And today well lived, makes
Yesterday a dream of happiness
And every tomorrow a vision of hope
Look well therefore to this day
Such is the salutation to the ever new dawn.

-- Kalidasa (AD 300-600)
Translated from Sanskrit

RESEARCH SUMMARY

A key determinant of lethality in infections by the malaria parasite *Plasmodium* is the rapid multiplication cycles in the blood of an infected individual. The range of erythrocytes invaded is directly correlated to parasite virulence. The 235kDa protein multigene family (*py235*) has been previously implicated in mediating host cell selection and invasion in the rodent parasite *Plasmodium yoelii*. Using *P. yoelii*, we defined the copy number of *py235* genes in parasites exhibiting different virulence. Quantitative expression of *py235* showed variations in the amount of *py235* expression, a mechanism the parasite utilizes to define its host cell repertoire, plays a direct role in adaptation of the parasite to a different host species and in virulence. In addition, we show that the parasite either downregulates its *py235* expression or switches to utilize a different *py235* profile to evade specific immunity. From this study, we have broadened our understanding of the diversity of the *py235* genes and how the parasite uses this gene family to mediate alternate invasion pathways, virulence and immune evasion.

1. INTRODUCTION

1.1 Malaria

1.1.1 Epidemiology

Malaria is the most important parasitic disease in the world. Today approximately 40% of the world's population, mostly those living in the world's poorest countries, is at risk of malaria. It is caused by the Apicomplexan parasite *Plasmodium*. There are four main malaria parasite species affecting humans—*Plasmodium vivax*, *P. malariae*, *P. ovale* and *P. falciparum*. *Plasmodium vivax* and *P. falciparum* are the most prevalent among the four.

The disease malaria was once more widespread but it was successfully eliminated by international interventions such as the elimination of mosquito vectors and antimalarial prophylaxis from many countries with temperate climates during the mid 20th century. Today malaria is found throughout the tropical and sub-tropical regions of the world. Recent estimates put numbers at 515 (range 300–660) million episodes of clinical *P. falciparum* malaria in 2002 (Snow et al., 2005). It is estimated to cause one million deaths annually, of which 75% occur in children under the age of 5. Up to 90% of all annual clinical cases of malaria occur in Africa alone. These estimates are up to 50% higher than those reported by the WHO and 200% higher for areas outside Africa, indicating that the disease

prevalence is actually higher, especially in Southeast Asia and South America. The indications of the spread of *P. falciparum* malaria into new regions of the world and its reappearance in areas where it had been eliminated are worrying concerns. The economic burden of malaria is immense, with a growth penalty of 1.3% per year in some African countries (World Health Organization (WHO) statistics) (Snow et al., 2005). It has been estimated to cost US\$ 12 billion GDP lost in primarily sub-Saharan Africa per annum (WHO). There are thus significant differences in GDP rates between countries with and without malaria.

1.1.2 The disease

Typically, malaria is an acute febrile illness that manifests as periodic febrile paroxysms occurring every 48 or 72 hours with afebrile asymptomatic intervals (Gilles, 1996). The severity of the clinical episodes depends on the species of *Plasmodium* that causes the infection and the preexisting immunity of the patient (which depends on the history of infection of the patient). If drugs are not available for treatment or if the parasites are resistant to them, the infection can progress rapidly to become life-threatening. Malaria pathology is predominantly caused by the infection and destruction of red blood cells (anaemia). *Plasmodium falciparum* malaria can also result in complications such as cerebral malaria, hepatic and renal dysfunction, hemoglobinuria, electrolyte instability, acidosis, hypoglycemia, pulmonary oedema, circulatory collapse and intravascular coagulation. Perinatal mortality, low birth weight and maternal anemia are also a major concern in infected pregnant women and their unborn children (Gilles,

1996). Long term effects of the disease in the young include brain damage after suffering from severe malaria or learning impairments.

1.1.3 Intervention and control

The existing control methods for malaria include combination chemotherapy, spraying of insecticides and the use of insecticide treated bed nets. Insecticide resistance of the mosquito vector and drug resistance of the parasite have been the primary causes of the spread of this disease. New drugs and an efficient vaccine are a dire requirement in affected countries. The successful development of a malaria vaccine that can lead to long term sustainable reduction in the global burden of malaria is limited by our understanding of the complex biology of the causative parasite, *Plasmodium*. Understanding host-parasite interactions at molecular levels and utilizing the successful sequencing of the genomes of a number of different related *Plasmodium* species that infect various hosts other than humans (Carlton et al., 2002; Gardner et al., 2002) has now made it possible to examine the biology of the parasite using this information.

1.2 The parasite

1.2.1 Life cycle of *Plasmodium*

Plasmodium parasites are obligate intracellular organisms. The life cycle of a typical parasite (Fig 1-1) involves various complex stages within different host cells. Both an arthropod vector and a vertebrate host are vital for this cycle. The initial bite during a blood meal of an infected female *Anopheles* mosquito releases up to 100 asexual sporozoites into the bloodstream of the host (Fig 1-1A), which quickly migrate to the liver and transmigrate through resident dendritic cells and a number of hepatocytes and then successfully invade a hepatocyte (Carrolo et al., 2003; Mota, Hafalla, and Rodriguez, 2002; Mota et al., 2001; Mota and Rodriguez, 2001). In the hepatocyte, the asexual parasite undergoes multiple rounds of mitotic division and forms 10000 to 30000 liver merozoites. Upon maturation, the infected hepatocyte ruptures, releasing these merozoites into the bloodstream. Liver merozoites quickly invade erythrocytes in the blood. In the asexual blood stages (Fig 1-1B), *Plasmodium* undergoes a series of distinct morphological stages. The first stage after invasion is called the ring stage. As this parasite develops, it eventually becomes what is called a trophozoite where DNA replication begins. The parasite undergoes mitotic division and forms the third morphological stage, a schizont which contains 8 to 32 invasive merozoites. The number of merozoites each schizont contains is species dependant. These merozoites, upon release from a mature schizont, can invade other uninfected erythrocytes, continuing the asexual erythrocytic cycle. The release of the merozoites from the schizonts coincides with the characteristics fever cycles in the infected host. Some of the merozoites after

invasion develop due to an as yet unknown trigger to form sexual stage male and female gametocytes. These are taken up by a mosquito during the next blood meal. Once in the mosquito (Fig 1-1C), the male and female gametes emerge from their host cells. The male gamete exflagellates then fertilizes the female gamete and form the diploid zygote. The zygote develops to the motile diploid ookinete which traverse the midgut epithelium and develops as an oocyst with thousands of asexual sporozoites (Meis et al., 1992). Mature sporozoites upon rupture from the oocyst migrate into the salivary glands of the mosquito (Golenda, Starkweather, and Wirtz, 1990; Vanderberg, 1974; Vaughan, Noden, and Beier, 1992) and are released in the next blood meal of the next available vertebrate host.

Figure 1-1. Life Cycle of *Plasmodium*

The life cycle of *Plasmodium* involves a mosquito vector to transmit parasites to an animal host. In the animal host there is a hepatic stage asexual cycle (A) and multiple rounds of asexual cycles in the erythrocytes (B). Differentiated sexual stage parasites are picked up by the mosquito in a blood meal and undergo fertilization, development and mitosis to produce asexual sporozoites in the mosquito (C). (Adapted from www.uni-tuebingen.de)

1.2.2 Invasive forms of Plasmodium spp.

During the different life cycle stages of *Plasmodium* the parasite needs to invade a range of different host cells. The ookinete needs to penetrate the mosquito midgut endothelium (Fig 1-1C); the sporozoite is required to invade both the mosquito salivary gland (Fig 1-1C) as well as the hepatic cells of the vertebrate host (Fig 1-1A); the merozoite invades erythrocytes (Fig 1-1B). Since it is at the erythrocytic stage of the parasite's life cycle that causes the disease symptoms typically associated with malaria, this stage will be most important to this study. The invasive forms of the malaria parasites are like all members of the phylum Apicomplexa (Levine, 1971) characterized by a polarized morphology, and a unique set of organelles: the rhoptries, micronemes and dense granules that play a crucial role in the invasion process (Fig 1-2). The exception is the ookinetes of *Plasmodium* that do not have rhoptries. The actual reason for this is unknown, but may be due to the fact that the ookinete is the only stage of the parasite which does not form a parasitophorous vacuole membrane (PVM) after invasion. The contents of the apical organelles give the invasive parasite the ability to recognize an appropriate host cell, invade such a cell effectively and develop successfully within it.

Figure 1-2. Apical Organelles in *Plasmodium*.

Organelles playing a role in invasion in an apicomplexan parasite (Bannister and Dluzewski, 1990) are the micronemes, the rhoptries and the dense granules.

1.3 Variations in range and efficiency of cells invaded among *Plasmodium* species

Among the different *Plasmodium* species, the range and efficiency of erythrocytes invaded by the merozoite varies (Table 1-1) (Garnham, 1966). This phenomenon of parasites having different preferences for erythrocytes of different stages of maturity suggests that different recognition signals may be required for invasion of the host cell. Among the human malaria causing parasites, *P. vivax* invades reticulocytes while *P. falciparum* invades all ages of erythrocytes. Parasite numbers seen in *P. vivax* infections are generally low compared to *P. falciparum* infections possibly due to the fact that reticulocytes make up only 1% of the total number of host erythrocytes. This leads to a distinct difference in pathology between these two parasites, with *P. falciparum* generally leading to a more severe potentially life-threatening disease while *P. vivax* gives a less severe and rarely fatal clinical picture.

In *Plasmodium yoelii*, which infects rodents, a similar pattern exists, where the typically avirulent 17X YA line (hereafter called YA) (Jarra and Brown, 1989) preferentially invades reticulocytes and causes a mild self limiting infection, while the virulent 17X YM line (hereafter called YM) (Yoeli et al., 1975) invades erythrocytes of all ages and causes a fatal infection in experimental BALB/c mice. It is clear (from Table 1-1) that there is a direct link between the range of erythrocytes invaded and resultant pathology of the disease. The molecular mechanism involved in erythrocyte invasion thus is important in understanding host cell specificity of the parasite and subsequent virulence.

Species	Host cell type	Infection pathology
<i>P. vivax</i>	Reticulocytes	Mild in humans
<i>P. malariae</i>	Mature erythrocytes	Mild in humans
<i>P. falciparum</i>	All ages	Severe * in humans
<i>P. ovale</i>	Reticulocytes	Mild in humans
<i>P. berghei</i>	Reticulocytes	Severe * in mice
<i>P.y. yoelii</i> 17X	Reticulocytes	Mild in mice
<i>P.y. yoelii</i> 17X YM	All ages	Severe in mice
<i>P. vinckei vinckei</i>	Mature erythrocytes	Severe in mice
<i>P. chabaudi</i>	Reticulocytes and mature erythrocytes	Severe * in mice
<i>P. knowlesi</i>	Reticulocytes	Mild in simians
<i>P. cynomolgi</i>	Reticulocytes	Mild in simians

Table 1-1. Host cell preference and infection pathology between *Plasmodium* species.

This table indicates that a link may exist between host cell preference and severity or lethality of infections. Asterisks indicate exceptions. In the case of *P. falciparum*, mild infections and chronic persistence of parasitemia exist in asymptomatic patients. The severity of *P. berghei* infections is complicated at low parasitemias by cerebral complications. In *P. chabaudi*, a severe pathology is seen, but the host animal is often able to clear parasites and survive an infection. The infections in natural hosts can vary in outcome to milder infections.

1.4 Invasion of erythrocytes

1.4.1 Cellular events in invasion

Within a mammalian host, the parasite has to overcome two major hurdles in the invasion process. First it has to migrate quickly to its host cell in order to evade immune attack, and second, it has to recognize the hepatocyte or erythrocyte where it can develop and multiply. This is the only time when the invasive parasite is exposed directly to the immune system. To the parasite, this means that it has to effectively invade an appropriate cell rapidly and effectively before immune attack. From a clinical point of view, the rate of successful invasion of erythrocytes by *P. falciparum* merozoites is a major factor in the pathogenesis of malaria, since it has a direct bearing on asexual blood stage densities and dynamics. The process of *P. falciparum* erythrocytic invasion is easily amenable to laboratory studies as compared to the invasion of hepatocytes or barriers in the mosquito. A consequence of this is that most of the data on host cell invasion by *Plasmodium* are obtained for processes during erythrocyte invasion.

Invasion of host cells by *Plasmodium* is a complex process involving many ligand receptor interactions, and multiple checkpoints exist before the parasite commits to invasion of the selected cell (Barnwell, 1999; Cowman and Crabb, 2006; Dvorak et al., 1975; Gaur, Mayer, and Miller, 2004; Preiser et al., 2000b). The main steps in the erythrocyte invasion process shown below (Fig 1-3) are: (1) initial merozoite binding, reorientation and deformation of erythrocyte, (2) formation of a junction (the irreversible commitment of the parasite to invasion) and (3) parasite entry. The entire process takes only about one minute, and implies

an efficient invasion process to ensure minimal risk of elimination by immune attack.

Figure 1-3. Steps in erythrocyte invasion

The malaria parasites *Plasmodium* undergoes three main steps in erythrocyte invasion: initial merozoite binding, reorientation and deformation of erythrocyte, formation of a junction (the irreversible commitment of the parasite to invasion) and parasite entry (Chitnis and Blackman, 2000)

1.4.2 The molecular basis of invasion

There are many ligands and receptors that are important in erythrocyte invasion. Table 1-2 summarizes a recent review of some of these proteins in *P. falciparum* that are important in merozoites. Due to space and relevance constraints, in this section, only the important proteins that mediate host cell recognition and selection will be described.

Location	Protein	Function and features
Surface proteins	MSP1	Band 3 ligand, associates with MSP6 and 7. Polymorphic
	MSP 2, 4, 5, 10	Potential species specific (Pf) functions, mostly unknown.
	ABRA	Putative protease
	S antigen	Function unknown
	GLURP	Function unknown
	MSP3	Weakly associated with surface MSP9
	MSP6	Weakly associated with MSP1
	MSP7	MSP1 binding protein. Involved in normocyte invasion in <i>P. berghei</i>
	SERAs (3,4,5,6)	Serine repeat antigens. Contain cysteine protease domains (mostly schizont expression)
Cap proteins	MCP-1	Merozoite Cap protein 1. Facilitates attachment or movement of junction along cytoskeletal network
Microneme proteins	AMA-1	Mediates the apical reorientation of merozoite during the invasion process.
	MTRAP	Motor associated protein, binds to aldolase, thrombospondin related
	ASP	Apical sushi protein. GPI anchored. Contains adhesive "sushi" domain.
	SUB2	Subtilisin like serine protease (MSP1 and AMA1 processing "shedase")
	EBLs	Binds receptors on erythrocytes
	Pfalciparum RH4 (RBPH)	Unknown function.
Rhoptry proteins	RAMA	Associates with rhoptry complexes
	Rap (1,2,3)	Low molecular weight complex. Only RAP1 involved in trafficking
	RhopH (1,2,3)	High molecular weight complex. Function with SERAs
	MAEBL	Unknown. Invasion of salivary gland and hepatocytes by sporozoites
	RBPHs	Binds erythrocytes
Dense granules proteins	RESAs	ring infected erythrocyte surface antigens. Associated with spectrin
	RIMAs	Ring membrane antigen

Table 1-2. Properties of important merozoite proteins in *Plasmodium*.

Proteins are grouped according to their localization. These are found in *P. falciparum*, and may be expressed in other *Plasmodium* species. (Adapted from Cowman et al 2006)

1.4.2.1 Early interactions with the erythrocyte surface

Early interactions are thought to be between surface proteins on the merozoite and unknown receptors on the erythrocyte surface. These mediate a low affinity, reversible interaction between parasite and erythrocyte. Molecules found on the surface include the merozoite surface proteins (MSPs) 1-10 (Black et al., 2003; Black et al., 2001; Drew, Sanders, and Crabb, 2005; Holder et al., 1985; Marshall et al., 1997; Marshall, Tieqiao, and Coppel, 1998; McColl et al., 1994; Mills et al., 2002; Oeuvray et al., 1994; Pachebat et al., 2001; Trucco et al., 2001; Weber et al., 1988), proteases of the serine-repeat antigen (SERA) family (Delplace et al., 1987; Knapp et al., 1989) and other glycosylphosphatidylinositol (GPI)-anchored proteins non-covalently associated in complexes. (Goel et al., 2003; O'Donnell et al., 2000; Sanders et al., 2005). The most abundant protein is MSP1, which has been shown to bind erythrocytes through human Band-3 receptor (Goel et al., 2003; Perkins and Holt, 1988). After binding to the erythrocyte, the merozoite reorients itself such that the apical end is in contact with the erythrocyte membrane to ensure closer interactions between ligands at the apical end of the merozoites and receptors on the erythrocyte (Dvorak et al., 1975).

Apical membrane antigen (AMA)-1 is an apical organelle protein essential early in invasion (Mital et al., 2005; Triglia et al., 2000). Although micronemal in localization, it is discussed here since it is involved in the early interactions of the merozoite to the host erythrocyte. It is a type I integral membrane protein with a 3' cysteine rich region, a tandem duplicated 5' cysteine-rich region and 16 cysteine

residues that confer a unique structural characteristic to the ectodomain (Cheng and Saul, 1994; Peterson et al., 1989). AMA-1 function appears in the apical reorientation during the invasion process (Mitchell et al., 2004). Antibodies against AMA-1 have been shown to block invasion (Mitchell et al., 2004), as an intermediate interaction that occurs between the initial weak interactions the MSPs have with the erythrocyte and the tight ones that the micronemal proteins make with the erythrocyte (Alexander et al., 2005; Lebrun et al., 2005).

1.4.2.2 Late interactions

Once reorientation has occurred micronemal and rhoptry proteins mediate specific interactions with receptors on the host erythrocyte that lead to tight junction formation. After commitment, invasion rapidly proceeds with the discharge of the contents of the micronemes, rhoptries and finally dense granules. To complete the process of invasion, merozoite serine proteases cleave erythrocyte membrane protein Band3 and cause a localized disruption in the cytoskeleton, thus allowing the parasite to enter the host cell (McPherson et al., 1993; Roggwiler et al., 1996). The junction moves from the apical pole to the posterior end of the merozoites as the parasite enters the erythrocyte through an actin-myosin motor (Keeley and Soldati, 2004). Merozoite cap protein 1 (MCP-1) is located at the attachment site formed between the apical region and erythrocyte (Klotz et al., 1989). It appears to participate in merozoite invasion by facilitating attachment or movement of the junction along the cytoskeletal network (Hudson-Taylor et al., 1995; Klotz et al., 1989).

A parasitophorous vacuole membrane (PVM) forms in the junction area, and the junction becomes a small annulus where the parasite moves through as it enters the expanding parasitophorous vacuole. Entry into erythrocytes involves a series of signaling events through β -adrenergic receptors via GTP binding proteins (Harrison et al., 2003). Upon completion of the parasite entry, the tight junction disappears and the parasitophorous vacuole membrane and erythrocyte membrane will fuse and separate, completing the entry process. The content of the dense

granules is thought to be discharged only after the parasite has completed its entry and is implicated in the modification of the host cell. This completes the invasion process and allows the parasite to reside and multiply in the erythrocyte (Cowman and Crabb, 2006; Sherman, 1999). The rhoptries and micronemes proteins that mediate the specific recognition interactions leading to junction formation are crucial to this study and will be discussed in detail.

1.5 Apical organelle proteins

Merozoites have an ability to discriminate between different types of erythrocytes due to specific ligand-receptor interactions. While a subset of proteins such as MSP1 and AMA1 seem to mediate the initial interaction between a merozoite and erythrocyte (Holder and Freeman, 1982; Peterson et al., 1989), it is during the subsequent steps immediately before junction formation when the specific host cell recognition observed in the different parasites occurs.

The apical organelles and the proteins in them relevant to this study will be discussed below (Fig 1-4). Apical proteins are highly conserved among the invasive forms of other Apicomplexan parasites such as *Toxoplasma*, suggesting an essential structural and functional conservation across the Phylum. Among the various *Plasmodium species*, there are also homologs of the rhoptry and microneme proteins expressed indicating conserved receptor recognition specificities.

Figure 1-4. Proteins in the apical complex and erythrocyte receptors involved in invasion.

1.5.1 Microneme proteins

Micronemes are small fusiform organelles that vary in shape and numbers in different species of *Plasmodium* (Dubremetz et al., 1998). They are situated in the apical end close to the rhoptry duct. Discharge of micronemal contents are associated with specific recognition and adhesion of the parasite to the host cell (Carruthers and Sibley, 1999).

1.5.1.1 The Erythrocyte Binding Like (EBL)/Duffy Binding Protein (DBP) family

At least one member of the EBL family of proteins have been shown to be expressed in all *Plasmodium* parasites analyzed to date, suggesting an important conserved function. This protein family has been implicated in the specific attachment and selection of a host cell in erythrocyte invasion.

All the EBLs are characterized by conserved exon-intron structures, splicing boundaries and contain two extracellular regions that have conserved cysteines and hydrophobic amino acid residues (Adams *et al.*, 1992) (Fig 1-5). This suggests a conserved three-dimensional structure of this domain, and a common evolutionary origin (Michon et al., 2002). Sequence analysis of the extracellular domain of the DBP (discovered first in *P. knowlesi*) can be divided into 6 regions (I to VI) based on amino acid homology to the *P. knowlesi*, *P. vivax* and *P.*

falciparum DBP (Adams *et al.*, 1990). Two cysteine rich domains (in regions II and VI) contain conserved cysteines and aromatic amino acid residues. Erythrocyte binding assays have shown that the Duffy binding ligand (DBL) domain within region II is involved in the binding to the Duffy antigen (Chitnis *et al.*, 1996; Chitnis and Miller, 1994; Ranjan and Chitnis, 1999).

The DBL domain

DBL domains have been identified in two groups of parasite ligands, the variant *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP-1) (Peterson, Miller, and Wellems, 1995; Su *et al.*, 1995) and the superfamily of Erythrocyte Binding-Like (EBL) proteins expressed in the invasive stages of many *Plasmodium* species (Adams *et al.*, 2001; Adams *et al.*, 1992). Members of the EBL family are found in the micronemes of the merozoite (and one paralogue, MAEBL, exists in rhoptries, which will be discussed in the next section on rhoptry proteins). (Table 1-3 and Fig 1-5).

The DBL domain is a conserved cysteine rich domain approximately 35kDa that has been shown to directly mediate binding to host cell receptors (Chitnis *et al.*, 1996; Haynes *et al.*, 1988; Miller *et al.*, 1976; Miller *et al.*, 1975; Wertheimer and Barnwell, 1989). Furthermore, recent work using site directed mutagenesis has given a more detailed map of the essential amino acids that mediate the specific interactions between this domain and its corresponding Duffy receptor (Vanbuskirk, Sevova, and Adams, 2004).

Figure 1-5. Schematic diagram of the EBLs.

Sequence comparisons between members showed high overall conservation denoted by DBL domains between the EBLs. Cysteine rich regions (C-Cys) and Transmembrane (TM) regions are shown.

Organism	Ligand	Receptor	Erythrocyte binding properties	Antibodies	References
<i>P. falciparum</i>	RH1	sialic acid residues trypsin resistant (Y)	shown	block binding	(Rayner <i>et al.</i> , 2001)
	RH2a	Unknown	not shown	block invasion	(Triglia <i>et al.</i> , 2001)
	RH2b	chymotrypsin sensitive, indep of other members (Z)	shown	block invasion	(Triglia <i>et al.</i> , 2001)
	RH3	Unknown	NA (psuedogene)	NA (pseudogene)	(Taylor <i>et al.</i> , 2001)
	RH4	Unknown	not shown	ND	(Kuneko <i>et al.</i> , 2002)
	RH5	Unknown	Not shown	ND	(Stubbs <i>et al.</i> 2005, Cowman and Crabb 2006)
	EBA175	Glycophorin A	sialic acid residues	block binding and invasion	(Jakobsen <i>et al.</i> , 1998; Sim <i>et al.</i> , 1994)
	EBA 181 (JESEBL)	Trypsin resistant chymotrypsin sensitive sialic acid dependant	not shown	ND	(Gilberger <i>et al.</i> , 2003b)
	EBA 165 (PEBL)	Unknown	NA (psuedogene)	NA (pseudogene)	(Triglia <i>et al.</i> , 2001)
	EBA 140(BAEBL)	Glycophorin C	Gerbich positive erythrocytes	block binding, inhibit fig-rec int	(Maier <i>et al.</i> , 2003; Narum <i>et al.</i> , 2002)
<i>P. vivax</i>	EBA1	Unknown	not shown	ND	(Peterson and Welles, 2000)
	RBP1	Unknown	shown	ND	(Galinski <i>et al.</i> , 1992)
	RBP2	Unknown	shown	ND	(Galinski <i>et al.</i> , 1992)
<i>P. yoelii</i>	DBP-1	Duffy antigen	shown	block invasion	(Miller <i>et al.</i> , 1979; Wertheimer and Barnwell, 1989)
	Py235	Unknown	not shown	block invasion	(Freeman <i>et al.</i> , 1980; Holder and Freeman, 1981)
	EBL	Unknown	not shown	ND	(Prasad <i>et al.</i> , 2003)
<i>P. knowlesi</i>	DBP α	Duffy antigen	sialic acid independent	block invasion of human, an partial rhesus	(Adams <i>et al.</i> , 1990; Singh <i>et al.</i> , 2002)
	DBP β	Unknown	not shown	ND	(Adams <i>et al.</i> , 1990)
	DBP γ	Unknown	not shown	ND	(Adams <i>et al.</i> , 1990)
<i>P. reichenowi</i>	RH (5 members)	Unknown	Not shown	ND	(Rayner <i>et al.</i> 2004)
	EBL (EBA 175 and EBA 140)	Unknown	Not shown	ND	(Ozwaru <i>et al.</i> 2001, Baum <i>et al.</i> 2003)

Table 1-3. Features of the EBL and RBPH family members

This is a current review of the known details of each EBL and RBPH. Each organism has at least one member of the EBL and RBPH family. The corresponding receptor if known is stated, along with the erythrocyte binding properties and the effect of antibodies. ND indicates not done.

1.5.1.1.1 *P. vivax* Duffy binding protein.

P. vivax only invades human reticulocytes that are Duffy positive (Kitchen, 1938; Mons, 1990). The unique restriction of *P. vivax* to Duffy positive erythrocyte is mediated by a single copy gene that codes for the 140kDa DBP (Fang *et al.*, 1991). This parasite-encoded ligand specifically binds the Duffy blood group antigen on human erythrocytes (Haynes *et al.*, 1988). Subsequent work has shown that DBP specifically interacts with a 35 amino acid long region of the Duffy receptor (Chitnis *et al.*, 1996) and that this interaction is independent of sialic acid. Competition experiments with purified Duffy glycoprotein or pre-treatment of erythrocytes with a monoclonal antibody against a Duffy determinant (Wertheimer and Barnwell, 1989) indicated that DBP plays a role after merozoite reorientation during the invasion process (Miller *et al.*, 1979). High levels of antibodies against DBP are detected in patients exposed to repeated doses of *P. vivax* (Fraser *et al.*, 1997), and there is some evidence that there is some protection mediated by these antibodies. In *P. vivax* field isolates only a limited sequence variability in the DBL domain of DBP has been observed (Tsuboi *et al.*, 1994). None of the observed changes lead to alternate receptor specificity, but rather play an important role in immune evasion (Vanbuskirk *et al.*, 2004). Structural motifs in this region have also been elucidated for receptor binding (Singh *et al.*, 2003).

1.5.1.1.2 *P. knowlesi* DBPs

P. knowlesi can invade both human and rhesus erythrocytes. *P. knowlesi* invasion of human erythrocytes requires the Duffy antigen (Mason et al., 1977; Miller, 1975; Wertheimer and Barnwell, 1989), but its invasion of rhesus erythrocytes is Duffy independent, demonstrating the ability of the parasite to utilise other simian specific erythrocyte receptors for invasion. Erythrocyte binding assays identified 135kDa Duffy Binding Proteins (PkDBPs) of *P. knowlesi* (Miller et al., 1979). Antibodies against PkDBP were cross reactive with the PvDBP (Haynes et al., 1988). Unlike in the case of *P. vivax* it soon became clear that there were multiple members of DBPs in *P. knowlesi*. At least three PkDBPs, termed α , β and γ were discovered (Adams et al., 1990; Adams et al., 1992) all sharing conserved DBL domains with 70% homology to that of *P. vivax*. Only the DBL domain of PkDBP α has been shown to bind to the Duffy receptor while the DBLs of the other two proteins are thought to bind to so far uncharacterised receptors specific to the simian erythrocytes (Chitnis and Miller, 1994). Antibodies raised against region II of PkDBP α , effectively inhibit invasion of human erythrocytes as well as partially inhibit invasion of rhesus erythrocytes by *P. knowlesi* merozoites (Singh, Puri, and Chitnis, 2002). Recently, a knockout of PkDBP α has shown that this protein is essential for junction formation in human erythrocyte invasion, but is non essential in invasion of rhesus erythrocyte (Singh et al., 2005), indicating that *P. knowlesi* may use alternative receptors on rhesus erythrocytes to form a junction and invade by Duffy independent pathways. The expansion of DBPs in *P. knowlesi*

indicates that parasite may utilise additional receptors for invasion of multiple host species.

1.5.1.1.3 *The P. cynomolgi EBLs*

The simian parasite *P. cynomolgi* is thought to be phylogenetically more closely related to *P. vivax* than to *P. knowlesi* (Escalante and Ayala, 1994; Waters, Higgins, and McCutchan, 1991) and this is supported by the fact that the one EBP gene so far sequenced has the highest homology to the DBP of *P. vivax* (Okenu *et al.*, 1997; Ozwara *et al.*, 2001). On the other hand Southern blot analysis has shown that unlike *P. vivax*, *P. cynomolgi* has at least two genes encoding EBP (Okenu *et al.*, 1997; Ozwara *et al.*, 2001). This would suggest that *P. cynomolgi* may also exhibit alternate invasion pathways, as do *P. knowlesi* and *P. falciparum* (see below).

1.5.1.1.4 *P. falciparum EBLs*

Unlike *P. vivax* *P. falciparum* can invade Duffy negative and positive erythrocytes equally well and is known to utilise a number of receptors on the erythrocyte surface (Miller, McAuliffe, and Mason, 1977); (Dolan, Miller, and Wellems, 1990; Dolan *et al.*, 1994; Mitchell *et al.*, 1986; Perkins and Holt, 1988). It has been shown that the ability of *P. falciparum* to utilise a wider range of erythrocyte receptors is at least in part due to the expansion of the number of EBL genes.

The members

Even before the completion of the *P. falciparum* genome-sequencing project multiple distinct EBL members had been identified. Currently, there are five micronemal members called EBA175 (Camus and Hadley, 1985), BAEBL (EBA140) (Lobo et al., 2003; Maier et al., 2003; Mayer et al., 2002), JESEBL (EBA181) (Michon et al., 2002); PEBL (EBA165) (Triglia *et al.*, 2001); EBL1 (Peterson and Wellems, 2000), and one paralog MAEBL (discussed in the next section on rhoptry proteins). MAEBL does not contain a DBL domain indicating that MAEBL is not involved in DBL mediated erythrocyte binding. EBA165 appears as an untranslated pseudogene as it contains a number of frame shift mutations in one strain of parasites and no evidence for a protein product has been obtained to date (Triglia *et al.*, 2001). The *P. falciparum* EBLs are all located in the subtelomeric regions of different chromosomes (Gardner *et al.*, 2002) regions that contain many genes associated with parasite virulence and disease associated pathology (Craig and Scherf, 2001; Freitas-Junior et al., 2000; Hernandez-Rivas et al., 1997; Rubio, Thompson, and Cowman, 1996).

Conserved domain structure

All *P. falciparum* EBLs have duplicated DBL domains (Termed F1 and F2), unlike the single DBL domain in PvDBP (Sim et al., 1994) (Fig 1-5). This should indicate that both domains are capable of equal binding activity. It is interesting however that so far only the F2 region has been shown to have any binding activity, though F1 region augments the binding strength when expressed

together with F2 (Ockenhouse et al., 2001; Sim et al., 1994). Experiments involving mutation analysis and cocrystallization of the DBL domain with a sialated glycan had identified the receptor binding domain of this region (Tolia et al., 2005). The F1 and F2 helical domains form dimers that establish interdomain channels, and shown to constitute a binding cleft, which possibly mediates different interactions from the DBPs of other parasites.

Receptor binding specificities

Like for the case of the PvDBP receptor binding specificities have been shown for four EBLs of *P. falciparum*. Using a range of enzyme treatments of erythrocytes it was clearly demonstrated that EBA175 binding to erythrocytes was dependent on the sialic acid components of Glycophorin A (Camus and Hadley, 1985), indicating that Glycophorin A is a receptor for this protein. This was ultimately confirmed by the direct disruption of EBA175 in the parasite using gene knock outs. In this case the parasite was unable to utilise Glycophorin A as a receptor during the invasion process (Duraisingh et al., 2003a; Reed et al., 2000). Using similar approaches EBA140 was shown to bind to Glycophorin C (Lobo et al., 2003; Maier et al., 2003; Mayer et al., 2002) and EBA181 binds to an uncharacterized erythrocytic sialoglycoprotein (Gilberger et al., 2003b). In these knockout experiments, the parasites were still able to invade erythrocytes efficiently (Gilberger et al., 2003a; Kaneko et al., 2000; Maier et al., 2003; Reed et al., 2000). The presence of sialic acid dependency of EBA175, EBA181 and EBA140 indicate a related binding pocket that may be useful in intervention

studies (Adams et al., 2001). Additional evidence for the direct role of EBLs in erythrocyte binding came from the observation that antibodies against EBA175 and EBA 140 can inhibit erythrocyte binding and invasion (Jakobsen *et al.*, 1998; Narum *et al.*, 2002; Sim, 1998).

In PvDBP, sequence variation of the DBL domain observed in field isolates lead to different antigenic variants instead of leading to a change in the receptor specificity (Vanbuskirk et al., 2004). In the EBLs of *P. falciparum*, a different reason for sequence variation possibly exists. It has been suggested that polymorphism in EBA140 may have arisen in response to mutations in the human erythrocyte receptor (Mayer et al., 2002) (Maier et al., 2003). Polymorphisms in EBA140 and EBA181 allow for variations in binding characteristics to different erythrocyte receptors (Mayer et al., 2002; Mayer et al., 2004). Indeed it has been shown that a single amino acid change in the binding domain of EBA140 is sufficient to change the receptor recognition (Mayer et al., 2002).

The expression of multiple EBLs is also seen when culture conditions are manipulated. The treatment of erythrocytes with enzymes like neuraminidase (to remove sialic acid) or trypsin (to remove other protein components) does not completely ablate invasion (Camus and Hadley, 1985); (Dolan, Miller, and Wellems, 1990; Dolan et al., 1994; Gaur et al., 2003; Rayner et al., 2001). While overall parasite invasion on initial exposure to these treated erythrocytes is greatly reduced, over a period of time a few parasite strains are able to adapt and are able to invade these erythrocytes with similar efficiency. Interestingly, if culturing in the presence of enzyme treated erythrocytes is subsequently removed the parasite

slowly reverts back to the Glycophorin A dependent pathway. There is a lot of evidence that many cultured parasites mostly use the EBA175/GlyA interaction, with a minority of cultured parasites exhibiting alternate ligand-receptor combinations (Duraisingh et al., 2003a; Duraisingh et al., 2003b; Gilberger et al., 2003a; Maier et al., 2003; Stubbs et al., 2005; Triglia et al., 2005). These observations using laboratory cultured parasites somewhat contrasts with some of the observations made recently using parasites directly obtained from infected individuals. Field isolates from India, and Brazil show that mostly alternate ligand receptor combinations are used for invasion while similar studies performed in the Gambia and Tanzania indicate the prevalence of the conventional EBA175/Glycophorin A pathway utilized by these parasites (Baum, Thomas, and Conway, 2003; Jakobsen et al., 1998; Lobo et al., 2004; Okoyeh, Pillai, and Chitnis, 1999).

Other functional role of EBL

It is important to note that the invasion machinery involves more than the EBLs binding to an appropriate receptor. The first evidence of an additional role for these large EBL proteins was shown when it was observed by mutating EBA175. While the disruption of the 3'-cysteine rich region or the transmembrane region had no measurable effect on either the level of EBA175 protein expression or its subcellular localization (Reed et al., 2000). The role of the cytoplasmic domain of EBA175 was shown when the cytoplasmic domain of another parasite protein, thrombospondin-related anonymous protein (TRAP), expressed in invasive

sporozoites replaced the downstream functions associated with invasion (Gilberger et al., 2003a). The functional homology of EBA175 and TRAP cytoplasmic tails suggests that at different invasive stages, similar ligand-receptor interactions link these proteins to the invasion machinery.

1.5.1.1.5 *P. reichenowi* EBLs

Even though *P. reichenowi* is considered in evolutionary terms most closely related to *P. falciparum* it can not invade human erythrocytes. This is despite the fact that ortholog of EBA175 showing 80% predicted amino acid identity to the *P. falciparum* gene has been identified in *P. reichenowi* (Ozwara et al., 2001). In addition, a homologue of EBA140 has been identified using specific primers to the *P. falciparum* gene in PCR amplification of *P. reichenowi* DNA. The gene fragment isolated has a 92% deduced amino acid identity to *P. falciparum* EBA140 with a duplicated DBL domain (Baum, Thomas, and Conway, 2003). The high sequence identity of the *P. reichenowi* EBLs to its closely related *P. falciparum* counterparts would suggest that they should be able to bind to human erythrocytes, experimental data indicates that this is not the case. A possible explanation for this comes from modeling the crystal structure of PfEBA175 with the unique residues in the *P. reichenowi* homologue reveals differences in glycan binding sites that could explain the distinct host cells specificity of these parasites (Chattopadhyay et al., 2006) (and Julian Rayner personal communication), indicating that the sugar moieties on the receptor account for the different invasion properties between these parasites.

1.5.1.1.6 *The P. yoelii, P. berghei, P. vinckei and P. chabaudi homologs of EBLs*

A single copy gene containing a DBL domain was identified based on its shared characteristics with PvDBP, PkDBPs and EBA175 in *P. yoelii* (Carlton et al., 2002; Prasad et al., 2003). This EBL has been shown to bind erythrocytes through its DBL domain (Prasad *et al.*, 2003), but not much else has been elucidated about this gene.

1.5.1.2 Other microneme proteins

Subtilisin like proteases (such as PfSUB1 and 2) (Barale et al., 1999; Blackman et al., 1998; Hackett et al., 1999; Harris et al., 2005), have been found in micronemes and dense granules. PfSUB1 is a serine protease thought to be involved in the removal of the coat on merozoites (Bannister et al., 1986), and processing of MSP-1 and AMA-1 early in invasion and during tight junction formation. Studies in *P. berghei* show that PbSUB accumulation correlates with more efficient maturation of MSP-1 and AMA1 and a higher multiplication rate of *P. berghei* in vitro (JC Barale personal communication), implicating this protein as a major virulence factor. .

1.5.2 Rhoptry proteins

The rhoptries are club shaped membrane bound vesicles that lie as pairs at the apical pole of the parasite. They are organized into 2 structures comprising each of a bulb and a thin necked duct. The rhoptries discharge their protein during adhesion of the merozoite to the host cell surface. The tips of the ducts fuse with

one another along with the merozoite membrane (Aikawa et al., 1978), and the contents are also responsible for the formation of the parasitophorous vacuole membrane (PVM) during invasion. This could explain why ookinetes do not have a rhoptry, as no PVM is seen after invasion of the midgut wall by ookinetes.

1.5.2.1 The reticulocyte binding protein homologs (RBPHs)

The Reticulocyte Binding Protein homologues (RBPHs) is a family of proteins that are found in all parasite species tested to date (Freeman, Trejdosiewicz, and Cross, 1980; Holder and Freeman, 1981) (Galinski et al., 1992), (Kaneko et al., 2002; Rayner et al., 2000; Rayner et al., 2001; Taylor et al., 2001a; Triglia et al., 2001; Tsuboi et al., 1994) (Rayner et al., 2004) (Table 1-3). Some of these proteins have been shown to directly bind erythrocytes while others bind indirectly to erythrocytes. Unlike the DBPs, no binding region has been identified as yet for these proteins. The characteristic of this family of proteins is that all members of the RBPH family (except *P. falciparum* PfRH4) share an approximately 500 amino acid high homology region initially identified when comparing the *P. yoelii* PY235 and the *P. vivax* PvRBP (Keen et al., 1994) (Fig 1-6) Overall sequence homology between the different members is low but they contain a number of additional conserved blocks of amino acids which clearly identify them as members of this gene family (Fig 1-6). The expression pattern and apical location of all members is consistent with a role of these proteins during the invasion process.

Figure 1-6. Schematic diagram of the RBPHs.

Orange region: 500 amino acid homology between members. SS: signal sequence. TM: transmembrane region. A cysteine rich region is also indicated.

1.5.2.1.1 The RBPHs in *Plasmodium yoelii* (PY235) and other rodent malaria parasites

Role in invasion and virulence

The 235 kDa rhoptry protein (PY235) of *P. yoelii* was first identified using monoclonal antibodies prepared from mice resistant to the virulent YM strain of *P. yoelii* (Freeman, Trejdosiewicz, and Cross, 1980) (Holder and Freeman, 1984b). Two of these antibodies were able to confer protection in passive transfer experiments by modulating the infection restricting the YM parasites to predominately reticulocytes, thereby ensuring the survival of the host mouse strain (Freeman, Trejdosiewicz, and Cross, 1980) (Fig 1-7). One of these antibodies has been shown to recognize at least a single member of PY235 protein family (Ogun et al., 2006).

Figure 1-7. The effect of antibodies on parasite challenge

Typical YM infections involve the rapid multiplication of parasites. Passive transferred anti PY235 monoclonal antibody protects against the lethal effect of YM infections, causing reduced parasitemia rates and clearing of the parasite from peripheral blood. Immunization with PY235 yields a better protection against YM, and these parasites are more reticulocyte restricted.

Immunofluorescent microscopy and subsequently immuno-electron microscopy showed that these antibodies recognized a protein located in the rhoptries of the *P. yoelii* merozoites (Freeman, Trejdosiewicz, and Cross, 1980; Oka et al., 1984). Vaccination of BALB/c with the immuno-purified 235 kDa protein also protected against a subsequent challenge with the *P. yoelii* YM, converting these fulminating infections to a reticulocyte restricted and self limiting

infection where peak parasitemias reached lower levels than the passive transfer experiments (Holder and Freeman, 1981) (Figure 1-7). This suggested that the antibody to this protein protected against challenge infection by preventing the entry of parasites into mature erythrocytes without affecting reticulocyte invasion. Further evidence for the involvement of PY235 in red cell recognition came from the demonstration that the protein can directly bind erythrocytes. The potential receptor is neuraminidase resistant, chymotrypsin and trypsin sensitive (Ogun and Holder, 1996; Ogun *et al.*, 2000).

The *py235* genes

Screening of an expression library using antibodies specific to *PY235* led to the identification of the gene coding for this protein (Keen *et al.*, 1990). Once the gene had been identified it became rapidly apparent that multiple different copies of *py235* were present in the genome of *P. yoelii*. The copy number was estimated to be between 20-50 based on PCR analysis and hybridization experiments (Borre *et al.*, 1995; Khan *et al.*, 2001) somewhat higher than the 12-14 copies identified by the incomplete *P. yoelii* strain 17X 1.1 genome sequencing project (Carlton *et al.*, 2002). The sequence comparison of *py235* members showed high overall conservation with regions of variability interspersed throughout the coding region (Khan *et al.*, 2001; Narum *et al.*, 2001a). Chromosome mapping has shown that all *py235* genes are located in the subtelomeric region of some chromosomes (Owen *et al.*, 1999). In both YM and YA parasites, the *py235* genes were located in the subtelomeric regions of chromosomes 1, 5, 6, and 10. An additional locus on

chromosome 13/14 was found in YA. Immunofluorescence reactivity of anti PY235 antibodies to other malaria parasites indicated that the protein may be conserved in other rodent *Plasmodium* species (Holder and Freeman, 1984b). This was subsequently confirmed by sequencing the *P. berghei* and *P. chabaudi* genomes. A similar copy number is found in *P. berghei* while there seem to be fewer members of this gene family in *P. chabaudi* (www.sanger.ac.uk).

Py235 gene expression

Since antibodies against PY235 alter a normally virulent infection to an avirulent one, one fundamental question that arises is whether there are any differences in the expression of *py235* between a typically virulent and an avirulent parasite. Some differences in the *py235* repertoires and transcription profile have been observed between the YM and YA lines (Khan et al., 2001; Preiser and Jarra, 1998), and the subject of this thesis is in large part to address this question. From the study of the transcriptional profile of *py235* in single parasites, we have further insights on the significant role *py235* plays in parasite biology. A new form of clonal phenotypic variation had been described in which a single schizont produces merozoites that transcribe distinct members of *py235*. This has been postulated to enable the parasite to adapt to variations in the host cell environment and evade host immunity (Snounou, Jarra, and Preiser, 2000) (Gruner et al., 2004; Preiser et al., 1999b).

Py235 in other stages

Interestingly, the role of *py235* is not just limited to erythrocyte invasion as analysis of *py235* transcripts of different life cycle stages of *P. yoelii* indicated that distinct members of *py235* are expressed in the sporozoite, hepatic schizont and erythrocytic schizont development stages (Preiser et al., 2002). The role of RBPHs and the EBLs may be important in all invasive stages of the parasites as expression of EBA175 has been observed in sporozoite stages of *P. falciparum* (Gruner et al., 2001). Immunofluorescence analysis using antibodies recognizing PY235 proteins confirmed the differences between pre-erythrocytic and erythrocytic PY235 proteins. Antibodies against sporozoites stage PY235 were also found to specifically inhibit hepatocyte invasion. It is clear from this work that parasite proteins once thought to solely have a role in merozoite invasion now have to be regarded in a different light as they may have a broader role in the different invasive stages of the parasite.

1.5.2.1.2 *Plasmodium vivax* RBPs

P. vivax is restricted to invading reticulocytes (Kitchen, 1938; Mons, 1990). The recognition of the Duffy antigen by this parasite is mediated by the Duffy Binding Protein, and the recognition of reticulocytes is mediated by another set of proteins called Reticulocyte Binding proteins (RBP) 1 and 2. (Galinski *et al.*, 1992). The PvRBP proteins have been localized to the apical end of the merozoite but it is not yet clear whether they are located in either the micronemes or rhoptries. PvRBP1 and 2 have been postulated to form a complex that mediates adhesion and recognition of the reticulocyte (Galinski *et al.*, 1992). Sequence comparison with the *py235* genes of *P. yoelii* showed that *PvRBP-1* and *PvRBP-2* shared weak but significant sequence identity as well as structural features, including net charge and hydrophobicity (Galinski, Xu, and Barnwell, 2000). The difference in copy number of the RBPHs between *P. yoelii* and *P. vivax* is intriguing. If confirmed, this could explain the different biological properties observed in the two parasite species.

1.5.2.1.3 *Plasmodium falciparum*

Due to the low sequence homology and the lack of any cross reactive immunological reagents between the different *Plasmodium* species it was difficult to establish whether members of RBPH were also present in *P. falciparum*. Only once the *P. falciparum* genome sequence became available was it possible to identify members of the RBPH family in this parasite (Gardner *et al.*, 2002).

The members

To date a total of six RBPHs have been identified in *P. falciparum*, the reticulocyte binding protein homologue 1 (PfRH1) also named normocyte binding protein 1 (NBP1) (Rayner et al., 2001; Triglia et al., 2005; Tsuboi et al., 1994), the reticulocyte binding protein homologues 2a and 2b (PfRH2a and 2b) (Rayner et al., 2000; Triglia et al., 2001), the reticulocyte binding protein homologues 3 (PfRH3) (Taylor et al., 2001b), the reticulocyte binding protein homologue 4 (PfRH4) (Kaneko et al., 2002; Stubbs et al., 2005), and the reticulocyte binding protein homologue 5 (PfRH5) (Cowman and Crabb, 2006; Stubbs et al., 2005). PfRH1 as well as PfRH2a and 2b seem to locate to the rhoptries of the merozoite PfRH4 seems to have a more micronemal location. Whether this would indicate a functional difference still needs to be determined. There is some evidence that at least a small proportion of PfRH2a is moved to the apical pole of the merozoite after schizont rupture (Triglia et al., 2001) which is something that has previously been observed for PY235 as well (Ogun and Holder, 1994; Triglia et al., 2001). PfRH3 may be a pseudogene since frameshifts in the gene sequence exist, and no protein has been found expressed during the erythrocytic part of the life cycle (Taylor et al., 2001b), though there is some evidence that it may be expressed in sporozoites (Florens et al., 2002). The expression of PfRH3, PY235 and the *P. falciparum* EBA175 indicates collectively that the RBPHs and EBL proteins play a role in multiple invasive stages of *Plasmodium* parasites. Upon examination of the *P. falciparum* RBPHs it appears that different parasite strains and isolates display

some sequence variation between the same members of the family ranging from a few amino acid changes to large deletions (Taylor, Grainger, and Holder, 2002). Furthermore, some members were found to be completely absent in some strains (Duraisingh *et al.*, 2003b), indicating a selection pressure to increase diversity. The transcription and expression pattern of PfRBPH varies between different parasite lines (Duraisingh *et al.*, 2003b; Taylor, Grainger, and Holder, 2002). *PfRH3* transcription is upregulated in the absence of *RH2b* in one strain of *P. falciparum* (Baum *et al.*, 2005). This is not a compensatory upregulation as a sequential disruption of both *RH2b* and *RH3* in the parasite leads to similar invasion rates as a single *RH2b* disruption alone. Collectively, all the data point so far to the fact that in *P. falciparum*, (and *P. yoelii*) there is a huge expansion of the RBPHs, and at least some members may be functionally redundant.

Host cell specificity

In both *P. yoelii* as well as *P. vivax* the RBPH are believed to play an important role in the recognition of specific receptors on the surface of the red blood cell. Direct binding of the protein(s) to the erythrocyte has been shown and in the case of *P. yoelii* antibodies against PY235 have been shown to inhibit invasion (Fig 1-4). PfRH1 has been shown to directly bind a sialic acid containing trypsin resistant receptor on the erythrocyte and antibodies raised against this protein inhibit merozoite invasion of trypsin treated erythrocytes (Rayner *et al.*, 2001). RH4 binds a neuraminidase and chymotrypsin resistant receptor (Baum *et al.*, 2005). So far direct binding of PfRH2a or 2b to erythrocytes has not been demonstrated although there is evidence that antibodies can differentially inhibit invasion in some parasite strains (Triglia *et al.*, 2001). The most convincing evidence for how the parasite uses the RBPHs came from gene knockout experiments showing that PfRH-2b recognizes a unique chymotrypsin sensitive receptor and that it functions independently of other members (Duraisingh *et al.*, 2003b). Information available concerning the receptors of PfRH2a is limited though it is likely that each member of this gene family recognizes a unique receptor on the erythrocyte surface.

1.5.2.1.4 *Plasmodium reichenowi* RBPHs

Comparison of the RBPH gene family from the chimpanzee parasite *P. reichenowi* shed further light on the evolution as well as conservation of these parasite ligands. Like its close relative *P. falciparum* it contains 5 corresponding members of this gene family (Rayner *et al.*, 2004). Overall the genes are conserved but there are some notable differences with the homolog of *PfRH-1* having a large number of deletions as well as some insertions that lead to over 200 stop codons. This suggests that the longest reading frame in *PrRH-1* is only 645 base pairs in length compared to the more than 8kb of the corresponding *P. falciparum* gene. The *PrRH2b* gene on the other hand is highly conserved while for *PrRH2a* only the C-terminal end is conserved. *PrRH-3* lacks the stop codons found in the *PfRH-3* ortholog distinguishing it from the *P. falciparum* pseudogene. The overall conservation of *PrRH-4* is approximately 86% identity to the *P. falciparum* ortholog. The fact that both *P. reichenowi* and *P. falciparum* express diverse members of RBPHs may indicate that these parasites are both under selection pressure (Rayner *et al.*, 2004).

1.5.2.2 Other rhoptry proteins

Other rhoptry proteins are the rhoptry associated proteins (RAP)-1, 2 and 3 (Howard et al., 1998; Schofield et al., 1986). These exist in the low molecular weight complexes that may have an as yet undefined role in merozoite attachment and invasion of erythrocytes. RAP-1 is relatively non- polymorphic among the RAP proteins (Howard and Peterson, 1996) and has been shown to be involved in trafficking of RAP2 to the Rhoptries, but the disruption of this trafficking process does not lead to any changes in the invasion phenotype (Baldi et al., 2000). The RhopH 1, 2 and 3 proteins make up the high molecular weight complex which is thought to function with the serine repeat antigen (SERA) (Perkins and Zieffer, 1994). RhopH-3 is one such protein that is localized in the parasitophorous vacuole and plasma membrane of newly infected red blood cells (Sam-Yellowe, 1992; Sam-Yellowe, Shio, and Perkins, 1988). Antibodies to RhopH proteins showed partial protection to infection (Siddiqui et al., 1987). The RAP and RhopH proteins have been also found in the rodent *Plasmodium* (Hienne et al., 1998) (Kaneko et al., 2001; Ling et al., 2004).

A highly conserved paralog of the EBL proteins, MAEBL has been found in all *Plasmodium* species so far analysed (Michon et al., 2002). MAEBL, unlike the other EBLs, is located in the rhoptries of the merozoite indicating that it may have evolved a unique role in erythrocyte invasion (Noe and Adams, 1998); (Noe, Fishkind, and Adams, 2000). MAEBL is unique in that it does not contain a DBL domain but instead is a chimeric paralog sharing identity to AMA-1 within the

tandem duplicated cysteine rich domains (termed M1 and M2 domains) and the carboxyl cysteine rich domain of EBL (Blair *et al.*, 2002; Kappe *et al.*, 1998) (Fig 1-5). The duplicated cysteine rich domains conserved with the AMA 1 regions have previously been shown to have erythrocyte binding activity (Fraser *et al.*, 2001; Kappe *et al.*, 1998). A chimeric protein in the apical complex, MAEBL has been shown to be crucial in salivary gland invasion, as MAEBL knockouts parasites are blocked at invasion (Kappe *et al.*, 1998; Kariu *et al.*, 2002). In rodent parasite studies, MAEBL is crucial in hepatocyte invasion as antibodies block invasion in this stage (Preiser *et al.*, 2004; Preiser *et al.*, 2002).

1.5.3 Dense Granule proteins

These spherical organelles have densely granular interiors, seen by electron microscopy, and are situated in the cytoplasm between the rhoptries and merozoites nucleus. After invasion these move to the merozoites surface and release their contents by exocytosis from the parasite plasma membrane into the parasitophorous vacuole, and are thus important in entry of the parasite into the erythrocyte. Molecules such as the ring infected erythrocyte surface antigens (RESAs) which is associated with spectrin (Berzins et al., 1986; Foley et al., 1991) and Ring membrane antigen (RIMA) (Trager et al., 1992), emerge from the dense granules and are found in newly infected and developed ring stage parasites.

1.6 Alternate pathways for invasion

The parasite possibly utilizes multigenes (multiple genes that have a potential to perform similar functions in a single parasite) to allow it to maintain infection in the midst of a changing environment. The parasite has evolved to utilize alternative ligand-receptor interactions to deal with these changes encountered. We can already appreciate the complex nature of the invasion machinery, and need to note that no single ligand-receptor interaction can mediate the entire process. Alternative pathways are the series of complex interactions that utilizes various unique ligands and receptor combinations to mediate attachment, selection and invasion of a suitable erythrocyte, while allowing the parasite to evade elimination by the imminent immune system.

The changing environment can come in many forms. The most obvious is through variations in the erythrocyte receptors. In the human host variations in the host receptor can range from the complete absence of a receptor like the Duffy blood group antigen in people of African ancestry or Glycophorin B seen in some populations (Fraser, Giblett, and Motulsky, 1966; Miller *et al.*, 1975) to mutations in receptors like Glycophorin C (Lobo *et al.*, 2003; Maier *et al.*, 2003; Patel *et al.*, 2001). Also, the parasite can face preexisting immunity that can block a specific interaction or pathways, or acquired immunity to parasite antigens previously encountered by the immune system.

How a parasite utilizes different pathway is difficult to study *in vivo*, but a lot of data from *in vitro* cultured parasites allow us to speculate on the possibilities.

Modifying receptor repertoire

In cultured parasites, some parasite lines can compensate for the lack of suitable receptors by utilizing other more appropriate receptors. For example, after enzymatic modification of the red blood cell receptor, parasites that use the EBA175-Gly A interaction appear to use an alternate interaction. This is also seen after antibody mediated blocking of these interactions where parasites are able to continue invading *in vitro* (Camus and Hadley, 1985); (Dolan, Miller, and Wellems, 1990); (Dolan et al., 1994; Rayner et al., 2001) (Gaur et al., 2003). This could however be alternately explained by the effect variations in antibody efficiency has on its ability to block interactions in long term cultured invasive parasites.

Modifying ligand repertoire

Convincingly, transfection experiments have shown that the disruption of certain members (such as EBA175, EBA 140 and EBA 181) completely ablates the interaction of the parasite ligand (since it is no longer expressed) with its receptor forcing the parasite to use a different ligand receptor combination. Interestingly, these parasites do not suffer any loss in invasion efficiency (Gilberger *et al.*, 2003a; Kaneko *et al.*, 2000; Maier *et al.*, 2003; Reed *et al.*, 2000).

Receptor polymorphisms

When the parasite encounters polymorphisms of different receptors, it can also utilize different pathways to mediate a successful invasion. Analyzing field isolated parasites show that different ligand receptor combinations are selected for in infections of ethnically diverse hosts (Baum, Thomas, and Conway, 2003; Jakobsen et al., 1998; Lobo et al., 2004; Okoyeh, Pillai, and Chitnis, 1999; Pasvol, Weatherall, and Wilson, 1980). Modulation of the invasive potential can also be brought about by genetic diversity of the ligands. Sequence analysis of different parasite isolates has revealed there are extensive polymorphisms for EBL and RBPH genes (Nery et al., 2006; Taylor, Grainger, and Holder, 2002). The functional significance of these polymorphisms, in terms of receptor recognition or invasion profiles, is yet to be demonstrated. The polymorphic residues in EBA 140, EBA 181, and substitutions and deletions in the *P. falciparum* RBPHs are good examples (Mayer et al., 2002) (Mayer et al., 2004; Taylor, Grainger, and Holder, 2002).

1.7 Interplay between RBPHs and EBLs:

Most of the data to date has highlighted that specific pairs of ligand receptor combinations can be ablated, and parasites are capable of using alternate ligand receptor combinations. It is possible that a mechanism for red blood cell recognition and invasion based on distinct combinations of two or more ligand-receptor interactions, would offer a good solution for maintaining a broad invasive potential at a minimal cost in genetic complexity. It is also then possible that a situation where the combined ligands are selected from the two families (RPBH and EBL) would be optimal. This hypothetical scheme has the potential to account for a number of features associated with malaria infections. First, reliance on at least two distinct host receptors for target cell identification would help ensure that the parasite will predominantly invade the cells suitable for its development. Second, a defined combination between two ligands, one from the RPBH and the other from the EBL families, is likely to target a different set of host receptors from those targeted by another combination. Thus, each RBPH/EBL pair would potentially define a distinct invasion pathway.

At present it is not known whether the EBL and RBPH interact with their respective receptors in separate independent events, or whether this occurs in the context of a macromolecular complex comprising both ligands. It has been recently proposed that the *P. falciparum* RBPH have an initial sensing function that serves to characterize the erythrocyte as suitable (Duraisingh *et al.*, 2003b). This initial positive interaction would then lead to the recruitment of the high affinity ligands, such as EBA175 or another member of the EBL family (Figure 1-8), ultimately

resulting in the release of micronemal contents and junction formation which initiate the process of red blood cell invasion. This is consistent with current understanding of red cell invasion by *P. vivax* where reticulocyte identification precedes recruitment of the DBP (Galinski and Barnwell, 1996), and in line with the proposed mode of action of the *P. yoelii* Py235 protein (Khan, Jarra, and Preiser, 2001). It must be stated that establishing the timelines of the numerous steps of a process normally completed within minutes is fraught with technical and interpretational hurdles. Irrespective of the finer details, disruption of either the interaction mediated by RBPH as well as EBL would significantly inhibit merozoite invasion.

Figure 1-8. Sensing by RBPHs and recruitment of EBLs.

P. falciparum RBPH may have an initial sensing function that serves to characterize the erythrocyte as suitable. This initial positive interaction would then lead to the recruitment of the high affinity ligands, such as EBA175 or another member of the EBL family resulting in the release of micronemal contents and junction formation which initiate the process of red blood cell invasion. NU: Nucleus; Mn: Microneme; Rh: Rhoptry; DG: Dense granule.

P. falciparum has five distinct EBLs and five different RBPHs that could be combined with each other and give the parasite the potential to utilize a total of 25 different invasion pathways. Similarly while *P. yoelii* may only have a single EBL it has around 14 different RPBHs (*py235s*) again giving rise to 14 possible unique pathways. If different domains of the same protein (EBL or RBPH) are capable of exhibiting receptor recognition, the number of possible pathways will be greater than this. Current experimental methods have yet to test if this is possible.

All *Plasmodium* species analyzed appear to express at least one member of RBPH and EBL. Parasites that have a broader range of invasion pathways appear to have an expanded diversity of both the RBPHs and EBLs. Collectively this indicates that both an EBL and an RBPH member are required in invasion in the *Plasmodium spp.* Differences in the expression profile of these genes in the different *Plasmodium spp.* may indicate differences in how these genes are utilized. For example, in *P. vivax*, there is an absolute requirement for both RBPs and DBP, while in *P. falciparum*, multiple members appear to be expressed at the same time, though the parasite may use only some ligands to mediate interactions with a host receptor. We can also conclude that within each family, the proteins are functionally redundant, and have overlapping function as all the genes can be disrupted in different *P. falciparum* lines without a growth defect (Duraisingh et al., 2003a; Duraisingh et al., 2003b; Gilberger et al., 2003a; Maier et al., 2003; Stubbs et al., 2005; Triglia et al., 2005).

The interplay between the RBPH and EBL is classically demonstrated in experiments where activation of a sialic acid independent invasion pathway in *P. falciparum* laboratory clones is regulated by differential gene expression and silencing of a RBPH (*RH4*) in normal erythrocytes. (Stubbs et al., 2005) Selection of *RH4* activated parasites can be obtained by growing these parasites in continuous sialic acid depleted erythrocytes or by the complete destruction of the functional *EBA175* gene. Since *RH4* activation seems to be a direct response to the loss of *EBA175*, there is a direct implication of the interaction between the two families of invasion proteins. In this case the fact that both these proteins are in the micronemes may indicate a parallel compensatory role of *RH4* and suggests a spatial regulation of which these proteins are utilized.

1.8 Virulence and alternate pathways:

There appears to be a high amount of variation in disease severity observed in the field. Infections can range from being asymptomatic to fatal with case fatality rates in untreated non-immune individuals sometimes exceeding 20% (Alles, Mendis, and Carter, 1998). A lot of this variation can be attributed to prior exposure and acquired immunity, and definitely other factors contribute to the clinical outcome. This includes the species that cause the infection (typically *P. falciparum* causes the most fatal malaria), host age, nutrition, size of inoculum, socioeconomic factors, host genetics and parasite genetics (Baird, 1998; Greenwood and Armstrong, 1991; Marsh, 1992). The relative contribution of these factors have not been well quantified. While virulence has been defined as the ability of parasite to cause disease, indices of growth rate, parasitemias, clinical pathology, anemia or other indicators have been used to measure virulence. This is important as it is ultimately the virulence of each parasite that determines the burden of malaria. We have earlier discussed that the range and efficiency of host cells invaded by the asexual parasite has a direct implication on the virulence outcome of an infection. The variations in the expression of parasite ligands which lead to the parasite being able to invade a wide variety of erythrocytes would be a mechanism to increased virulence. To date, there is no evidence available in *P. falciparum* to show this, while in *P. yoelii*, there is some evidence of *py235* that suggests a role in these ligands and virulence (Freeman, Trejdosiewicz, and Cross, 1980).

Differences in *py235* exist between virulent and avirulent parasites (transcription and gene organization)(Khan et al., 2001; Preiser and Jarra, 1998), and immune pressure against PY235 leads to the normally lethal YM infections to convert to a milder form that can be cleared by the host (Figure 1-7) (Freeman, Trejdosiewicz, and Cross, 1980). While this does not create the direct link between PY235 and virulence, it suggests that the antibody to PY235 protein protected against challenge infection by possibly preventing the entry of parasites into mature erythrocytes without affecting reticulocyte invasion, indicating that virulence may be affected by the repertoire of cells invaded by these parasites. It is also possible from the passive transfer experiments that YM parasites are forced to either utilize an alternate pathway for invasion or be limited to invading a smaller subset of cells, and thereby directly impacting on virulence.

Further evidence from infections in Duffy knockout mice show that infections of erythrocytes lacking the Duffy antigen exhibited increased parasitemia rates, and thus were more susceptible to a non lethal *P. yoelii* strain than wild type mice (Swardson-Oliver et al., 2002), while a contrasting study showed that the knockout mouse was protected from a lethal *P. yoelii* strain, and converted the infection to a self-limiting one (Akimitsu et al., 2004). The latter case would suggest that a reduced range of host cells leads to a reduction in virulence. Taken collectively, the evidence would suggest that utilizing different ligand-receptor interactions, either in RBPHs or EBLs may explain variations in virulence. These observations also allow us to question if there is a link between *py235* and the Duffy receptor.

1.9 Role of the PY235 family in alternate pathways in *P. yoelii*

- The research model

The parasite appears to have the ability to use the diverse repertoire of invasion ligands to maximize its invasion potential. An expanded repertoire of *py235* genes allow the parasite be able to recognize a range of host receptors, it prevents elimination due to immune attack, and could exhibit differences in host range of cells invaded and virulence. The research presented in this thesis will address these hypotheses.

P. falciparum appears to express more than one member of the RBPHs and EBLs (Cowman and Crabb, 2006; Duraisingh et al., 2003b; Stubbs et al., 2005; Taylor, Grainger, and Holder, 2002), while *P. yoelii* appears to express only a single unique member in each merozoite. This indicates that *P. falciparum* and *P. yoelii* seem to have developed two different ways to maximize their invasion potential. An approach to achieve the goal of maintaining invasion despite heterogeneity in host receptors, while maintaining immune evasion is most clearly demonstrated in *P. yoelii*. Here each merozoite seems to express only a single member of RBL (*py235*), while at the same time at the population level maintaining distinct merozoites expressing different members of this gene family (Preiser et al., 1999a). This ensures that the overall invasion potential in the population is maintained as well as ensuring that the merozoites are also immunologically distinct. The parasite must express a mixture of invasion molecules that can maximize the potential for recognizing the correct host cell receptor to invade an available erythrocyte.

As the parasite matures, each schizont ruptures into multiple merozoites, each containing a distinct *py235* member. This was observed in transcription analysis of single micromanipulated parasites (Preiser et al., 1999a). This means that each schizont will produce a mosaic of different merozoites, each with the capacity to recognize different variants of a receptor. This mechanism of clonal phenotypic variation of *py235* maximizes the chances of at least some merozoites expressing the appropriate PY235 for successful invasion of the next host erythrocyte.

Preliminary results have indicated that within a single blood stage infection, the transcript level of different *py235* genes are different (Preiser, Unpublished data). This would indicate that there are variations in the number of merozoites that express one form of *py235* over another and therefore that the number of each merozoite variant produced in a single schizont is not the same. An imbalance of merozoites produced in a single schizont would have profound implications on parasite biology and the way the parasite utilizes the *py235* gene family. One way to explain this is to consider that merozoites expressing different variants of PY235 recognize different receptors or alternatively different variants of the same receptor. Depending on the strength of the interaction between a specific PY235 and a receptor on the erythrocyte a merozoite is more or less likely to successfully invade. Strong interaction would have a close to 100% success rate while weaker interactions would have a lower invasion frequency (Figure 1-9).

Figure 1-9. Schizont rupture and merozoite specificity to a host cell.

A schizont ruptures to produce merozoites each expressing a single different variant of PY235, and with different invasion efficiency. Colours indicate different variants of PY235.

It is possible that merozoites that have successfully invaded retain the genetic memory of the specific variant of PY235 it utilized and therefore have the potential to mature into schizonts that make more merozoites expressing this variant of PY235. An important feature of this model is that at some random frequency transcription of *py235* is switched in the developing schizont ensuring that a low level of merozoites expressing other PY235 variants is made. One result of such a model is that over a few replication cycles merozoites expressing the most successful PY235 variant will dominate at a population level while at the same time other less successful variants are maintained at lower levels (Fig 1-10).

Figure 1-10. Successfully invaded merozoites are selected for in subsequent cycles.

Merozoites expressing the most successful Py235 variant will dominate at a population level in subsequent cycles. This leads to a differential expression of Py235 in a population of schizonts.

We have just discussed that the utilization of an alternate invasion pathway can be observed using targeted gene disruption, antibody mediated blocking of interactions and enzymatic destruction of binding residues in either a specific RBPH or EBL. Studies so far in *P. falciparum* would indicate that most of the parasite ligands are expressed throughout the asexual cycle and the removal or blockage of any one receptor would lead to the utilization of the now appropriate parasite ligand without a detectable change in the actual expression pattern. Still the fact that parasites need to be adapted and selected would indicate that while the proteins are expressed in all parasites they may not be located in a functional position or in appropriate functional densities. During the adaptation process it is possible that the parasite rearranges or changes the location of the ligand to a now active position (Duraisingh et al., 2003b). This "biased" or selective exposure of a particular set of ligands, might arise during the genesis of the apical complex. In this way, loss of a particular ligand from the "active site" would liberate the place

for another ligand (Fig 1-11) and would consequently lead to a switch in the invasion phenotype. The alternative is that each merozoite is capable of only expressing a single ligand set at the “active site”, and it is only merozoites exhibiting the correct ligand-receptor combinations will invade and persist in a population. The notion of maintaining a limited number of parasite ligands in an accessible position would be consistent with a strategy of immune evasion for the merozoites a parasite from fully exposed to the host's humoral defences. Since the exposure of all the RBPHs and EBLs at the same time would potentially lead to a much more rapid acquisition of immunity capable of blocking all invasion pathways.

Figure 1-11. Genesis of the apical complex

Genesis of the apical complex. (A) A merozoite expressing many RPBHs and EBLs in the apical organelles. (C) Only one ligand set of the EBL and RBPH (either blue or red) is expressed at the site where the interactions with an erythrocyte take place. (B) Loss of particular ligands (blue) from the "active site" would liberate the place for another ligand (yellow).

While *P. falciparum*, *P. knowlesi* and *P. yoelii* appear to exhibit potential alternate invasion pathway, the absolute requirement of *P. vivax* for Duffy antigen makes it an ideal target for immune attack. In one report, it appears that the parasite appears to evade immunity by clustering polymorphic residues that are often under immune pressure away from the binding site, as in the case of PvDBP (Singh et al., 2006). This is further limited by the secretion of PvDBP from the micronemes which is probably followed by a very quick engagement to the Duffy antigen. This conformational change leads to the binding site being buried in a binding cleft and

thus be protected from immune attack (Singh et al., 2006). However, *P. vivax* DBL polymorphisms appear to be more extensive than shown in that report and are widely dispersed over the DBL domain (McHenry and Adams, 2006). To understand this further, cocrystallization of the DBL domain with its receptor is required.

We have discussed that *P. falciparum* appears to evade immunity through polymorphisms of the ligands, and concealed antigenic residues. In *P. yoelii*, a different mechanism may exist. The model proposed in Fig 1-9 and 1-10 will give the parasite a significant advantage as immunity to the dominant variant develops, it leads to the elimination of merozoites expressing this form of PY235 variant (Fig 1-12). The merozoites expressing other PY235 variants may be still able to invade, allowing the parasite to maintain its infection. In this situation merozoites with the next highest invasion efficiency would now form the dominant population until immunity against this variant leads to their elimination. As the invasion efficiency of merozoite is reduced the total number of parasites and therefore merozoites expressing a specific variant is reduced leading to lower amounts of antigen being presented in the host. This could possibly lead to an increase in the amount of time required for the host immune system to recognize these somewhat less invasion efficient merozoites and therefore these merozoites may not be eliminated as quickly (Fig 1-12).

Figure 1-12. Effect of immunity to dominant PY235 variant.

With host immunity to the dominant PY235 expressed, merozoites expressing dominant PY235 will be eliminated. Another less efficient member can be expressed, and will dominate during subsequent cycles.

There are a number of advantages to such an approach. In a case where only one type of *py235* gene product is always expressed within a population, the parasite is unable to invade different host erythrocytes, and if multiple *py235* genes are expressed per merozoite, there will be inactivation of merozoites by the host immune response. Thus a single schizont ruptures to produce merozoites with a mosaic of PY235 expressed (Preiser et al., 1999a). This allows the parasite to maintain infection and continue its transmission potential.

1.10 Chronic infections

Very little is known about the consequence invasion on the establishment of chronic infections. The benefit to the parasite to have such a large repertoire of PY235 proteins will enable the parasite to persist for a long time in the host, and establish chronic infections. This is observed in the natural host for *P. yoelii* infections, *Thamnomys*, where parasites persist for the life of the rodent (Landau and Chabaud, 1965). It is possible that a similar mechanism to utilize multigene families to establish persistent infections occurs in *P. falciparum*. The diagram below (Fig 1-13) shows the fluctuations of parasites seen in the blood stream of patients infected with *P. falciparum*. Individuals living in endemic areas, although clinically immune, often retain a persistent parasitemia, and asexual parasites are continuously switching to gametocytes and transmitted through mosquitoes (Good et al 2001).

Figure 1-13. Persistence of *Plasmodium* infections in a natural host.

Fluctuations of parasites in the peripheral blood of often asymptomatic individuals occur possibly by antigenic variation of multigenes. This allows for an effective immunity to any single variant and ensures the continued transmission of parasites during appropriate seasons. (Miller, Good, and Milon, 1994)

The persistence of infection is characterized by periodic peaks and dips in parasitemia, and can persist for as long as 480 days (Eyles and Young, 1951). The immune system is unable to control such persistence infections because of variant antigens, possibly including the RBPHs, as well as the EBL family, along with other variant surface antigens such as PfEMP1. The ability of *P. falciparum* as well as other *Plasmodium* species to establish long lasting chronic infections depends on the ability of at least some of the merozoites to successfully invade an erythrocyte. This occurs despite the fact that variations of red cell receptors like glycophorin A occur during the maturation of the erythrocyte and the development of host immunity to parasite encoded RBPHs and EBL.

2. AIMS OF THIS STUDY

From the introduction it is obvious that while alternate ligands are expressed, there appears to be no clear correlate between alternate ligands and virulence. Research in *P. yoelii* suggests that immunity to PY235 causes a change in the outcome of a virulent infection, and research in *P. falciparum* suggests that specific inhibition of individual ligand-receptor interactions does not completely ablate invasion, albeit no evidence of virulence of the selected or switched parasite has been shown. Since it is difficult to address many of these questions in *P. falciparum*, we will use the rodent malaria parasite *P. yoelii* to address the role of the RBPHs (*py235*) in virulence. In addition to its similarities to *P. falciparum*, the *P. yoelii* system is an attractive as a model because of the availability of diverse parasite strains single cloned from a wild isolate, and maintained in inbred animals. We also have the advantage of having a partially sequenced *P. yoelii* genome (albeit it is to a single strain 17X 1.1NL) and the ability to effectively measure virulence and multiplication rates of the parasite. Using this model in inbred mice allows us to manipulate immunity and other factors that may modulate virulence.

In this study, we will investigate the role of *py235* in the invasion process and selection of host cells in the asexual stages of the rodent parasite. The role of *py235* in alternate invasion pathways, virulence and immune evasion will be addressed. To do this, we will first evaluate the selective index (SI) (Simpson et al., 1999) as a reliable measure of virulence, and show the link between SI and virulence for *P. yoelii*. We will then attempt to validate the numbers of copies of the various

py235 genes and determine if the copy number plays a role in virulence. We will then determine the differences between the expression of these genes in YA and YM infections, using a real time RT PCR approach with appropriate controls. We will then study the differences in the expression of *py235* in experimental models of infection when we attempt to vary the invasion properties of the parasite by varying the host cells (by changing the host encountered by parasites) or study the ligand variations (when we attempt to use different parasites on a similar host animal), and study the changes this has on virulence and SI. We will show experiments where we modulate the immune effects on *py235* gene expression in some parasites to illustrate the behavior of modified ligand-receptor interactions on infections, virulence and SI.

The first chapter presented has illustrated the characteristics of the malaria parasite, the major molecular players in erythrocyte invasion and the evidence available on alternate invasion pathways, virulence and immune evasion by members of the EBL and RBPH families. The evidence discussed in Chapter 1 have led to the hypotheses of this study in this Chapter (2) to determine the role of *py235* in virulence, immune evasion and alternate invasion pathways. In Chapter 3 we will discuss the Materials and Methods used in this study in detail, so that any reproduction of the experiments can be performed easily. In Chapter 4 we establish the concept of SI, and use it as a reliable indicator of virulence in our study. We show the parasites that were used in this study, and its corresponding phenotype. Chapter 5 addresses the number of copies of *py235* that is present in multiple strains of *P. yoelii*, along with the potential involvement of copy number differences in *py235* in the virulence of these parasites. Chapter 6 demonstrates the

various ways the parasite utilizes the *py235* genes in response to changes in host cell repertoire and ligand repertoire, and shows the effect PY235 specific immune pressure has on PY235. Chapter 7 is a short study of how the parasite modulates *py235* in the absence of Duffy antigen using a Duffy knockout mouse model infected with *P. yoelii*. And finally Chapter 8 is a conclusion of all the work presented here in this dissertation and how it is significant to malaria research and intervention. Chapter 8 also opens the doors for future directives that are required for the continuance of this research. Appendices to give some background to the clones of parasites available, an alignment of the *py235* genes used in this study, the recipes of buffers, media and solutions and the Bibliography complete this dissertation.

3. MATERIALS AND METHODS

(Detailed recipes of solutions, buffers and media are in Appendix)

3.1 Parasite maintenance

Male BALB/c mice, bred specific pathogen free at the Nanyang Technological University Animal Holding Unit were infected with either cryopreserved stocks of parasites, or by syringe passage of 10^6 parasites in mice. *P. yoelii* 17X parasites strains include previously characterized YA (Jarra and Brown, 1989), YM (Yoeli et al., 1975), 1.1 (Landau and Chabaud, 1965; Weinbaum, Evans, and Tigelaar, 1976), Parents of a genetic cross between AC (Walliker, Carter, and Morgan, 1973), YM (Edinburgh)(Yoeli et al., 1975), and progeny 604, 605, 606, 607, 611, 612 (gift from Drs D. Walliker and R.Carter)(Walliker et al., 1976). The phenotype of these parasites in mice has been previously characterized (Holder and Freeman, 1984a; Landau and Chabaud, 1965; Walliker et al., 1976), and confirmed in our own experiments (Table 3-1 and Appendix). Adaptation experiments were performed similar to those in mice, with the exception of using an intravenous route of passage of parasites into young, 3 week old SPF Wistar Rats.

Experimental animal were monitored for infection by parasitemia monitored by thin blood smears stained with Giemsa (Sigma). Animals were sacrificed using Sodium Pentobarbitone and infected blood was collected as

previously described by cardiac bleeds into Heparin Saline. (Jarra and Brown, 1989).

Parasite / Animal	Host animal	Virulence phenotype
17X YA	BALB/c mouse	avirulent
17X YM	BALB/c mouse	virulent
17X 1.1 NL	BALB/c mouse	avirulent
YME (Edinburgh)	BALB/c mouse	virulent
AC	BALB/c mouse	avirulent
604	BALB/c mouse	virulent
605	BALB/c mouse	virulent
606	BALB/c mouse	intermediate
607	BALB/c mouse	virulent
611	BALB/c mouse	avirulent
612	BALB/c mouse	intermediate

Table 3-1. Characterization of *P. y. yoelii* parasite clones

Parasite clones grown in host animals gave one of three outcomes. An avirulent infection is where parasites are cleared from the host animal, and leads to the survival of the host. A virulent infection involves rapid parasite multiplication rates and is often lethal to the host. An intermediate phenotype involves also relatively rapid multiplication of the parasite and survival of 40-60% of the host.

3.2 Preparation of parasite DNA

Parasitized erythrocytes collected were washed once in 1X PBS by centrifugation at 2100rpm for 5 minutes at room temperature with low brake. Genomic DNA was extracted from mixed stage infected parasitized erythrocytes using the Invitrogen Easy DNA kit according to the manufacturer's protocol, and quantified using UV spectrophotometry at 260nm. Parasites from the genetic cross were also treated as above.

3.3 Isolation of Schizonts

Parasitized blood was washed twice in incomplete RPMI1640 (Invitrogen) by centrifugation at 2100rpm for 5 minutes at room temperature with low brake, and the buffer was aspirated to remove serum and buffy coat. Schizont stage parasites were resuspended in FBS (Hyclone) and layered on top of a 50-80% Nycodenz (Sigma) gradient made by using Nycodenz Working Solution and incomplete RPMI1640 in the same percentages previously layered in a 15ml centrifuge tube (Falcon) by centrifugation at 2100rpm for 20 mins at 21°C with no brake. Schizonts were collected from directly under the FBS layer and washed twice in 1X PBS.

3.4 RNA extraction and cDNA generation

RNA was extracted using the Qiagen RNAeasy kit directly from schizonts according to the manufacturer’s protocol, and quantified using UV spectrophotometry at 260nm. RNA was immediately used in subsequent steps and the remainder was transferred to -80°C for long term storage. DNase treatment was as performed (Table 3-2) at 37°C for 2 hours and the enzyme was deactivated at 75°C for 5 minutes.

Reagent	Amount
RNA (diluted to 10ug)	20ul
5X First strand buffer (Invitrogen)	12ul
25mM MgCl	9.6ul
100mM dNTPs	8ul
RNAsin (Promega 40U/ul)	4ul
DNase (Roche 10U/ul)	4ul
0.1mM DTT	4ul
H2O	2.4ul
Total	64ul

Table 3-2. Master mix for DNase treatment of Schizont RNA.

cDNA from this reaction was generated using random hexamers (Invitrogen) (Table 3-3) (Doolan, 2001). cDNA was stored at -20°C until use in the Real time PCR reaction.

Experiment	Reagent	Amount
cDNA synthesis -RT	DNase treated RNA	4ul
	(from DNase step above)	
	Random primers	0.5ul
	(Invitrogen 1ng/ul)	
	H2O	0.5ul
cDNA synthesis +RT	DNase treated RNA	60
	(from DNase step above)	
	Random primers	6
	(Invitrogen 1ng/ul)	
	SSII Reverse Transcriptase	6
	(Invitrogen)	

Table 3-3. Master mix for cDNA reaction using random primers.

3.5 Primer design and cloning

Unique primers were designed for eleven members of the *py235* genes (Table 3-4). The other three members in the database were unassigned contigs that exist as incomplete fragments of genes (Fig 3-1), and there were no identifiable regions that deemed each of these three contigs unique. Unique primers were designed using an algorithm where windows of 20 nucleotides were scanned against the whole gene family, in search of a suitable region within the stipulated size to serve as a unique amplified region (Zbynek Bozdech, unpublished algorithm). Only short regions of the eleven genes (size ranged from 132bp to 222bp) were amplified from genomic DNA extracted from 17X NL 1.1, YA and YM parasites (Table 3-5). β -actin was used as a control. The single amplified products (Fig 3-2) were then cloned into TOPO TA vector (Invitrogen) according to the manufacturer's protocol (Fig 3-3 and Table 3.6). The products amplified from both YM and YA parasites were sequenced to determine variations in the regions amplified.

Primer	Sequence 5' to 3'	Annealing temp and [Mg]	Amplified region (nt)
PYO3534 F	GATAGTGAGTACCATATTGTTTA	Temp 45°C	7722-7047
PYO3534 R	CAAGTATAATGATAATAATAATGA	Mg 2mM	
PYO3432 F	GTTATTTTGGTTATCTATAACGATTG	Temp 52°C	938-1098
PYO3432 R	CAAAATTTGTTAATACTATACGC	Mg 3mM	
PYO1365 F	CCATTGTACTGTCTATTTTCGTC	Temp 52°C	1320-1478
PYO1365 R	CTCGAATACAAAGTCGTTTAG	Mg 3mM	
PYO1185 F	GCATAGTATTAATGTATGCGTCTA	Temp 52°C	5277-5498
PYO1185 R	CACAACATGTAAATGATGTAAAATC	Mg 3mM	
PYO2104 F	GTAATTAGTTTTTTCTGAAGATTTGT	Temp 55°C	2410-2590
PYO2104 R	TGCAATTTTAGAACCAGCAAAGTATG	Mg 2mM	
PYO5054 F	AATCATTTTCTAATTGTTCGATAG	Temp 52°C	6082-6238
PYO5054 R	GATAATATTTTAGAAGCATC	Mg 3mM	
PYO6018 F	TGATATTGATACATTAAACCAAAAAATCG	Temp 60°C	1245-1395
PYO6018 R	TGGATCCTCGTTAAACATTGTTTTATTTGG	Mg 2mM	
PYO3184 F	CGTAATTCCTTTTATGCTGCTGATTTACAG	Temp 60°C	1745-1781
PYO3184 R	GGAAACAATTA AAAACCCCTTGAGGAAC	Mg 3mM	
PYO0649 F	CCTGAAGGGTATTTAAATTGTCTCC	Temp 60°C	3508-3639
PYO0649 R	GTGATATAAGAACAAAATCAAATGATTG	Mg 3mM	
PYO4930 F	GTTCTGAATCAATTTTCGTTTTATC	Temp 55°C	11384-11555
PYO4930 R	CACTAATAACAGTGATTATAACATCAACG	Mg 3mM	
PYO4630 F	CACTATTATGCTTTTGGGATTCTG	Temp 55°C	4264-4404
PYO4630 R	GTAAGAGTTATAAAAAAATATTTCTG	Mg 2mM	

Primer	Sequence 5' to 3'	Annealing temp and [Mg]	Amplified region (nt)
Actin F	TATGAAGGTATTGGTGAAAGATTAAC	Temp 45°C	2213-2364
Actin R	CACATTTGTTGAAATGTTGACAAAG	Mg 2mM	
18s rRNA F	TCAAAGTAACGAGAGCCCAATG	Temp 45°C	995-1140
18s rRNA R	ATTTTAAGCAACTCACTTGGC	Mg 3mM	
MSP1 F	ATGAAGGTGATTGGACTTTTATTTTCTTTCG	Temp 45°C	6156-6309
MSP 1 R	TTACTTGACTTTTTTGAAATAGTTCTAACCC	Mg 3mM	
EBL F	ATATCAGGTTAATGAATGTAAGG	Temp 45°C	3434-3583
EBL R	TCCGGTATTTTTATTGTGTCTGG	Mg 3mM	

Table 3-4. Primers used for PCR amplification.

(Continued)

Primers were designed to amplify regions unique to each *py235* gene. Control *P. yoelii* β - actin primers for DNA copy number quantitation and 18s rRNA for cDNA quantitation are also shown. To study other invasion genes, the rodent EBL and Merozoite surface protein (MSP-1) are also included in this analyses.

Figure 3-1. *py235* members.

Contigs from the 17X 1.1 genome database (www.PlasmoDB.org2004) containing partial or full length *py235* sequences. Full length sequence of E8 indicate that contigs PY04438 and PY06018 may be part of the same gene. Regions of homology shaded in black indicated.

Reagent	Amount
10X PCR buffer (15mM MgCl ₂ NEB)	1X
dNTPs (Invitrogen)	200uM
Specific Primers (Research Biolabs)	100ng each
Taq polymerase (NEB)	1U
H ₂ O	to 25ul
Genomic DNA (100ng)	2ul

Table 3-5. Master mix of PCR of gene specific *py235* gene fragments.

Figure 3-2. PCR products of *py235* genes and actin from YA parasites.

Regions of different *py235* members were amplified by gene specific PCR of *P. yoelii* YA genomic DNA. Products run on a 2.5% metaphor agarose gel show single bands of sizes ranging from132 to 222bp. *P. yoelii* b-actin as a positive control

Figure 3-3. TOPO TA cloning vector and inserted region

Reagent	Amount
PCR product	3ul
10X ligation buffer (Invitrogen)	1ul
PCR II vector (Invitrogen)	2ul
T4 ligase (Invitrogen)	1ul
H2O	to 10ul

Table 3-6. Ligation mix for TOPO TA cloning

3.6 Southern Blotting (performed by Peter Preiser)

1 µg of parasite genomic DNA from YM strain was digested with either BamHI, BclI, ClaI or EcoRI (New England Biolabs), and resolved on a 1% agarose gel. DNA in gels were depurinated and neutralized and then the gel was Southern Blotted. DNA was transferred to a nylon membrane (Hybond N+Amersham Pharmacia) after depurination and UV cross linked (Stratalinker Stratagene). Membranes were prehybridized (Perfect Hyb PLUS hybridization buffer Sigma) for 2 hours at 55°C. Probe was generated by PCR of parasite DNA with primers specific to PY04930 (Table 3-4) and labeled with α [³²P] (PrimeIt II Stratagene) according to the manufacturer's protocol. The probe was hybridized to the membrane at 60°C overnight, and next day washed with stringency wash buffers: 2XSSC/0.1%SDS for 15mins, followed by 0.5XSSC/ 0.1%SDS for 15mins and 0.1XSSC/ 0.1%SDS all at 55°C. The membrane was then exposed to X ray film at -80°C overnight and developed by autoradiography.

3.7 Real time PCR

The TA plasmids containing the 11 *py235* inserts were then used to generate standard curves for quantitative real time PCR from the range of 10ng down to 0.000001ng. To determine copy numbers of each gene, genomic DNA extracted from blood stage *P. yoelii* and each *py235* gene fragment was amplified using the same unique primers as standards to each *py235* variant, using Sybr Green Master mix (Applied Biosystems Inc.) (Table 3-7) and analyzed on a ABI 7000 thermocycler. Copy numbers were assigned to each gene by normalizing to the single copy gene, β actin. Primer sequences are in Table 3-4.

Reagent	Amount
Sybr Green (ABI)	7.5ul
H2O	6.4ul
10uM Primers each (Res Biolabs)	0.3ul
genomic DNA or cDNA	0.5ul
Total	15ul

Table 3-7. Real time PCR reaction mix

PCR on cDNA was performed as above (Table 3-7) and transcript amounts of each gene in each experiment was quantified relative to the standards, and normalized to parasite 18s rRNA. Other invasion molecules such as Merozoite surface protein 1 (MSP1) and the *P. yoelii* Erythrocyte Binding Like Protein (EBL) were also analyzed. Unless otherwise stated, p values (using the students T-test

available in Microsoft Excel) are <0.05 whenever statistical significance is mentioned.

3.8 Protein lysis and Western blotting

Parasitized blood was harvested as above. Schizonts collected were put in RPMI 1640 with 0.5% Albumax (Invitrogen) for 2 hours, and centrifuged at 2000rpm for 5 min to pellet mature schizonts. Schizonts were lysed in 1%NP40, 5mM EGTA, 150mM NaCl, 50mM Tris HCl (pH 8.2), 5mM EDTA with protease inhibitors (Roche), and proteins separated on a 4-12% Bis-Tris SDSPAGE (Invitrogen). Western Blots were performed as previously described (Doolan, 2001), using either polyclonal S6 antibody(Keen et al., 1994; Sinha et al., 1996) or monoclonal 25.77 antibody (Freeman, Trejdosiewicz, and Cross, 1980). Equal numbers of parasites were run on each lane (10^6 schizonts per lane). Parasite proteins recognized by hyperimmune serum from YA and YM parasite infected mice was used as a loading control along with antibody against previously characterized cytoplasmic protein g12 (MR4) (Wiser and Plitt, 1987).

3.9 Passive transfer experiments

Monoclonal antibody generating hybridomas were maintained in hybridoma culture medium (RPMI1640 with 25% FBS) until confluent. Supernatants were harvested by centrifuging the culture for 5 minutes at room temperature at 2900rpm to remove cells and debris, and supernatants filtered to remove debris. Secreted antibodies were purified on a 1ml HiTrap Protein G column (Amersham) by FPLC, and concentrated using an Amicon 10kDa centrifugation concentrator (Millipore). Control non protective anti PY235 25.86 antibody (Freeman) was also purified the same way.

Groups of 5 BALB/c mice were passively transferred with 0.5mg of protective anti-PY235 monoclonal antibody 25.77 or non protective 25.86 antibody or same volume of 1XPBS at Days -1, 0, +1 and +2. 10^7 mixed stage Parasites were passaged from a donor animal infected with YA into these passively transferred mice on day 0. Parasitemias were monitored until appropriate parasitemias and schizont collection, RNA extraction, cDNA synthesis and real time RT-PCR were performed as before.

3.10 SI determination

The selective index of parasite infections has been previously determined (Simpson 1997). It was thought to be a good indicator to determine the selectivity of a parasite to multiple invade erythrocyte. This is calculated according to the following equation:

$$\text{Selective index (SI)} = \frac{\text{Observed number of multiple infected cells}}{\text{Expected number of multiple infected cells}}$$

This is determined by counting parasitemias from Giemsa stained thin blood films for all the relevant experiments, along with counting the percentage of single infected, double infected, triple infected and quadruple infected (if applicable) ring and trophozoite stage cells in 300-1000 cells, depending on high or low parasitemias in each sample. Expected number of multiple infected cells is number of parasitized erythrocytes over total number of cells, and Observed number of multiple invaded cells is Number of multiple infected cells over number of total infected cells.

3.11 Duffy knockout mice challenge (In collaboration with Osamu Kaneko)

P. yoelii 17XL and NL strains were previously characterized and injected as before into donor C57/Bl6 wild type mice. Animals were sacrificed, and parasitized blood collected as before. Schizonts were harvested and RNA extracted and cDNA and Real Time PCR performed as before. 10^6 mixed stage parasites were also injected strain into groups of three wild type C57/Bl6 mice and

transgenic Duffy knockout C57/Bl6 mice (from Sekimizu) that have been previously characterized (Swardson-Oliver et al., 2002) (Dawson et al., 2000). Parasitemias monitored, schizonts harvested and RNA extracted and cDNA and Real Time PCR performed as before.

4. SELECTIVE INDEX AS A GOOD MARKER FOR VIRULENCE

4.1 Abstract

Previous studies have classified different clones of *P. yoelii* as virulent, avirulent or of intermediate virulence based on the ability of the host to survive an infection with a standard parasite inoculum (Walliker et al., 1976). While this classification is satisfying at the extreme ends of the spectrum it is unable to distinguish more subtle differences. In this section, we utilized the Selectivity Index (SI) of these parasites to correlate host cell specificity and virulence.

4.2 Introduction

Virulence has been classically defined as the ability of the parasite to cause a pathological disease in a host (Oxford English Dictionary). While previous reports measure virulence using indices of pathology, severity of disease symptoms, anemia levels, gross weight and gross anatomical changes (such as spleen size after infection), an alternative indicator is the survival of the animal and parasite multiplication rates. In our *P. yoelii* experimental model, we have observed avirulent infections where parasites reach 40-50% parasitemias and are cleared by the host, and virulent infections that rapidly increase in parasitemias to 70-80%, followed by the death of the animal. Intermediates exists where a typically mild or virulent infection is not observed. In this case, some infected

animals survive after exhibiting high parasitemias of around 70%, while some animals die. Parasites exhibiting intermediate virulence characteristics have been previously observed in various reports (Landau and Chabaud, 1965; Walliker, Carter, and Morgan, 1973; Walliker et al., 1976; Yoeli et al., 1975).

In *P. yoelii* virulence may be a direct reflection of the range of erythrocytes the parasite can invade. To develop a more quantifiable indicator of available erythrocytes for invasion and thereby virulence we investigated the Selectivity Index (SI) as a possible parameter. SI works on the basis that parasites are capable of invading a single erythrocyte multiple times, especially when the number of cells available for invasion is small. It uses the observed number of multiple invasions divided by the expected number of multiple invasions determined by a Poisson distribution to calculate the selectivity of a parasite to invade a single cell more than once. SI has previously been shown to be a reliable indicator of the erythrocyte repertoire different malaria parasites can invade (Simpson et al., 1999), with high SI indicating a limited number of erythrocytes available for invasion.

In specific and well-defined conditions (in laboratory animal infections), SI correlates with virulence of *P. yoelii*, especially when comparing across distinct clones and lines of *P. yoelii*. In *in vivo* systems, other indices of virulence may be skewed by immune responses of the host and subtle changes in the age, weight, diet, etc of the host animal. Using SI allows us to define virulence along with

parasitemias and survival of infected animals, and thus is especially useful in defining the limits of parasites exhibiting intermediate virulence.

4.3 Results and discussion

We have previously discussed that YM invades red cells indiscriminately, and causes a fulminating infection, while YA is reticulocyte restricted, and causes a milder infection. With this information, we expect selectivity to the erythrocyte population to differ in these parasites. We first determined the SI for *P. yoelii* clones YA (Jarra and Brown, 1989) and YM (Yoeli et al., 1975) at 5% parasitemia. To illustrate how parasites behave in a similar environment of cells available, we calculated SI on thin blood films of 5% parasitemia infections from these clones (Fig 4-1). Since YA parasites are limited in the repertoire of cells available to invade, these parasites are more likely to invade reticulocytes multiple times (Fig 4-1). This would lead to a high SI. YM parasites would continue to singly invade the broad range of cells, and give a low SI value.

Figure 4-1. Giemsa stained smears of YA and YM blood stage infections

Thin blood smears of Giemsa stained parasites from YA have more multiple invaded erythrocytes compared to the single invaded erythrocytes seen in smears of YM infections. Arrows indicate erythrocytes that are multiple invaded. Animals were screened to have equal starting reticulocyte load before infection using Brilliant Cresyl Blue stain (Giemsa).

We then studied the SI values of a range of parasites exhibiting variations in virulence to see if SI can be correlated to virulence. The parasites used were 17X 1.1 (Landau and Chabaud, 1965; Weinbaum, Evans, and Tigelaar, 1976) as well as the parents and a number of previously characterized progeny between a cross of a virulent YM (Edinburgh) and avirulent AC *P. yoelii* clone (Walliker et al., 1976). Progeny had been classified as either virulent, avirulent or of intermediate virulence. It is apparent from this analysis that there is a clear relationship between SI and virulence, when we look at all parasites studied (Table 4-1). Parasites that are considered virulent have low SI while avirulent have a high SI, parasites that were consider of intermediate virulence also had intermediate SI values.

Parasite	Virulence phenotype	Selective Index (range)
17X YA	avirulent	3.5 (3.2-3.8)
17X YM	virulent	0.1 (0.12-0.14)
17X 1.1 NL	avirulent	7.8 (7.5-8.1)
YME (Edinburgh)	virulent	0.26 (0.22-0.3)
AC	avirulent	2.4 (2.2-2.6)
604	virulent	0.18 (0.16-0.2)
605	virulent	0.13 (0.11-0.15)
606	intermediate	0.86 (0.83-0.89)
607	virulent	0.2 (0.18-0.22)
611	avirulent	3.6 (3.2-4)
612	intermediate	0.27 (0.24-0.3)

Table 4-1. SI values and ranges for parasites of varying virulence.

Intermediate virulent parasites exhibit intermediate SI values while avirulent parasites have high SI values, and virulent parasites have low SI values. SI values are expressed as mean SI, with the range of SI observed in multiple experiments in brackets.

It is interesting to note that the parasites exhibiting intermediates of virulence appear in selectivity closer to the virulent parasites, indicating that the leap from intermediate virulence (where anywhere from 50 to 80% infected animals die after infection), to virulence (where all animals die after infection) is a relatively small one. The subtle change between these parasites may mediate the drastic difference in outcome of death or survival of the infected mouse.

4.4 Conclusions

A higher SI signifies a limited repertoire of cells available for the parasite to invade and coincides with parasites causing a mild infection. The parasite's intrinsic multiplication potential is an important virulence factor in *P. falciparum* and *P. yoelii* malaria. The variations in the utilization of parasite ligands can contribute to the virulence of the parasite and outcome of the infection. It has been previously shown that parasites causing severe malaria exhibited unrestricted red cell invasion, whereas those from uncomplicated malaria were restricted to a smaller subset of red cells, indicating that parasites causing more severe disease were less selective and multiplied more at high parasitemias than those causing uncomplicated malaria in Thailand (Chotivanich et al., 2000).

While there are a lot of ways to study and define virulence, in this study we have opted to look at the parasite red cell selectivity index (SI) as an indicator. SI defines the range of erythrocytes available for invasion by the parasite. In the case of *P. yoelii*, virulence is a direct result of an expanded erythrocyte range, and the ability to invade mature erythrocytes, so this is an appropriate measure for our study. Previous estimates of virulence were done by assessing survival of the experimental animal, weight loss, anemia levels or the severity of disease symptoms in patients. We also need to exclude other parasite intrinsic factors that may cloud the virulent outcome such as cytokine inducing capacity, adhesion properties induced in the infected erythrocyte, cytoadherence and rosette formation.

The link between SI and virulence has been debated extensively in African malaria infections (Deans et al., 2006). Severity due to anemia is often clouded by cerebral and other complications of malaria, or the parasite strains differ in the mechanism of virulence used between Africa and Thailand. The African infections often are due to more than one strain of *P. falciparum* infecting a single patient, or when *P. ovale* and *P. malariae* are also present in populations. Unlike in Africa where the parasite almost never encounters a non-immune individual, in Thailand background immunity would be expected to be negligible due to the unstable transmission. This is why in Thailand virulence may be a direct result of expansion of the infection into a larger repertoire of cells (Chotivanich et al., 2000).

In our model, *P. yoelii* infections in rodents primarily exhibit virulence effects by rapid multiplication in the red blood cells and causes anemia in the infected host. SI can be measured best when the host environment is stable, when parasite conditions are stable. We are able to control for these by using inbred littermate mice as hosts and using clonal populations of fixed numbers of parasites to infect the host. SI is thus a reliable indicator of host range and virulence for our studies.

5. DIFFERENCES IN THE COPY NUMBER OF THE *py235* GENE FAMILY IN VIRULENT AND AVIRULENT LINES OF *PLASMODIUM YOELII*

Iyer, JK, Fuller, K., and Preiser, PR (2006)

Differences in the copy number of the *py35* gene family in virulent and avirulent lines of *Plasmodium yoelii*. Mol Biochem Parasitol 150: 186-191

5.1 Abstract

Differences in *py235* sequence and copy number have been proposed to be responsible for the differences in invasion phenotype seen in the avirulent *P. yoelii* YA line and the virulent YM line. The newly available genome sequence data for the avirulent *P. yoelii* 17X NL 1.1 (albeit incomplete) has now made it possible to investigate this further. A number of approaches including real time PCR were used to determine the exact copy number of individual *py235*. Except for two *py235* genes in YA and one in YM, where differences were seen, there are no other differences in *py235* copy number between the two lines and 17X NL1.1. Analysis of progeny of a genetic cross between YM and an avirulent strain AC yield similar limited variations in copy number. This study shows that the copy number of *py235* in the analyzed *P. yoelii* strains is significantly lower than previous estimates and much more in line with the published genome sequence. The lower copy number as well as the limited difference of *py235* in the virulent lines make it highly unlikely that these are the factors contributing to the differences in invasion observed.

5.2 Introduction

5.2.1 Diversity of the *py235* family

The diversity of the *py235* genes has been notoriously difficult to determine. Data has only been gathered from the genomes of three cloned lines, YA, YM and 17X 1.1NL. 5X coverage partial sequence information is only available for 17X 1.1 NL (Carlton et al., 2002), which makes it difficult to determine if the repertoire is greater or smaller in different strains. Diversity occurs within the *py235* repertoire of a given cloned line and between those of different lines. The exact repertoire of this family in the genome in any cloned line is unknown, and complex subsets have been proposed based on the analysis of different regions of the gene.

Evidence for the diversity of the *py235* repertoire was derived initially from an experiment where phages with a *P. yoelii* expression library were screened using hyperimmune sera, and gene fragments corresponding to *py235* were discovered (Keen 1990). Southern blotting indicated that multiple copies (at least 3) were present in the genome. The second evidence for diversity came from sequence analysis of a 1kb central region amplified from line YM genomic DNA (Borre et al., 1995). The resultant 101 clones of this fragment was analyzed by restriction fragment length polymorphisms (RFLP), and separated into seven variants. Further heterogeneity in the form of varying sequences and point mutations (mostly non-synonymous) was discovered when the 3' end of these variants were sequenced. This data indicated that there were possibly more than 50 different *py235* genes in the YM genome. The second evidence for broad diversity was determined on YA and YM genomes using a tri-peptide repeat region at the

conserved 3' end of the gene (Preiser and Jarra, 1998). Genomic and cDNA templates were used, and results indicated six groups of different repeats. Two were specific to each clone and two shared between YA and YM. The third evidence for diversity was discovered in the YM line, where sequence polymorphisms were seen in the C terminus repeat region, a central region and large fragments in the N terminus for the gene (Narum et al., 2001b). This was determined using cDNA and sequencing. The fourth evidence was through the sequencing of the complete 8kb full length of five of the *py235* genes, and these were cloned from the YM line (Khan et al., 2001). Comparing the sequence of these complete *py235* genes indicated that the 5' end was more diverse than the conserved 3' end. Genes were classified into 4 groups and used to show (through RFLP) that these genes had subtle differences. With the final evidence from the sequencing results from the 17x 1.1 NL strain (Carlton et al., 2002), there were 14 different *py235* genes in that genome, though unassigned contigs may mean that this number can be underestimated. This diversity is shown in a schematic representation (Fig 5-1) (Gruner et al., 2004)

Figure 5-1. Diversity of *py235* genes

py235 gene organisation (schematic representation), a single intron is present at the 5-end of the 8kb gene. The region with high homology to the *P. vivax rhp-2* gene is highlighted in dark green; the polymorphic repeat region at the 3'-end (highlighted in red) and the variable region towards the 5'-end of the gene (highlighted in pink) were exploited for the RT-PCR expression analyses. A partial repertoire of *py235* genes has been obtained for parasites of the 17X line clones YMorA (in yellow, below the centre), by direct sequencing of PCR or RT-PCR products. Five full-length *py235* genes have been characterised so far, but multiple types are known from detailed analysis of defined regions (indicated in the figure). The 5X coverage sequence data for the *P. yoelii* clone 1.1 (in blue above the centre), revealed 15 contigs which bear *py235* genes, though only six are full length. (Gruner et al., 2004) [1] Borre et al 1995, [2] Preiser et al 1998, [3] Narum et al 2001, [4] Khan et al 2001, [5] Carlton et al 2002 [6] Keen et al. 1994 [7] Keen 2000.

5.2.2 Genome organization of the *py235* family

The distribution of the *py235* genes among the chromosome was analyzed using Southern blots (Borre et al., 1995; Owen et al., 1999) in YM and YA parasites and Pulse field gel electrophoresis (Owen, 1994). A range of probes used indicated that *py235* genes were found in chromosomes 1, 5, 6 and 10 in YM and YA lines, and an additional copy was found in chromosome 13/14 in YA. This analysis unfortunately used the method of reading the intensity of different

hybridizing bands as multiple copies of *py235*, and is thus not a good method to determine copy number. Similar results were obtained using the probes designed to the different *py235* groups (Khan et al., 2001), confirming the limited diversity in chromosomal organization of these genes.

5.2.3 Biological implications of *py235* diversity

Biological data indicate that one or more *py235* genes in YM parasites are responsible for normocyte invasion, which leads to an increased repertoire of cells infected and thus increased virulence compared to YA parasites. Thus, differences in *py235* between YA and YM were thought to contribute to the differences in invasion phenotype seen in these lines. We have just discussed how studies focusing on these differences only detected limited diversity in sequence and copy number (Borre et al., 1995; Khan et al., 2001; Narum et al., 2001a; Narum et al., 2001b; Preiser and Jarra, 1998). Copy number estimates ranged from 20 to up to 50 copies of the approximately 8kb *py235* with YA containing at least one additional locus then YM (Keen et al., 1990). These numbers contrasted significantly from the predicted 14 copies detected in the completed genome sequence of the 17X NL 1.1 clone (Carlton et al., 2002). With conflicting data obtained from previous studies, it was important to resolve these differences by determining the exact copy number of different *py235* genes in 17X NL 1.1 using our method and then see if these genes were present in YA and YM. We also obtained during the course of this work five progeny exhibiting variations in virulence isolated from a genetic cross between YM and an avirulent line A/C (Walliker et al., 1976). This would

give us some insight into whether changes or variations in *py235* between the parasite lines could account for the differences in virulence.

5.2.4 The *py235* genes under study

For this study, we sought to determine if the number of duplicated *py235* genes plays a role in virulence. We were not attempting to fully characterize the diversity of the *py235* genes, and the tools available for such experiments are not available to us. The only available resource we have were the genes in the genome database. To determine the repertoire of *py235* genes to use from the database (Fig 5-2), we work with a few assumptions. *Py235* genes have a classic small exon1, a small intron and then a large exon2 structure, and typically have a 500 amino acid homology region, a transmembrane motif, and a cysteine rich region. For our analysis, we required that subset of genes to study should have at least the 500 amino acid homology region, and part of the other criteria to be studied. It is possible that these other *py235*-like genes sharing short regions of homology to the full length genes perform similar functional conservation (that is at present unknown). Similar to the chimeric MAEBL containing a DBL domain, these *py235*-like genes may not be implicated in blood stage recognition of receptors by merozoites. It would be interesting to obtain full length sequence information for these genes nevertheless.

Figure 5-2. *py235* genes in 17X 1.1NL database.

The *py235* genes that share the 500 amino acid homology regions (black) were chosen for this study. The other contigs exhibited too little sequence information for analysis.

5.2.5 Development of *py235* gene specific primers

Within the limitations of incomplete sequence data available, we were reasonably confident that the 14 genes chosen for the study represented the significant members of *py235* genes to study the duplication of these genes in virulent and avirulent parasites. It was important to first develop a set of specific primers for each of the *py235* found in the 17X 1.1NL genome database. Overall contigs containing both partial or full length *py235* sequences were identified in the

database (Fig 5-2) and the sequences were aligned with the previously characterized *py235* E8 gene using ClustalW. Overall homology of PY04438 and PY06018 to full-length sequenced *py235* (E8) would suggest that these two contigs are actually part of the same gene (Fig 3-1). This may also be the case for PY05995 and PY06381 with each of these contigs being part of the same gene as PY02033 or PY03432. We attempted to increase the number of genes that we could study by trying to link these contigs using PCR with unique primers designed against the ends of contigs PY05995, PY06381, PY02033 and PY03432. No products were obtained, indicating that these are likely to be four separate genes in the 17X 1.1 genome.

From the alignment it readily became apparent that there is high conservation between all members of this gene family making the design of unique primers difficult (See APPENDIX-*py235* alignment performed using ClustalW at www.embl.org). After careful manual inspection of the alignment we were able to identify 11 primer pairs that were specific for individual *py235* variants. The location within each *py235* the primer pair was designed against varied from gene to gene (Table 3-4). In all cases the size of the expected PCR product was in the range of 132 – 222 bp. It was not possible to design unique and specific primers to amplify PY02033, or even PY05995, PY04438 and PY06381. Overall we had specific primers for 11 out of a possible 14 genes.

5.2.6 Copy number determination of different *py235* using Southern Blotting (performed by Peter Preiser)

The PCR analysis with the gene specific primers would not be able to establish whether there were multiple *py235* that had a conserved region recognized but were diverged in other regions. Equally this analysis would not be able to establish whether there were multiple identical copies of *py235* in the genome. We thought that RFLP analysis of a specific *py235* using a range of different restriction enzymes predicted to cut either within the coding sequence or outside of it could shed some light onto this. Genomic DNA from both YM and YA were therefore digested with a range of different restriction enzymes. The DNA was separated, blotted and then probed with a PCR product obtained with primers specific for PY04930 (Table 3-4). Based on the 17X 1.1 genomic sequence fragments of 6.8 kb, 5.7 kb, 3.36 kb and 6.8 kb were expected when genomic DNA was digested with BamH1, Bcl1, Cla1 and EcoR1 respectively (Table 5-1). In both YM (Fig 5-3) and YA (data not shown) these fragments were clearly identified. In addition a number of other bands were seen in the different restriction digests. These bands correspond to the expected restriction fragments from other known *py235* genes that have regions of similarity to the probe. These bands however can be attributed to the cross reactivity of the probe to other member of *py235*. While the PCR primers were specific to a unique gene the region amplified still showed a high level of overall sequence homology to other *py235* (Table 5-1). For example in the case of PY04930 there is still a 92% sequence identity to

PY06018. This high identity would lead to significant cross hybridization to other gene fragments even at high stringency hybridizations and washing. All fragments seen in the Southern blot could therefore be attributed to cross reactivity with no additional new band being identified. While this analysis would indicate that PY04930 is only present once in either YM or YA it is still possible that other homologous genes produce bands that are very large and are therefore not resolved by agarose gel electrophoresis (bands > 23.1 kb are seen in Fig 5-3). It is clear that RFLP analysis on its own is not able to completely resolve the issue of copy number and variation between the different parasite lines, and would lead to inconclusive results in the determination of the copy number in virulence. For some of the *py235* genes, it may be possible to utilize probes designed against UTR regions and intron sequences to determine copy number, as an alternative method. However for our study we decided to utilize the quantitative PCR approach, as this would yield more pertinent information, and we had the reagents to address copy number differences among *P. yoelii* strains with variations in virulence.

Figure 5-3. Southern Blot of YM genomic DNA probed with PY01365 specific PCR product (performed by Peter Preiser).

Genomic DNA was digested with a range of restriction enzymes individually, and probed with the PCR product of primers specific to PY04930. Expected fragments of 6.8 kb, 5.7 kb, 3.36 kb and 6.8 kb when genomic DNA was digested with BamH1, Bcl1, Cla1 and EcoR1 respectively are indicated with a star in the short exposure (a). In addition a number of other bands can be seen in the different restriction digests, with hybrids of up to 88% homology detected. Long exposure (b) shows these bands.

Gene	Amplified product	BamHI	BclI	ClaI	EcoRI
	Homology (%)				
PY04930	100	6.8	5.7	3.36	6.8
PY06018	92	>6.9	>6.5	3.4	5.7
PY00649	90	>9.17	4.59	1.69	3.99
PY04630	89	>12	4.53	3.33	5.1
PY03432	89	>5.065	>1.815	>3.195	>2.950
PY02104	88	>5.214	>5.745	3.908	5.124
PY03184	88	>7.061	NA	>6.957	>5.434
PY05054	78	NA	NA	NA	>3.796

Table 5-1. Expected fragments of RFLP Southern Blot.

17X NL 1.1 sequences were compared to probe specific to PY04930, and expected fragments tabulated based on percent homology and size of restriction fragments. NA: not applicable as these enzymes do not cut within sequenced regions of the genes.

5.2.7 *Specificity of the primers*

To assess the specificity of each primer pair we performed PCR using genomic DNA from 17X 1.1, YM and YA parasite clones. In all cases only a single specific band of the predicted size was detected (Fig 3-2), as was also seen for the single copy gene β -actin. These results would indicate that the primers have the expected specificity and that all genes seen in 17X 1.1 are also found in YM and YA. This was subsequently checked by sequencing these amplified products.

Multigene families present difficulties in distinguishing orthologous and paralogous relationships, and the *py235* genes are no exception to this. One limitation to our PCR based approach is that primers designed against strain 17X 1.1 genome information may pick up only one member of *py235* in strain 17X 1.1, but in other parasite may amplify regions from more than one *py235* gene (Figure 5-2). Primer pair A and B may amplify a region unique to gene A, but also pick up an identical sequence in Gene B in another parasite line that has undergone divergent evolution of its *py235* genes (Figure 5-4). Alternatively, in another parasite line, the primers may amplify parts of Gene C, but miss out the variations in the region between the primers. This could lead to an overestimation of copies of genes (as in a case where Genes A and B are in the same organism) or an inaccurate classification of copies of genes (if Gene C is present along with gene A in one organism). At least for YA and YM parasites, we confirmed that all the primers did indeed only amplify a single sequence, and each PCR product was cloned and a number of clones were sequenced. For every PCR product only a

single sequence identical to the sequence of 17X 1.1 could be seen (data not shown). The lack of both size and sequence variation for any of the PCR products would indicate that *py235* genes are highly conserved between different parasite lines. It would be worthwhile to determine the variations (if any) in the *py235* sequence obtained from paralogs in the other strains used later in this study, to fully determine the repertoire of *py235* genes in these parasites.

Figure 5-4. Amplification of multigene families in different organisms.

Primers specific to a single *py235* may amplify similar sequences in other *py235* genes that are not sequences in some parasites. The region between the primers should also be analyzed to ensure that the regions are indeed unique to just a single gene.

5.3 Results and discussion

5.3.1 Copy number determination of different *py235* using real time PCR

We first attempted to determine the copy numbers of these 11 genes in 17X NL 1.1. Quantitative Real time PCR was performed using 17X NL 1.1, YM and YA genomic DNA as well as cloned target sequences as standards. All results were compared to β -actin which served as a single copy gene reference. The real time PCR shows that the number of *py235* genes in 17X 1.1 coincides with the number obtained in the published genome, where each *py235* is present only as a single copy. There were however some clear differences between YM and YA, only a single gene (PY01365) exist as two copies in YM while all the other genes are found as a single copy (Table 5-2). This contrasts with YA where PY01365 is present as three copies, while PY05054 and PY6018 are present as two copies. All other *py235* genes in YA are found in single copies (Table 5-2). These results are consistent with previous data showing that the overall copy number of the *py235* genes is higher in YA than in YM. Interestingly, the overall copy number of *py235* seen in YM is much closer to that of the avirulent 17X NL 1.1 reference strain than YA. This would indicate that *py235* copy number is not necessarily a factor in parasite virulence.

Gene	Actin	PY0 1365	PY0 5054	PY0 3432	PY0 3534	PY0 2104	PY0 1185	PY0 4630	PY0 4930	PY0 3184	PY0 0649	PY0 6018
Size PCR product	252	159	157	161	176	181	222	141	212	147	132	151
copy no YA (15)	1	3	2	1	1	1	1	1	1	1	1	2
copy no YM (12)	1	2	1	1	1	1	1	1	1	1	1	1
Copy no 17X1.1 (11)	1	1	1	1	1	1	1	1	1	1	1	1
Copy number YME (11)	1	1	1	1	1	1	1	1	1	1	1	1
Copy number AC (12)	1	1	1	1	1	1	1	1	1	1	1	2
Py235 variants						Y11182 [1] (100%) Y11186 [1] (99%) AF31492 0 [2] (100%)		U19979 [4] (99%) U19985 [4] (99%) AF31492 1 [2] (99%) AF31528 2 [2] (99%) AF31528 3 [2] (99%) AF31528 8 [2] (99%)	Y11181 [1] (86%) Y11184 [1] (99%) Y11185 [1] (86%) AF32344 2 [3] (100%) U19988 [4] (99%) U19989 [4] (99%) U19990 [4] (99%) AF31528 0 [2] (99%) U19991 [4] (100%) U19992 [4] (99%)	AF32344 3 [3] (99%) AF32344 4 [3] (99%) U19980 [4] (99%) U19987 [4] (99%) AF31527 7 [2] (99%) AF31528 0 [2] (99%) AF31528 4 [2] (99%) AF31528 6 [2] (99%)	U19981 [4] (100%) U19983 [4] (100%) AF31492 2 [2] (100%) AF31527 9 [2] (93%)	U19982 [4] (100%) U19984 [4] (99%) U19986 [4] (96%) AF31527 8 [2] (99%) AF31528 1 [2] (99%) AF31528 5 [2] (99%) AF31528 7 [2] (99%) AF31538 0 [2] (99%) AF31538 1 [2] (99%) AF31538 2 [2] (99%) AF31538 3 [2] (99%)

Table 5-2. Copy number of *py235* genes in parasites.

Total *py235* copy number for each strain indicated in brackets, with individual gene copy number tabulated for each strain. Results indicate that YA has a higher *py235* copy number than YM. Reference strain 17X 1.1 and two other strains YME and AC also show variations in copy number of PY06018. *py235* variants from other studies on YM and YA DNA with homology to 17X1.1 genome data shown. Round brackets () indicate percent homology to the sequenced strain. Square brackets references sequences from studies as indicated. [1] Preiser et al 1998, [2] Narum et al 2001, [3] Khan et al 2001, [4] Borre et al 1995.

We have discussed that previous work using PCR and RFLP based analyses of both YM and YA had given a copy number estimate of around 25-50 *py235* per genome. Even with the additional four *py235* copies detected in YA there is a significant discrepancy between these results. One possibility is the automated assembly of the 17X NL 1.1 genome was unable to distinguish some of the highly homologous *py235* genes, thereby underrepresenting the overall copy number. Alternatively, it is possible that the PCR and RFLP based analysis overestimated copy number. Artifacts can be generated through low fidelity PCR using a non-proofreading Taq polymerase. This could introduce polymorphisms, and lead to an overrepresentation of the actual copy number. To investigate this further we compared the different sequences and approaches used. Of the six *py235* genes predicted to have multiple copies in the genome of YM or YA only one, PY06018, has two copies as determined by real time PCR (Table 5-2). PY2104 is a single copy gene and this is consistent with the available sequence data for YM/YA with the only difference seen being due to a confirmed sequence variation in YM (Preiser and Jarra, 1998). For PY04630 real time PCR again shows only a single copy in both strains compared to two variants seen by PCR (Borre et al., 1995) and three by RT-PCR (Narum et al., 2001b) in YM. None of the sequences including a genomic clone obtained from YM give a perfect match to this gene, most likely reflecting clone specific variations. The often single nucleotide changes seen in the different PCR and RT-PCR clones could reflect errors introduced by the enzyme and may not actually reflect different *py235* genes in the genome. Using a similar analysis for the other multiple sequences seen in the different studies it became

apparent that PCR artifacts may account for all observed differences. Furthermore, it is clear that minor sequence variations exist between the different parasite strains accounting for the fact that often sequences are only >99% identical. While PCR errors could account for most of the differences it is clear that some difference can not be explained by this. DNA clones Y11181/Y11185 (Preiser and Jarra, 1998), AF315279 (Narum et al., 2001b) and U19986 (Borre et al., 1995) while having the highest identity to PY04930, PY00649 and PY06018 respectively, clearly represent different members of *py235*. The best explanation would be that these sequences do not necessarily represent multiple copies but actually represent some of the sequence gaps still existing for a number of different *py235* in the database (Fig 5-1). We do not know if there are any significant differences in the organization and sequence between YA, YM and the 17X NL 1.1 *py235* genes. The higher copy number of *py235* in YA compared to YM is consistent with previous studies.

5.3.2 The py235 repertoire in a genetic cross indicates that virulent and avirulent *P. yoelii* strains are very similar

The *py235* copy number data obtained from the three different lines gave no indication that variations in any of the known *py235* genes are linked to a difference in parasite phenotype. To determine whether this was also the case for other *P. yoelii* clones, we extended our analysis to a genetic cross between parental lines YM (from an older stabilate from Edinburgh) and AC (an avirulent line). The parents of the genetic cross also share identical numbers of copies of the *py235* genes, with a single exception in PY06018 duplicated in the avirulent AC line (Table 5-3). Interestingly, YM (Edinburgh) initially thought to be identical to the YM clone originally used in this study shows a difference, with YM (Edinburgh) having only a single copy of PY01365 compared to two copies seen in YM. Whether this increase in copy number has developed as a result of continuous selection, random duplication or deletion events after more than 20 years of continuous culture in different host mice or actually reflect two different clones cannot be determined at the moment.

Analysis of five progeny of the cross by quantitative real time PCR as before show that the avirulent clone 606 has a similar duplication in PY06018 to clone AC, unlike all the other virulent and intermediate clones (Table 5-3). Acquisition of the duplicated PY06018 gene during recombination following the cross is inherited like the virulence trait previously reported (Walliker et al., 1976). Whether the maintenance of this duplication is in anyway linked to the avirulent phenotype is unlikely as 17X NL 1.1 has only a single copy of this gene.

Clone	Pyrimethamine sensitivity	SI	Actin	PY0 1365	PY0 5054	PY0 3432	PY0 3534	PY0 2104	PY0 1185	PY0 4630	PY0 4930	PY0 3184	PY0 0649	PY0 6018
604 Virulent (11)	Resistant	0.18	1	1	1	1	1	1	1	1	1	1	1	1
605 Virulent (11)	Sensitive	0.13	1	1	1	1	1	1	1	1	1	1	1	1
606 Avirulent (12)	Sensitive	0.86	1	1	1	1	1	1	1	1	1	1	1	2
607 Intermediate (11)	Sensitive	0.2	1	1	1	1	1	1	1	1	1	1	1	1
612 Intermediate (11)	Resistant	0.27	1	1	1	1	1	1	1	1	1	1	1	1

Table 5-3. Copy numbers from the progeny of a cross between YME and AC

The cross yielded five progeny with varying virulence. Virulence measured by Selective Index (SI), parasitemia rates (Appendix) and survival of host animals. Pyrimethamine sensitivity shows the differences of the progeny from the YM (Pyrimethamine sensitive) and AC (Pyrimethamine resistant) parents.

5.4 Conclusions

It seems that the gene copy number of *py235* does not correlate with virulence in *P. yoelii*, albeit our study has only addressed the copy number for 11 out of 14 *py235* genes, and with more *py235* sequence information available in the future from work done in 17X NL 1.1 and other parasites, we may be grossly underestimating the true variations in these genes. With the current knowledge of these genes, it appears that our results demonstrate that less virulent parasite clones (with the exception of 17X NL 1.1) contain more copies of *py235* than do virulent clones. One can speculate that increased copy number of *py235* give the parasite more flexibility and adaptability as well as an increased capacity to evade host immunity. This would allow the parasite to establish more long lasting infections, thereby increasing the likelihood of transmission. Whether specific amino acid differences observed in corresponding PY235 members in virulent and avirulent parasites are sufficient to account for the difference in phenotype still needs to be established. Previous work done on BAEBL or JESEBL (members of the EBL family of *P. falciparum*) have shown that even a single amino acid change in the binding region can lead to the recognition of a different receptor (Mayer et al., 2002; Mayer et al., 2004). The lack of the complete sequence of all the different *py235* of both virulent and avirulent parasites as well as the lack of information on which regions within PY235 mediate the binding to the erythrocyte receptor will make it difficult to satisfactorily resolve these issues in the near future.

It is important to note that at this point, there are a lot of singletons, and contigs in the *P. yoelii* genome database that resemble the *py235* gene. The 11 genes chosen for this study and subsequent analyses in this dissertation exhibit close resemblance to a classical *py235* gene, defined by a two exon, one small intron structure, with conserved cysteine residues and a transmembrane domain. Performing a BLAST search of these known *py235* genes against the singleton database has yielded many sequences that may be *py235*, but are more likely other genes that share some sequence similarities with the other *py235* genes.

From this work, we suggest that the copy numbers of *py235* in YA and YM have been overestimated. This highlights the fact that variations in copy number between the different parasite lines exist and these differences may contribute to significant biological differences. Currently, genome data is not only available for *P. falciparum* and *P. yoelii* but also a range of other *Plasmodium* species and this serves as an invaluable resource. Importantly, this work presented in this study shows that especially in the case of multigene families, important variations between different parasite strains can exist and these differences need to be taken into account to ensure the correct interpretation of any results.

6. VARIATIONS IN THE EXPRESSION OF THE 235 kDa RHOPTRY PROTEIN OF *PLASMODIUM YOELII* MEDIATE HOST CELL ADAPTATION AND IMMUNE EVASION

Iyer JK, Preiser PR (2006).

Variable expression of the 235kDa rhoptry protein of *P. yoelii* mediate host cell adaptation and immune evasion. (Submitted)

6.1 Abstract

One of the key determinants of virulence during malaria infections is the rapid asexual multiplication of the parasite in its host. Here we show that variations in the amount of transcript and protein of *py235* are a mechanism that the parasite uses to define its host cell repertoire. High levels of *py235* transcription leads to a wider range of erythrocytes invaded and therefore increased virulence. This is observed in adaptation of the parasite to a different host species and in virulent parasites. In contrast, to evade PY235 specific immunity, the parasite either downregulates *py235* transcription thereby decreasing the host cell repertoire and virulence, or switches to utilize another variant of *py235*, and maintain infection. These results show a new mechanism where variations in the quantity of parasite transcript define the parasite host cell repertoire and enable it to evade host immunity.

6.2 Introduction

Since variations in the copy numbers of the *py235* genes that were studied in the previous chapter do not seem to have a profound effect as a predictor of virulence, we explored whether variations in expression correlate with virulence. In this chapter, we have analysed the transcription pattern of PY235 in virulent and avirulent parasites using quantitative Real time RT PCR. Once this was established, we investigated changes in patterns of expression of *py235* in host cell selection and immune evasion. In *P. yoelii*, *P. vivax* and *P. falciparum* RBL proteins have also been shown to bind to uncharacterized receptors on the erythrocyte (Duraisingh et al., 2003b; Galinski et al., 1992; Ogun et al., 2000; Rayner et al., 2001). These findings indicate that PY235/RBL recognizes unique receptors on the surface of the erythrocytes and thereby determines host cell specificity. While the receptor for PY235 remains unknown, we have designed experiments to possibly alter the host cell repertoire to illustrate the effect of host range on *py235* expression. We then studied the effect of specific immunity to PY235 and the subsequent changes in *py235* expression in challenged parasites. Using the model previously described (Figs 1-9, 1-10 and 1-12), we expect significant changes to the expression of the *py235* genes with each of these pressures, namely changes in the host cell repertoire and immune system, that illustrate how the parasite uses *py235* to maintain infection and not lose its transmission potential in the midst of a changing environment.

6.3 Results and discussion

6.3.1 *Variations in the levels of transcription of different Py235 genes.*

We have previously discussed the evidence available linking PY235 with parasite virulence and host cell selection. In order to investigate this further we established the transcription profile of all the 11 different *py235* in parasite clones displaying differences in virulence as well as in parasites encountering a different host cell environment. BALB/c mice were infected with either *P. yoelii* YA or YM and blood stage schizonts were harvested for RNA extraction and subsequently used for quantitative RTPCR analysis. Due to the high sequence similarity between the different *py235* as shown in the alignment (Appendix-Alignment) it was difficult to develop gene specific probes suitable for quantitative Northern blot analysis. We therefore used a quantitative Real Time RT-PCR method to accurately and consistently determine the transcription levels of different *py235* members. This approach and primers has already been successfully used to estimate the copy number of the different *py235* genes in the different *P. yoelii* clones utilized in this study, as shown in Chapter 5 (Iyer, Fuller, and Preiser, 2006). Controls were chosen based on those that were transcribed in constantly across similar numbers of schizonts used from different parasites. Transcript amounts of histone, fructose biphosphate aldolase, β -actin and 18s rRNA were found to give similar results. For consistency, all data presented here is normalized to parasite 18s rRNA,

6.3.1.1 Transcription profile of *py235* in YA parasites in a BALB/c mouse

Both in terms of absolute amounts as well as when expressed as percentages, we obtain a pattern of the *py235* transcription profile in each parasite. A number of characteristics of this profile is seen in YA infections in BALB/c mice. Transcription levels observed for the different genes is reproducible in different experiments, with PY01365 consistently being transcribed at the highest and PY05054 at the lowest level. The transcription pattern is also reflected at the protein level with a dominant member of PY235 being detected by anti-PY235 specific antibodies on a Western blot using a polyclonal antibody raised to a region conserved in PY235 called S6 (Keen et al., 1994; Sinha et al., 1996) (Fig 6-1 inset). However there may be more than a single member depicted by this single band seen on a gel. Without mass spectrometry analysis it is difficult to assume that this band corresponds to PY01365, but from this Western Blot, it is clear that multiple members of PY235 are expressed. The data here is consistent with our model that a unique member of *py235* will dominate at the population level after subsequent rounds of replication in a suitable host.

Figure 6-1. *Py235* profile of YA parasites harvested from BALB/c mice.

Quantitative Real timePCR of a selection of *py235* genes from YA schizonts from BALB/c mice. Results shown are normalized to 18s rRNA and expressed as percent of total *py235* transcribed for 11 genes. Data indicates that a differential expression of *py235* is seen with PY01365 as the dominant transcript. Inset: Western Blot of lysate of schizont extract from the same parasites with polyclonal S6 raised to a conserved region shared among the PY235s as a primary antibody. This shows that multiple members of PY235 are expressed, some at higher levels than others.

6.3.1.2 Transcription profile of *py235* in YA parasites in a Rat

Based on our model, we would expect that changes in the host cell receptor would lead to merozoites expressing another variant of PY235 dominating at the population level. While the receptor for PY235 is not known, we would expect that different host mouse strains would express variations in host receptor. To explore whether host cell variation could lead to a change in the dominant *py235* expressed we first adapted *P. yoelii* YA to a range of inbred animals, including CBA mice, C57/Bl6 mice and outbred Swiss mice. No significant changes in infections or *py235* profiles were observed in all these cases, indicating that a common receptor for *py235* may be shared among these hosts, or that the dominant protein is capable of functional binding the different receptor(s) on the new host cells.

A change in the species of host animal may then elicit a change in the host receptor repertoire, and hence we chose to infect rats instead of mice. When we adapted *P. yoelii* YA to Wistar Rats, changes in infections and *py235* transcription were observed. Only 40-60% of young rats became infected when inoculated with *P. yoelii* YA harvested from a donor mouse indicating that unlike in mice, *P. yoelii* has a difficulty establishing an infection in this seemingly less suitable host. Adapted parasites harvested from donor rats on the other hand were able to infect all new acceptor rats. The parasites retained their ability establish infections when parasites were passaged back and forth between rats and mice across two generations. Similar to the infections in mice, blood stage schizonts from YA infected rats were harvest for RNA extraction.

There are some noteworthy changes in the *py235* transcription profile in rat adapted YA compared to that seen in mice. PY01365 is an even more dominant transcript in rat adapted parasites (>50% in rats compared to 32% in mice) with PY06018 levels increasing from 12% to 20% of total message in the rat (Figure 6.2). Importantly PY01365 and PY06018 constitute > 70% of the total *py235* message in YA parasites harvested from rats compared to approximately 40% in mice (Compare figures 6.2, 6.1 and 6.4). Correspondingly, there is a significant reduction in transcripts from PY03534, PY01185 and PY02104 in YA parasites obtained from rat.

Figure 6-2. *py235* transcription profile of YA parasites harvested from young Wistar Rats.

Quantitative real time PCR of transcribed *py235* genes in YA parasites harvested from Wistar Rats. Results shown are normalized to 18s rRNA and expressed as percent of total *py235* transcribed for 11 genes. Error bars indicate data obtained for three infections. The data shows that there exists a differential expression of the *py235* genes, with PY01365 dominant. The dominant transcripts also appear to contribute a higher percentage of total *py235* transcribed than in the previous figure (Fig 6-1).

6.3.1.3 Transcription profile of *py235* in YM parasites in a BALB/c mouse

Since changes in *py235* expression may reflect both host cell adaptation as well as virulence we investigated the expression pattern of *py235* in the highly virulent *P. yoelii* YM clone. This parasite is able to invade erythrocytes indiscriminately and this is reflected by the very low SI of 0.1, previously discussed in Chapter 4. We first observed that the transcription profile of *py235* in YM is very similar to YA (Fig 6-3) with PY01365 still being the dominant transcript. However, there are some important differences with PY03184 now making a substantial contribution to *py235* transcription levels (16% in YM, 2-3% in YA mice/rats), while PY01185, PY02104 and PY04360 were significantly reduced. Western blot analysis using again the PY235 specific polyclonal antibody S6 confirms this result with at least one dominant PY235 variant being detected (Fig 6-3 inset), although this needs to be confirmed by Mass spectrometry.

Figure 6-3. *py235* transcription profile of YM parasites harvested from BALB/c mice.

Quantitative Real time PCR of total transcribed *py235* from YM parasites show that a differential expression of *py235* is observed, with PY01365 as the dominant member. Results shown are normalized to 18s rRNA. Inset: Western Blot of Lysate of Schizont extract from the same YM parasites with polyclonal S6 as a primary antibody against conserved regions shared among the *py235* genes. Results indicate that more than one member is expressed of this family and at different levels.

6.3.1.4 Comparison of *py235* transcription profile in YA parasites in BALB/c mice, YA parasites in Rats and YM parasites in BALB/c mice.

When we compare all the three profiles, we see that the subtle differences observed in transcription may contribute to the differences seen in virulence between these infections (Fig 6.4). Statistical significant changes are indicated by a star compared to YA infections in mice. PY02104 is not transcribed in YM parasites, or transcribed below detection levels compared to YA parasites. Expression of PY03184 is high in YM mice compared to YA parasites. Rat infected YA expresses higher levels of PY01365 than mouse infected YA parasites. These subtle differences could account for phenotypically unique invasion properties of these parasites.

Figure 6-4. *py235* transcription profile of YA Mouse, YA Rat and YM Mouse. Quantitative Real time PCR of total transcribed *py235* from three parasite infections show a difference in the total *py235* repertoire transcribed and possibly utilized. Asterisks (*) indicate statistically significant changes to expression compared to YA parasites in BALB/c mice ($p < 0.05$). Results shown are normalized to 18s rRNA. Inset: Western blotting using S6 antibody on YA and YM parasites where equal numbers of schizonts were loaded on each lane.

The data obtained so far fits into our model of the differential expression of *py235* genes in schizonts (Fig 6.5), where parasites maintain infection despite heterogeneity of receptor encountered. Although our initial prediction was that parasites may use different ligands (proportions of ligands expressed or combinations of ligands) for each receptor encountered, it seems that the data shows that when the host receptors are assumed to be altered (compare rat and mouse infections), the parasite transcribes a unique profile of *py235* variants, and when the receptor is the same, but the parasite different (compare YM and YA infections in mice), the parasite transcribes yet another unique profile of ligands. This indicates that the parasite at the population level is able to compensate for the lack or change in receptor when it encounters a different host. However different parasites in a similar host use other ligand profiles to invade. The dominant ligand remains the same (PY01365), indicating that a common receptor may be shared among the host animals, or that the parasite is able to bind the different receptor with the same dominant ligand.

Figure 6-5. Model for differential expression of PY235 in schizonts.

A schizont ruptures to produce merozoites each expressing a single different variant of PY235, and with different invasion efficiency. Colours indicate different variants of PY235. Merozoites expressing the most successful PY235 variant will dominate at a population level in subsequent cycles. This leads to a differential expression of PY235 in a population of schizonts.

6.3.1.5 Clustering of genes to mild and virulent parasites

We analysed the *py235* transcription profile of a number of previously characterized progeny between a cross of a virulent YM (from Edinburgh) and avirulent AC *P. yoelii* clones (Walliker et al., 1976). Progeny had been classified as either virulent, avirulent or of intermediate virulence as in Chapter 4. The analysis of the transcription profile of these parasites did not yield significant differences. PY01365 was still the dominant gene transcribed in these parasites (data not shown), however, when we analysed the contribution of each *py235* gene to the sum total of all transcribed *py235* clustering among the virulent or avirulent parasites, we obtained some interesting results. This was measured using the same quantitative Real time PCR data used to determine *py235* transcript amounts, and plotted on a graph to show contribution of each gene to overall *py235* transcribed (Figure 6-5)

Figure 6-5. Contribution of specific *py235* genes to overall *py235* profile.

Real time PCR results of *py235* transcribed amounts were measured as before and overall contribution of all genes to *py235* was plotted. Only in (A) PY03432, (B) PY05054 and (C)PY04630 shown above are there any clear clustering of expression to mild and virulent parasites. This was performed for three animal infections. (D) indicates a hierarchical clustering of this analysis.

From this data it is possible that PY03432 and PY05054 may be associated with the mild phenotype and PY04630 may be associated with the virulent phenotype. How the lesser transcribed genes affect virulence still needs to be elucidated, and whether these lesser transcribed (and possibly expressed) variants of *py235* can be considered true virulence factors, can only be addressed with more

virulent, avirulent and intermediate strains, and an analysis of the protein expression levels, localization and use of these proteins in invasion. Specific antibodies are required for this to be analyzed, and must remain as a future directive. It is however interesting to speculate that these lesser transcribed members may be expressed as proteins that complex with the dominant *py235* variant, and act together, to determine virulence outcome. The complex formation could be similar to one suggested for *P. vivax* (Galinski et al., 1992)

6.3.2 Upregulation of Py235 transcription in virulent and adapted parasites

We have shown that there were a number of changes in the *py235* transcription profile in both YA parasites adapted to rats and YM parasites. It was also important to establish whether there might be changes in the overall expression level of total *py235*. We reanalyzed the data to determine the overall *py235* transcript amounts in parasites normalized to 18s rRNA amounts. Surprisingly, dramatic differences in amount of overall transcript levels between parasites was observed when analyzing total *py235* transcript in the different infections (Figure 6-6 and 6-7).

6.3.2.1 Upregulation of *py235* transcribed in rat adapted YA parasites

A dramatic difference is observed when comparing the total amount of *py235* expression in the adapted parasites (Fig 6-6). It is clear that YA upregulates total *py235* nearly 100 fold in rats as compared to mice. This variation in transcript level changes rapidly as the parasite is switched between the two hosts (where *py235* levels drop again to lower amounts when switched back to mice) suggesting that epigenetic factors rather than specific mutations play an important role. The rapid change in establishment of the infection in the new host would also imply that the parasite may utilize a mechanism of sensing the host environment in order to ensure a rapid response to *py235* transcription. Parasite multiplication rates remain predictable, and so indicative of a mechanism of switching to enhanced *py235* transcription, not selection, and no delay in obtaining parasites was seen after infecting the host animal intravenously with 10^7 parasites. The need for YA to adapt to rats indicate that rat erythrocytes are more difficult to invade than mouse erythrocytes, possibly due to variations in the PY235 receptors utilised during the invasion process. From our data it is possible that only PY01365 and maybe PY06018 are efficient parasite ligands in rats, though this should be tested when protein expression differences can be determined.

We postulate that upon selection, the merozoites expressing the most utilized ligand will be selected for and persist in the population, consistent with our model, while possibly diluting the profile of gene expression of the other ligands. The other ligands may play a role in interacting with the dominant ligand, but this remains speculative. The reliance on these two PY235 members alone is though

not sufficient to ensure efficient invasion and it appears that in order to achieve successful invasion of rat erythrocytes, parasites increase their levels of *py235* ligand and expand its available repertoire of cells to include rat cells. Our data indicates that the parasite may have an intrinsic ability to regulate its *py235* levels to adapt to different hosts encountered, ensuring its transmission potential in the new host environment.

Figure 6-6. Total *py235* transcripts amounts in adapted YA parasites.

Quantitative Real time PCR of total transcribed *py235* from YA parasites show that an upregulation of *py235* is seen in parasites adapted to and harvested from rats. Total transcript amounts are calculated as the sum of all *py235* transcripts normalized to 18srRNA, and results are relative to matched number of schizonts where cDNA was derived from. The *py235* levels reduce again when parasites are passaged back into mice, but remain high in subsequent rat to rat passages.

6.3.2.2 Upregulation of *py235* transcribed in mouse YM parasites

We then reevaluated our data to calculate the transcript amounts of *py235* in the truly virulent parasite YM and compared it to YA parasites in BALB/c mice. The absolute amounts of total *py235* is seen in YM with 100-1000 fold higher levels being detected as compared to YA (Figure 6-7), an upregulation similar to the results observed in YA after adaptation to rats. The mean selective index for these parasites is also shown.

Figure 6-7. Total *py235* transcribed in YA and YM parasites from BALB/c mice

Quantitative Real time PCR of total *py235* transcribed in YA and YM schizonts show an upregulation of *py235* levels in the more virulent YM parasites. A lower SI value in the YM parasites signify that a wider repertoire of cells are available for this parasite, compared to the YA parasite. Total *py235* transcribed is measured as sum of all *py235* transcripts normalized to 18srRNA for matched numbers of schizonts where cDNA was generated from.

From this, it is clear that high *py235* levels may correlate with low SI. To determine whether we are observing a general upregulation of invasion molecules in the virulent YM parasite, we also compared the transcription levels of *P. yoelii* specific merozoite surface protein (MSP)-1 and the erythrocyte binding protein (EBP) and found no change in transcription levels of these genes between YA and YM parasites (Fig 6-8). This indicates that the transcription upregulation is specific to the *py235* genes.

Figure 6-8. Total amount of other invasion genes transcribed by YA and YM parasites.

Quantitative Real time PCR of EBL and MSP1 in YA and YM parasites normalized to 18s rRNA in five experiments. Results show that the levels of EBL and MSP1 did not differ significantly in YA and YM parasite derived schizonts.

Importantly the upregulation of PY235 was also reflected at the protein level with Western blot analysis of matched samples (Fig 6-9) using monoclonal 25.77 antibody against at least one member of PY235 (Holder and Freeman, 1984b; Ogun et al., 2006) indicating a 3-5 fold increase in total PY235 in YM, compared to YA. Hyperimmune serum recognizing other parasite proteins that are expressed at equal levels in both YA and YM parasites, and this was used as a loading control. There is a lack of suitable loading controls for *P. yoelii* that we had available to us. To ensure equal loading, we also matched equal numbers of YA and YM schizonts loaded onto each lane.

Figure 6-9. Western Blot of YA and YM schizont lysates with 25.77 Monoclonal antibody

Results show that YM parasites express more PY235 protein than YA parasites. Parasite protein recognized by hyperimmune serum was used as a loading control.

6.3.3 *SI and py235*

From this data it became clear that upregulation of overall *py235* may be a mechanism by which the parasite is able to increase its host cell repertoire and therefore virulence. We therefore investigated whether there was a direct relationship between SI of different *P. yoelii* strains and the amount of *py235* expression using 17x NL 1.1 and the parasites from the genetic cross. The total amount of *py235* transcription for each parasite was then determined and correlated to SI. It is clear from these data that there is an inverse relationship between *py235* levels and SI confirming our initial observations that high levels of *py235* may enable merozoites to invade a wider range of erythrocytes. We analysed the *py235* transcript amounts of a number of previously characterized progeny between a cross of a virulent YM and avirulent AC *P. yoelii* clones (Walliker et al., 1976). Progeny had been classified as either virulent, avirulent or of intermediate virulence as in Chapter 4. The total amount of *py235* transcription for each parasite was then determined and correlated to SI (Fig 6-10 and appendix). It is clear from this data that there is an inverse relationship between *py235* levels and SI confirming our initial observations that high levels of *py235* enable merozoites to invade a wider range of erythrocytes. The table of SI and virulence phenotype for each parasite clone or line is shown in Chapter 4.

Figure 6-10. Relationship between mean SI and amount of *py235* transcribed.

X-axis is the total level of *py235* transcribed in each schizont population determined by Quantitative real time RT-PCR. Y axis is the SI calculated for each parasite strain. An inverse relationship exists between SI and parasite *py235* levels. Phenotype of parasite virulence is indicated. Intermediate virulence refers to an atypical course of infection which cannot be classified as either virulent or avirulent (mild) and in such cases, some infected mice are able to clear extremely high parasitemia. See appendix for this data tabulated.

6.3.3 *Py235* expression under immune pressure

From the model of clonal phenotypic variation, we propose that merozoites expressing the dominant PY235 ligand will not be able to persist due to specific immune attack after subsequent rounds of multiplication. We predict that the parasite population is selected to one with a subset of merozoites expressing a different dominant ligand. We tested this hypothesis by using passive transfer experiments and challenges with YA and YM parasites.

6.3.3.1 Immune modulation of *py235* in YA parasites

It has been shown that specific immunity against PY235 can modulate an infection (Fig 1-12). Passive transfer with anti-PY235 specific monoclonal antibody 25.77 and immunization with purified antigen can convert a lethal infection into a mild self-limiting one, when challenged with YM and also reduce the peak parasitemias and multiplication rates of YA infections when the experimental animal is passively transferred with 25.77. (Fig 6-11).

Figure 6-11. Passive transfer experiment with Monoclonal antibody 25.77 and challenge with YA parasites

Parasitemias increase rapidly in YA infections in BALB/c mice (Black). Passive transferred mice upon challenge with YA parasites are protected from this rapid rise in parasitemia and are usually cleared at lower peak parasitemias (green) than the control. End points of the graphs indicate point of schizont harvest for subsequent experiments

To study how PY235 expression changes under immune pressure, we first studied the transcription profile of *py235* in mice infected with YA in the presence and absence of protective PY235 antibodies (Freeman, Trejdosiewicz, and Cross, 1980). We observed no significant differences between the profile of *py235* transcribed in the two experiments, with PY01365 again being the dominant member expressed, contradicting our hypothesis. However the relative abundance of *py235* transcripts in the parasites from the protected animals was significantly reduced (Fig 6-12). These data show that down regulation of *py235* is associated with the ability of the parasite to evade the host immune response while at the same time impacting on the invasion potential of the merozoites (and thus lower

parasitemia rates). The fact that there is a direct correlation between *py235* transcript levels and available host cell repertoire (SI) would suggest that the lower parasitemia rates in the protected animals is due to a lower invasion potential of the merozoites. To establish whether these merozoites indeed have a reduced invasion potential we determined the Selectivity Index (SI) of the parasite in the presence and absence of the monoclonal antibody. There is a significant increase in the SI in parasites that are grown in the presence of the protective monoclonal antibody (Figure 6-12) suggesting that merozoites with low levels of *py235* are limited in the erythrocytes that they can invade. One way to explain this is by arguing that the parasite has evaded the immune system by reducing the repertoire of cells invaded and reducing its virulence potential. It is still possible that under a longer term, constant specific immune attack against one single variant of PY235, a different dominant member will be expressed, but the reagents (antibodies that are specific for each variant) are currently unavailable to test this.

Figure 6-12. Down regulation of *py235* upon passive transfer with 25.77 in YA parasites.

Quantitative real time PCR of total *py235* transcribed show a downregulation of *py235* levels in protected animals challenged with YA, compared to the no antibody control. SI indicates mean selectivity index of each experiment, SI= 3.5 in the no antibody control experiment and 10.2 in protective 25.77 experiment. This shows that a higher selectivity (more multiple invasions) is observed in the protected infections.

6.3.3.2 Immune modulation of *py235* in YM parasites

A different phenomenon occurs in YM parasites. When mice were passively transferred with 25.77 and then subsequently challenged with YM parasites, the parasitemia curves were significantly changed, changing a lethal infection into a non-lethal one. (Fig 6-13). Interestingly, unlike in YA, there was no change in the SI between these two populations of parasites, indicating that the repertoire of cells being invaded by YM parasites were not affected by the antibody.

Figure 6-13. Parasitemia curves for passive transfer experiment with 25.77 and YM challenge.

Parasitemias increase rapidly in YM infections in BALB/c mice (data presented in black), and usually lead to the death of the infected animal. Passive transferred mice upon challenge with YM parasites are protected from this rapid rise in parasitemia and are cleared at lower peak parasitemias (Data presented in green) than the control. End points of the graphs indicate the point of schizont harvest for subsequent experiments

This lack of change in SI led us to investigate the *py235* transcribed levels in this experiment. There was no subsequent change in *py235* transcribed total amounts between these populations of parasites, coinciding with the lack of difference in SI (Fig 6-14)

Figure 6-14. Total *py235* transcribed in passive transfer experiment with protective antibody 25.77 challenged with YM.

Quantitative real time PCR of total *py235* transcribed show no change in total *py235* levels in protected animals challenged with YM, compared to the no antibody control. SI values remained at a low selectivity index of 0.1 for both experiments.

As there was no change in *py235* amounts, we investigated the profile of the individual genes in this experiment. Interestingly, the profile between the two parasite populations differed. The dominant transcript in the protected parasites switched to PY06018, while transcript levels of PY01365 along with most of the other *py235* genes were reduced (Fig 6-15). This "switching" to another dominant member was statistically significant ($p < 0.005$), and could indicate that YM parasite utilizes another mechanism for evading the immune system, by varying the *py235* it can use to invade, and forcing the parasite to be less lethal to the animal. This fits into our model, showing that under immune pressure, another variant of *py235* dominates at the population level (PY06018 dominates instead of PY01365). It is also important to note that although the monoclonal antibody used (25.77) was shown to recognize one dominant PY235 variant (Ogun et al., 2006), it appears to inhibit the expression of multiple members in subsequent cycles. This indicates that the antibody is crossreactive to multiple PY235 variants.

Collectively, our data shows that the parasite can use two mechanisms of immune escape, downregulating entire *py235* transcript levels or converting to another member of *py235* dominating a the population level.

Figure 6-15. *py235* transcriptional profile of protected 25.77 and control infections with YM parasites.

Quantitative Real time PCR of the 11 *py235* genes normalized to parasite 18s rRNA show that the differential expression of members of *py235* is observed in both experiments. Of significance, note the level of PY06018 parasites from the protected animals dominating instead of PY01365, signifying a switch in the ligand utilization by these parasites ($p < 0.05$).

6.4 Conclusions:

How merozoites adapt to variations in host cell receptors and immunity is still not completely clear. Even less is known about parasite factors that contribute to differences in host cell repertoire and thereby virulence. Evidence from work initially done in *P. yoelii*, and subsequently supported by studies on the *P. falciparum* RBPHs, show that this family of proteins mediate unique redundant invasion pathways that are important in virulence and immune evasion (Preiser et al., 2000a; Snounou, Jarra, and Preiser, 2000). Furthermore at least in *P. yoelii* PY235 has been implicated as a major virulence factor, since antibodies specifically targeting PY235 affect the outcome of a lethal infection (Freeman, Trejdosiewicz, and Cross, 1980; Holder and Freeman, 1981; Holder and Freeman, 1984b). The results presented here give substantially new insights into how variations in *py235* expression contribute to parasite virulence as well as immune evasion.

In *P. yoelii*, individual merozoites only express a single member of *py235* (Preiser et al., 1999a) and recent work using mass spectrometry has identified PY01365 as one of the dominant PY235 detected by the monoclonal antibody 25.77 (Ogun et al., 2006). Taken together with our data reported here, this would indicate that during the infection in BALB/c mice the parasites makes more merozoites expressing *PY01365* than any other *py235* member. These findings are consistent with our hypothetical model on how PY235 plays a role in host cell selection and immune evasion. This mechanism would ensure that there are

always some merozoites that can invade albeit at reduced efficiency. To maintain transmission in the midst of immunity to dominant variants, the parasite may adopt successive cycles of invasion utilizing merozoites expressing less efficient *py235* variants. This could contribute to the fluctuations in parasitemia seen in chronically infected patients with *P. falciparum* (Miller, Good, and Milon, 1994), as we discussed in the Introduction, though this remains speculative.

When we compare these different parasites, consistent with this model there is a significant reduction in the PY235 members that contribute to the efficient invasion of rat erythrocytes, possibly reflecting a reduction of suitable receptors present in rat erythrocytes. It is also possible that small variations of PY235 sequence leading to subtle differences in receptor affinity or recognition may be a contributing factor for the observed differences in YM *py235* expression, though this has not been tested in our system. The differential expression of PY235 in all the parasites analyzed suggests the uniqueness of each variant of this family to each receptor encountered on an appropriate host cell for invasion. The lesser dominant variants may also interact with the dominant variant of PY235 to form a complex, much like what is postulated for the *P. vivax* RBPs (Galinski et al., 1992), and allow the parasite to exhibit differences in invasion potential, host cell range and virulence.

P. yoelii adapting to utilize different receptors found on rat erythrocytes is analogous to previous work in *P. falciparum* has shown that upregulation of PfRH4 expression is associated with the parasite utilizing different invasion pathways

(Stubbs et al., 2005). Unlike in *P. falciparum*, where the changes in expression profile are very precise, a more subtle mechanism seems to be operating in *P. yoelii* with the changes being observed in a selected subset of *py235*. It is clear that the mechanisms observed in both *P. falciparum* as well as *P. yoelii* are conceptually similar with differences observed between the two parasites species most likely reflecting the multifactorial environment encountered by the parasite being maintained in vivo.

While changes in expression of different *py235* variants are a mechanism by which the parasite can adapt to differences in the erythrocyte receptors encountered our data shows an additional mechanism by which merozoite invasion potential is increased: by the expression of higher levels of PY235. When YA adapts to rat it is not sufficient to select suitable PY235's that can utilize the rat specific erythrocyte receptors alone, but in addition also requires significantly more *py235*. It is the combination of the two factors that enable YA to successfully adapt to invade rat erythrocytes. YM parasites also increase the amount of PY235 to be able to invade a wider range of erythrocytes within the same host.

The difference in host cell specificity between virulent and avirulent parasite strains correlate with the differences identified for *py235* amounts. Differences in transcript amounts and protein amounts are commonly observed, due to a range of reasons, such as translational regulation, post-translational processing, and some level of protein degradation at the time of sample collection.

It is probable that small changes in protein amounts can contribute to significant changes in phenotype (REFS: Scacheri PC. PNAS Feb 17 2004 V 101#7), and our results indicate that an upregulation of *py235* transcribed (and expressed) correlate with increased virulence, due to an increase in the host cell repertoire (denoted by a lower SI).

One way to demonstrate how this works is to look at the fundamental differences between reticulocytes and normocytes. Erythrocytes possess no DNA and are therefore unable to synthesize new proteins, including receptors on its surface. During the life span of a host erythrocyte receptors molecules on the surface are continuously lost or degraded and other receptors like senescence factors become exposed (Ballas et al., 1986; Berlin, 1975; Clark, 1988; Killmann, 1964) (Woolley et al., 2000). A normocyte will thus have less appropriate receptor molecules than a reticulocyte, and be potentially more difficult to invade. For the merozoite this would mean that more mature erythrocytes have potentially a lower receptor density or contain receptors with reduced affinity. Virulent parasites express more PY235 and are therefore able to efficiently recruit and bind to the limited receptors found on the normocyte surface. This allows the virulent parasite to invade a wide repertoire of erythrocytes. In contrast, less virulent parasites are limited in the repertoire of cells available. Thus under flow conditions, the parasite may not be able to have enough time to make a tight contact with the normocyte receptors. The binding to reticulocytes should still be efficient, since the receptor densities are high enough to establish a tight contact quickly, and commit to invasion (Fig 6-16). The same logic could apply to the rat erythrocytes. Host

receptor variations on rat erythrocytes could significantly reduce ligand-receptor affinities and make it more difficult for YA parasites to invade. YA parasites that have an increased ligand density are thus selected for in the rat. This compensation of reduced affinity by a high ligand density will allow the parasite to invade rat cells.

Figure 6-16. Model for mechanism of normocyte invasion of YM parasites compared to YA parasites.

YA parasites expressing low levels of PY235 are unable to bind to the limited number of receptors on the normocyte surface to ensure commitment to invasion. YM parasites express more PY235 and are thus able to recruit sufficient receptors to ensure binding and invasion of normocytes.

Less virulent parasites (such as YA), which express lower amounts of PY235, would therefore have a narrower range of cells to invade than virulent parasites (such as YM), and this is confirmed by the SI, as seen in Chapter 4. In the field, it has been observed that *P. falciparum* causing severe malaria are capable of invading a wider repertoire of host cells (thus a reduced SI) in vivo than those causing uncomplicated malaria (Chotivanich et al., 2000), which also could be a reflection of higher ligand densities in these more pathogenic parasites. Density dependent ligand-receptor interactions have been shown to lead to increased downstream responses. A similar phenomenon is seen in the process of Trophocytosis, where sustained intracellular signalling (Hudrisier et al., 2005) is mediated by a prolonged T cell engagement due to high ligand densities on antigen presenting cells. Other evidence illustrating a direct relationship between ligand affinity and receptor density with internalization of a microorganism has also been established previously where *Staphylococcus aureus* coated with high affinity ligands for integrins were more effectively engulfed by cultured cells than those coated with low affinity and low densities of ligands. (Tran Van Nhieu and Isberg, 1993).

In this study we have shown that the ability of the malaria parasites to invade a wider range of erythrocytes and thereby increase virulence is not necessarily due to the presence or absence of a unique parasite ligand (virulence factor). Instead variation in the amount of a parasite ligand alone is sufficient to change invasion properties of merozoites. The underlying virulence factors are those modulating expression levels of invasion ligands.

Specific immune attack on the *py235* proteins would be a fundamental problem for these labile merozoites. Our data suggests that the parasite has the intrinsic ability to evade immune attack by one of two mechanisms. Either by down regulating its *py235* levels, or by switching to another *py235* variant dominating in the population. This is observed in the case of YA and YM parasites, respectively. The down regulation of *py235* expression limits the parasite to a smaller repertoire of cells. This is reflected in the reduced SI. This may limit parasite population size and yet still allow the parasite to maintain the infection through a low level parasitemia. It is possible that under continuous immune pressure against a dominant *py235* variant, this may allow the parasite to switch to other *py235* variants and establish a chronic infection, though this is not tested in our assay. YM parasites under specific *py235* antibody attack, may utilize another variant, which is not under immune attack, without a marked reduction in the efficiency of invasion. The observed change from a lethal to a self limited infection may be due to the reduction of the total parasite load. In this case, parasites expressing a dominant ligand will be cleared. The remaining parasite levels (of parasites expressing another ligand such as PY06018) are low enough for the host animal to clear the infection (leading to a difference in the virulence, if measured by survival of the infected mouse). Alternatively, if each merozoite expresses a complex of PY235 proteins, specific immune attack may eliminate merozoites expressing one combination. This could be by targeting at least one member of the complex. Merozoites that express other combinations of PY235 proteins may not be targeted and persist for a few cycles, and then are cleared by the immune

system. The lack of change in the SI indicates that YM parasites are not limited in their repertoire of cells, regardless of the PY235s expressed. The phenomenon of switching to another invasion pathway, discussed earlier is also seen in *P. falciparum*. This may be what happens in persistent infections of *P. falciparum* with asymptomatic patients having fluctuations of parasites in their peripheral blood. To avoid immune attack, the parasite may ensure that infection is persistent by altering the expression of ligands that mediate invasion.

Our data would suggest that both genetic and epigenetic factors play an important role in the ability of the parasite to expand its erythrocyte repertoire suitable for invasion and virulence. The observation that *P. yoelii* virulence is inherited in a Mendelian fashion strongly supports a genetic factor, while intermediate virulence and the up and down regulation of *py235* in response to host cell variation or immune pressure supports an epigenetic component as well. Our data suggest that the parasite may possess an intrinsic ability to regulate its *py235* levels to adapt to different hosts encountered, ensuring its transmission potential in the new host is maintained. The rapid changes of *py235* levels when switching between different hosts would indicate that the parasite is able to sense its host environment and regulate transcription accordingly. Phenotypic selection of a subgroup of a population that undergoes a constant low level switching between ligands may also be happening. Either mechanism employed, this would be the first evidence that the malaria parasite is able to sense and actively respond to its external surrounding.

The results presented here for the first time show that quantitative variations in parasite invasion ligands is associated with malaria virulence. While more global variations in parasite transcription have been observed in response to merozoite specific immunity (Shi et al., 2005), this is the first direct demonstration that the down regulation of a parasite invasion ligand is a possible merozoites immune escape mechanism. Importantly, other parasite molecules involved in parasite adhesion or that are under immune selection pressure could employ a similar mechanism of expression regulation.

7. THE ROLE OF PY235 IN THE DUFFY INDEPENDENT AND DEPENDANT PATHWAY OF ERYTHROCYTE INVASION

7.1 Abstract

Parasites have been shown to use Duffy antigen as a receptor to invade erythrocytes. While parasites such as *P. vivax* have an absolute requirement for the Duffy antigen for erythrocyte invasion, *P. knowlesi* and *P. yoelii*, use an as yet uncharacterized pathway to invade rhesus or mouse erythrocytes respectively. The ligand or receptor for these interactions are unknown. In this chapter, we study the involvement of *py235* and the *P. yoelii* *EBL* transcripts in the Duffy dependent and independent pathway, by challenging Duffy knockout animals with two *P. yoelii* clones that have different virulence. Our study shows that *py235* and *EBL* profiles and transcript amounts remain unchanged in parasites harvested from the Duffy knockout animals, compared to wild type animals. This indicates that *P. yoelii* *py235* and the *EBL* pathways of invasion do not include the Duffy blood group antigen as a receptor.

7.2 Introduction

The intimate relationship between parasite and host has selected for certain traits in the host that confers resistance to *Plasmodium*. The resistance of Duffy blood group protein negative individuals of West African descent to *P. vivax* infections is a classic example (Miller et al., 1975).

The Duffy blood group antigen glycoprotein, where homologs are found also in mice is a member of the seven transmembrane protein chemokine family. It binds both CXC and CC chemokines and is also called Duffy antigen receptor for chemokines (DARC) (Chaudhuri and Pogo, 1995; Chaudhuri et al., 1993; Chaudhuri et al., 1994; Horuk et al., 1993). *P. vivax* and the related simian malarial parasite *P. knowlesi* both use Duffy blood group protein as the receptor to invade human erythrocytes (Chaudhuri and Pogo, 1995). Duffy negative human erythrocytes, expressed predominantly in persons of African ancestry are resistant to invasion by both *P. vivax* and *P. knowlesi* (Miller et al., 1976) (Miller et al., 1975). While both parasites are absolutely dependant on DARC for invasion of human erythrocytes, *P. knowlesi* appears to use an alternative pathway to invade rhesus monkey erythrocytes. Receptors for this pathway are at present unknown (Haynes et al., 1988; Miller et al., 1976; Miller et al., 1975; Wertheimer and Barnwell, 1989). The presence of alternate invasion pathways in *P. knowlesi* may be similar to how *P. falciparum* is believed to use EBLS and RBPHs that exist for *P. falciparum*.

The *P. vivax* and *P. knowlesi* parasite ligand for the Duffy receptor is the Duffy binding protein (DBP), a 135kDa member of the EBL family. Region II of the *P. vivax* DBP was shown to bind erythrocytes in vitro (Chitnis et al., 1996; Chitnis and Miller, 1994). A 35 amino acid peptide from the extracellular domain of the human Duffy antigen blocked the binding of *P. vivax* and *P. knowlesi* DBP Region II and Duffy positive erythrocytes. The effect this blocking has on invasion has not been tested in vivo.

In mice, *P. yoelii* uses the Duffy receptor for erythrocyte invasion. The region of that is involved in binding and invasion by the *P. yoelii* homolog of the DBP has been identified (Xu, Niu, and Chaudhuri, 2006). However, its cognate erythrocyte receptor is unknown. The use of a Duffy knockout animal has greatly enhanced the study of this interaction.

The Duffy knockout animal had been previously generated by homologous recombination with a gene targeting vector (Dawson et al., 2000; Fukuma et al., 2003; Luo et al., 2000). Knockout mice were similar in size, embryonic development, health and neurological behaviour compared to wild type mice, and the gross and histological anatomy of the thymus, spleen, lung, brain also were similar (Luo et al., 2000). The Duffy antigen found to be functionally redundant in mice, except for a possible role in the neutrophil migratory process (Luo et al., 2000).

Using the Duffy knockout animal model in *Plasmodium* infections, a few key observations were made. Mouse Duffy antigen is essential for normocyte invasion, but there appears to be a Duffy independent pathway for reticulocyte invasion by *P. yoelii* (Swardson-Oliver et al., 2002). (Luo et al., 2000; Xu and Chaudhuri, 2005). In this case, the absence of DARC reduces normocyte invasion to almost negligible amounts, but only slightly affects reticulocyte invasion, leaving low parasitemias. This phenomenon may be specific to just *P. yoelii*, as infecting these DARC knockout animals with *P. chabaudi* does not show any changes in invasion rate (Swardson-Oliver et al., 2002), and *P. berghei* infections have not been tested.

The lethal strain of *P. yoelii* 17XL was used in another study to determine the susceptibility of Duffy knockout and normal mice to infection. Results from that study indicate that Duffy knockout mice were protected from the lethal effect (Akimitsu et al., 2004). One way to explain these observations is to imagine that in the case of normal mice infections, the initial invasion of erythrocytes may be through mouse Duffy protein, as the reticulocyte content of uninfected mouse blood is very low (<1%). After malaria induced anemia causes the increase of reticulocyte counts, the parasite may switch to use the Duffy independent pathway to continue invasion of reticulocytes.

Information on the binding characteristics of the ligand used in the Duffy independent pathway was obtained from a few experiments. The binding of

merozoites released soluble proteins from *P. yoelii* to erythrocytes was reduced in Duffy knockout mouse erythrocytes, compared to normal mouse erythrocytes, indicating that Duffy protein may be the receptor for many of these proteins (Xu, Niu, and Chaudhuri, 2006) including possibly PY235. Since the *py235* gene family is responsible for host cells specificity and its corresponding receptor has not been identified, we wanted to investigate the role whether *py235* played a role in the invasion of Duffy positive cells.

In this study we follow the changes in the transcription of *py235* and the *P. yoelii* EBL in the Duffy invasion profile. The *P. yoelii* EBL protein, containing a DBL domain that was identified based on its shared characteristics with PvDBP, PkDBPs and EBA 175 in *P. yoelii* (Prasad *et al.*, 2003), may be another potential candidate protein for mediating the interaction with Duffy antigen. We utilized a lethal strain 17XL and another non lethal strain 17X NL of *P. yoelii* in normal or Duffy knockout C57/Bl6 mice (which was what was available at the time of experimentation).

7.3 Results and discussion

7.3.1 *Py235 profiles in 17X NL and L parasites in wild type mice*

We first performed a transcription analysis of schizont stage parasites from the donor C57/Bl6 animals, to get a baseline profile of the invasion molecules during infection. As before, 11 *py235* genes were analyzed and results obtained normalized to 18s rRNA. The results of the profile show very few differences between the virulent 17XL and non lethal 17X NL lines (Fig 7-1), though these profiles differ from that of 17X YA and YM done in Chapter 6. The dominant transcript seems to be in this case PY06018, with PY01365 at a lower level. Two interesting observations are seen. Firstly, the percentage of PY01365 expressed is higher in the lethal parasite than in the non lethal parasites. Second, PY02104 is expressed in the avirulent NL line only, albeit at a low amount, similar to what was seen in YA parasites in the previous chapter. Statistically significant differences in the profile are indicated with an asterisk (* $p < 0.05$).

Figure 7-1. Transcription profile of 17X NL and L lines of *P. yoelii*

Quantitative real time PCR analysis of *py235* transcripts normalized to parasite *18s rRNA* indicate only slight changes in the profile between lethal (17XL) and non lethal (17X NL) *P. yoelii* schizont stage parasites harvested from wild type C57/Bl6 mice. Statistically significant differences in the profile are indicated with an asterisk (* $p < 0.05$).

7.3.2 Py235 profiles in 17X NL and L parasites in Duffy knockout mice

The Duffy Knockout C57/Bl6 mice were challenged with either 17XNL or 17XL parasites harvested from the donor wild type C57/Bl6 mice. Schizont stage parasites from the Duffy knockout animals were analyzed for *py235* profile as before. Our hypothesis is that if *py235* plays a different role in the Duffy independant and dependant pathways, this will be reflected in the transcription profile of *py235*, either by amount or variant of *py235* used. However, we see that there is no change in the transcription profile between both 17X NL (Fig 7-2) and 17X L schizonts (Fig 7-3) collected from Duffy knockout animals and wild type animal infections. The minor difference shown in the *py235* profile and amounts between the knockout and wild type animals are not statistically significant (p values >0.1). This indicates that the *py235* assessed in this study are unchanged in the Duffy independent and dependent pathways, and are not the ligand for Duffy.

Figure 7-2. 17XNL profile in Duffy knockout and control animals

Quantitative real time PCR analysis of *py235* transcripts normalized to parasite *18s rRNA* indicate only negligible changes in the profile between non lethal (17X NL) *P. yoelii* schizont stage parasites harvested from wild type C57/Bl6 mice and Duffy knockout C57/Bl6 mice.

Figure 7-3. 17XL profile in Duffy kncokout and control animals.

Quantitative real time PCR analysis of *py235* transcripts normalized to parasite *18s rRNA* indicate only negligible changes in the profile between lethal (17X L) *P. yoelii* schizont stage parasites harvested from wild type C57/Bl6 mice and Duffy knockout C57/Bl6 mice.

7.3.3 P. yoelii EBL transcription in 17X NL and L parasites from wild type and Duffy knockout mice

We investigated if there were any changes in the expression of the *P. yoelii* EBL, and if its interaction with the erythrocyte receptor would be affected by the lack of Duffy antigen, we tested the transcription profile of this gene (Figure 7-4), and found that no significant changes in its expression in both parasites from the wild type and knockout mice. This suggests that the EBL is also not the receptor for Duffy antigen, since the assumption is that EBL function is essential for merozoite invasion of an erythrocyte, similar to *P. vivax*.

Figure 7-4. EBL expression in parasites harvested from normal and Duffy knockout mice.

Quantitative real time PCR analysis of EBL transcripts normalized to parasite *18s rRNA* indicate no changes in the amount of transcript between lethal (17XL) and non lethal (17X NL) *P. yoelii* schizont stage parasites harvested from sets of 5 wild type C57/Bl6 mice and Duffy knockout C57/Bl6 mice. Slight variations in numbers indicate variations in each animal infection.

7.4 Conclusions

The role of *py235* and EBL have been investigated in this study. We observe no significant differences in the transcription profile or amounts of *py235* and EBL in Duffy knockout and wild type mice, indicating that these ligands may not be the corresponding ligand for the Duffy receptor, and they do not appear to play a role in the Duffy invasion profile.

It is noteworthy that this analysis cannot be compared to those in previous chapters, as the parasite clones available for this study were different, and the host animal background (C57/Bl6) also different. We were unable to get the knockout animals in a BALB/c background. We are unable to conclude about the host cell selectivity and immune evasion mechanisms, as significant experimental differences also exist in this study compared to those in previous chapters. The treatment of host animals with phenylhydrazine to increase their repertoire of reticulocytes available could lead to a high number of cells available for the mild parasites to invade, competing with the large repertoire of cells that the lethal parasite has available.

We are also unable to conclude on the immunity to *py235* and EBL in this study as the immune responses of Duffy antigen knockout mice and wild type mice to the invasion of parasites may be different. Previous data have shown that knockout animals express lower amounts of plasma concentrations of some

chemokines compared to wild type mice (Fukuma et al., 2003). Parasitemia in these knockout mice can be suppressed by the stimulation of the immune response by the induction of leukocytes (Akimitsu et al., 2004; Dawson et al., 2000). While this very preliminary study eliminates *py235* and the EBL as the sole ligand for Duffy antigen in *P. yoelii*, it may be interesting to see which other parasite genes are expressed in a whole *P. yoelii* genome microarray and determine if other parasite proteins are altered when the parasite encounters a Duffy knockout host cell.

8. CONCLUSIONS AND FUTURE DIRECTIVES

8.1 Summary of study

The purpose of this study was to understand the role of *py235* in virulence, immune evasion and selection of alternate invasion pathways in *P. yoelii*.

8.2 Copy number determination

This work has contributed to our understanding of the diversity of *py235* in various *P. yoelii* strains. We show that although variations in the genes may exist between the strains of *P. yoelii*, copy numbers of these genes do not correlate with virulence. These experiments highlight the limitations of the use of the partially sequenced 17X1.1 genome for the analysis of the other parasite clones available for study. It has been suggested that polymorphisms in EBA140 and EBA181 can account for variations in binding affinities and erythrocyte receptor specificities and ultimately lead to differences in the invasion phenotype. (Mayer et al., 2002; Mayer et al., 2004). This could be relevant in the case of the *py235* genes. It is difficult to obtain sequencing data from multiple parasites. However full length sequencing of the *py235* genes between parasites may highlight the diversity in other regions of these genes. Some of these genes have been sequenced and shown to have significant diversity within a strain of *P. yoelii* (Green and Holder, 2000; Keen et al., 1994; Sinha et al., 1996). These differences could contribute to an increasing diversity of the *py235* multigene family among different parasites.

Pathogens with an increased ligand repertoire will have a selective advantage in a varying host environment. Parasites have a diverse range of genes (i.e. multiple *py235* genes), but also need to evolve a mechanism to take advantage of this diversity. This could be through varying the expression of these genes.

8.3 Virulence of parasites

We studied the expression profile of the *py235* genes in various host environments to see if this played a greater role in the invasion potential. Our *in vivo* study suggests that subtle variations in *py235* transcription may account for differences seen in virulence. A major contributing factor to virulence is the range of red blood cells invaded by a parasite. Hence, we correlated the selectivity of a host cell by *P. yoelii* using SI with *py235* levels, and argue that the significant factor contributing to parasite virulence is the abundance of ligand expressed. This upregulation is observed in virulent parasites, and parasites adapted to a different host. It would be interesting to see if the upregulation of parasite ligands is indeed a global phenomenon observed in other pathogen proteins, to induce a greater response, and greater virulence potential.

The utilization of expansions in the host cell repertoire and alternative invasion interactions may not be directly correlated to the classical cause of pathology seen in *P. falciparum* infections. This is difficult to study as in Africa, *Plasmodium* replication rates and induced anemia in the clinical picture is often

clouded by complications such as cerebral malaria. This makes the correlation of SI and severity difficult. African infections often present with severe complicated malaria, and individuals with pre-existing immunity to *Plasmodium* still exhibit complications such as cerebral malaria. One explanation for this is that cerebral malaria is in part mediated by the human immune system, though this remains speculative. Also, premunitive infections are common, and could be a continuous stimulus of the immune system through the existence of low level parasitemia in the bloodstream. In Thailand, a limited pre-existing immunity to malaria infections exist and anemia accounts for the virulence phenotype observed. This explains why SI values correlate very well with severity of malaria infections in Thailand, and not in Africa. It would be interesting to see if other *Plasmodium*, such as *P. vivax* parasites exhibit increased virulence, and then see if they show any marked differences in expanding the range of erythrocytes invaded.

8.4 Immune evasion

We have also shown association of two transcriptional phenomena the parasite exhibits whilst evading host immunity. It has an intrinsic ability to downregulate its *py235* ligand repertoire expressed to evade specific immunity, or switch to utilize another *py235* variant for invasion under the same immune pressure. These may be global mechanisms that the parasite can utilize to regulate the expression of other proteins. It would be worth studying if in chronic infections, the parasite expresses different variants of *py235* with immune pressure.

This can be studied as a chronic infection model exists in *P. yoelii* infections of the natural host *Thamnomys*.

A knockout of specific dominant *py235* variants in parasites would shed some light on the *py235* variants and how they may be used. The interplay of other genes during adaptation, immune pressure and upon encountering a host with a receptor knockout may force the parasite to use alternate pathways for invasion. Global variations that occur after these selection pressures may be studied using whole genome microarray analysis of the selected parasites. The interplay between the EBLs and RBPHs may be better demonstrated through experiments that target both families of protein, such as immunization with the rodent EBL and one or more members of PY235 and subsequent parasite challenge. It is important to have a wide range of reagents such as antibodies to the individual PY235 proteins, and technologies such as 2D gel electrophoresis and mass spectrometry to identify the proteins isolated from various parasites. It is also important to study the stoichiometry of the members that is essential for invasion, and determine if an initial sensing function of the PY235 is involved in forming tighter attachments by recruiting other proteins.

8.5 Zoonotic potential

The parasite encounters many variations in the host environment. While in the natural host, ligand-receptor affinities may be high, and ensure efficient invasion. However if the parasite encounters a new host repertoire, it may

upregulate its ligand repertoire to compensate for any lower affinity to a receptor. This could lead to an increased zoonotic potential. The adaptation of *Plasmodium* across species, or better known as the zoonotic potential of *Plasmodium* has been a commonly occurring phenomenon. While more than twenty-six species of *Plasmodium* are seen among primate populations, some of the ones closely related to the human malarias have been implicated and isolated as symptomatic malaria in humans in experimental, accidental or natural infections (Jongwutiwes et al., 2004; Kawamoto et al., 2002; Singh et al., 2004). Our data suggests that in such cross species adaptation, the selection of a parasite that has successfully upregulated its invasion ligand allows it to counteract the loss of the repertoire of cells available to invade. This could be mediated by sensing the changed host cell receptor encountered. Host cell specificity appears to be mediated by many possible ligand receptor combinations. Unlike parasites that have an absolute restriction for single specific ligand receptor combinations (such as *P. vivax*), *P. knowlesi* and *P. yoelii* appear to be more adaptable, possibly due to their intrinsic ability to modify their invasion ligand profiles and amounts. While this study measures successful invasion events as established infections, where parasites are seen to multiply effectively within cells, there are also many studies that show that *Plasmodium* can invade erythrocytes from other species that are not the natural host, but cannot develop further, and the reasons for which are to be further studied.

8.6 Selection versus switching

Parasites in a new and less suitable host environment can undergo one of two events. Part of the parasite population can be selected against by immune attack, leaving a preexisting subset of the parasite population that can still invade but is not targeted by the immune system. Alternatively, the parasite can actively sense its microenvironment and alter its protein expression. The difference between these outcomes is termed selection and switching respectively. This study may indicate that the parasite may have an ability to sense the host environment and take the necessary responses to switch to another phenotype. This appears apparent in the rapid downregulation of *py235* that occurs after rat selected parasites are reintroduced into a mouse. Within two cycles, the parasite has switched its expression levels of *py235* to lower amounts. Albeit a subpopulation with an imprinted genetic memory would always persist, this parasite still retains the rapid ability to infect rat cells in subsequent passages. Studies in *P. falciparum* have to date been limited in demonstrating this switching as all studies have been in parasites selected for a long period in continuous culture, without immune pressure.

8.7 Phenotypic variations of merozoites

How the parasite utilizes the EBL and RBPH proteins is interesting. In *P. falciparum*, it appears that the parasite has the ability to express multiple members of the RBPHs and EBLs. In this case, the spatial location of the EBLs and RBPHs may determine the diversity of invasion potential. In *P. yoelii*, a different picture may exist. In this case it appears that each merozoite can either express a distinct *py235* or it is also possible that the *py235* proteins may interact with each other to form a complex within each merozoite, much like what has been postulated for the complex of RBP1 and 2 in *P. vivax* (Galinski et al., 1992). The specific combination of PY235 proteins that exist in each merozoite can be different, and with the EBL, give rise to a mixed population of merozoites in each schizont. This mixed population may exhibit variations in host repertoire and invasion potential.

The dominant transcript and protein observed from our analysis and previous reports (Ogun et al., 2006) may be expressed in all merozoites, and with the lesser transcribed variants constitute the complex that mediates the selection of host cells range and invasion.

8.8 Significance of research

The implications of this study fundamentally lie in malaria treatment and control. Monitoring of clinical trials using selectivity index at least in uncomplicated cases of malaria anemia in Thailand, could assist in the therapeutic predictions of malaria severity and determine treatment options. This work also highlights the zoonotic potential of certain parasites due to the expansion of the gene families. We argue that these findings have important implications in the design of vaccines against malaria parasites. Instead of classically targeting multigene families as a collection of targets to design a vaccine against, we may be better suited to target the regulation factors of antigenic variation and immune evasion. Parasites may utilize multiple invasion pathways in *P. falciparum*, and are obviously different from the invasion profile in *P. vivax*, for example, and it may be a requirement to modify the design of vaccines for each parasite. The diversity and regulation of expression of multigenes that play a role in determining clinical immunity may also be characteristic of many other pathogens apart from malaria parasites, and thus affect vaccine strategies.

9. APPENDICES

9.1 General terms

Isolate: sample of parasites collected from a wild-caught animal in a unique occasion. An isolate may contain more than one species of parasite and more than one genetically distinct clone of a given species

Line: sample of parasites which have gone through a particular passage or treatment. Parasites in a line usually have certain characteristics in common, but are not necessarily genetically identical.

Clone: sample of parasites derived from an infection derived from a single haploid parasite, usually an asexual blood form or sporozoite.

9.2 Parasite profiles

The parasitemia curves for all the parasites used in this study are shown in the next section, along with data on the origin of each clone.

9.2.1 *Parasite origins*

Isolate 17X was a mild strain originally derived from the Thicket rat (*Thamnomys rutilans*) (Landau and Chabaud, 1965) in La Maboke Field station, Central African Republic in April 1965. This was maintained in the Museum National d'Histoire Naturelle in Paris and then sent to New York University and the London School of Hygiene and Tropical Medicine, and passed to the University of Edinburgh from these places, where they were single cloned and named a virulent clone YM and an virulent clone Line A (called here YA).

YA parasites were passed from LSHTM to Edinburgh to the National Institute of Medical Research (NIMR) where they were maintained in BALB/c mice.

YM uncloned parasites were derived from a stablate of 17X in NYU that suddenly underwent a transformation to a virulent one when a stablate was removed from deep freeze. The virulent line (an infected mouse, Yoelii's mouse YM) was sent to Edinburgh in 1972 and cloned out by serial dilution. Out of seven

clones, one YM (YME) was frozen and also sent to the NIMR where they were maintained in BALB/c mice.

Strain 17X 1.1 NL was obtained from the NIMR, and was also a single cloned parasite from the original 17X isolate, then sent to the Walter Reed Army Institute of Research (WRAIR), the National Institutes of Health (Maryland), and the Naval Medical Research Centre (NMRC, Maryland).

AC is derived from a cross between two mild *P. yoelii* lines denoted lines A and lines C, with different sensitivities to pyrimethamine and variants of the enzyme glucose phosphate isomerase (GPI).

The genetic cross was performed between clone YME and clone AC by injecting blood forms of mixed YME and AC parasites into the thick-ket rat. Gametocytes were taken up by *Anopheles stephensi* mosquitoes and transmitted to a mouse, and deep frozen. These parasites were cloned by serial dilution to yield many progeny, out of which clones 604, 605, 606, 607, 611 and 612 showed differences in pyrimethamine sensitivity and virulence. These were used for the study presented in this dissertation.

9.2.2 Parasitemia curves

Figure 9-1. Parasitemia curve YA.

Parasites are normally cleared after peak parasitemias at 20-50%

Figure 9-2. Parasitemia curve YM.

Infected animals are usually killed as parasitemia increases.

Figure 9-3. Parasitemia curve of 17X 1.1.
Parasites are cleared after peak parasitemias reach 20-40%.

Figure 9-4. Parasitemia curves of genetic cross

YM (Edinburgh) and AC parents and progeny 604,605, 606, 607, 611 and 612 of the genetic cross. Phenotypes of lethal or non lethal outcome are given in Table 4-1.

9.3 SI and parasite lines

The parasite lines used for this study differed in their SI for each experiment shown. Table shows the SI and corresponding *py235* level obtained from all parasites in the experiments in this dissertation.

Parasite / Animal	Host animal	Virulence phenotype	Selective Index (range)	py235 amount (ng)
17X YA	Balb/C mouse	avirulent	3.5 (3.2-3.8)	0.0050
17X YA	Balb/C mouse Passive transferred with MAb 25.77	avirulent	10.2 (10-10.4)	0.0005
17X YM	Balb/C mouse	virulent	0.1 (0.12-0.14)	1.6202
17X 1.1 NL	Balb/C mouse	avirulent	7.8 (7.5-8.1)	0.0160
YME (Edinburgh)	Balb/C mouse	virulent	0.26 (0.22-0.3)	1.4955
AC	Balb/C mouse	avirulent	2.4 (2.2-2.6)	0.1356
604	Balb/C mouse	virulent	0.18 (0.16-0.2)	0.5553
605	Balb/C mouse	virulent	0.13 (0.11-0.15)	0.3100
606	Balb/C mouse	intermediate	0.86 (0.83-0.89)	0.2154
607	Balb/C mouse	virulent	0.2 (0.18-0.22)	0.9002
611	Balb/C mouse	avirulent	3.6 (3.2-4)	0.0679
612	Balb/C mouse	intermediate	0.27 (0.24-0.3)	0.2353

Table 9-1. SI values and *py235* levels for each experiment.

9.4 py235 alignment

PY01185	1	ATGTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	66
PY05995	28	CTTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	84
PY01365	34	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY03534	61	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	120
PY05054	34	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY04630	34	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY00649	34	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY06018	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY02033	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY03432	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY03184	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY06381	31	CTTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	87
PY02104	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY04930	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY01185	10	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	66
PY05995	28	CTTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	84
PY01365	34	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY03534	61	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	120
PY05054	34	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY04630	34	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY00649	34	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY06018	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY02033	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY03432	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY03184	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY06381	31	CTTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	87
PY02104	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY04930	1	ATTTTCAAA	TTTATATCTAGAGTAATTTATGGGAAAA	AATTGACGGGAAAA	A	90
PY01185	67	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	125		
PY05995	85	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	143		
PY01365	91	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	149		
PY03534	121	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	180		
PY05054	91	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	149		
PY04630	91	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	149		
PY00649	91	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	149		
PY06018	91	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	149		
PY02033	1	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	149		
PY03432	1	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	149		
PY03184	36	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	94		
PY06381	88	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	146		
PY02104	1	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	94		
PY04930	36	AGGAATCATGATGACGAGTAAACAAATTTTATTCG	TACCAATATTTAAAGGAGTAA	94		
PY01185	126	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	180	
PY05995	144	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	198	
PY01365	150	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	204	
PY03534	181	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	239	
PY05054	150	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	204	
PY04630	150	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	204	
PY00649	150	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	204	
PY06018	150	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	204	
PY02033	1	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	1	
PY03432	1	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	1	
PY03184	95	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	149	
PY06381	147	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	201	
PY02104	1	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	1	
PY04930	95	TTTATATATTCGA	AACTTCTTCAAAATGA	AGAACAAATGATGA	149	
PY01185	181	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	230	
PY05995	199	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	248	
PY01365	205	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	257	
PY03534	240	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	293	
PY05054	205	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	263	
PY04630	205	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	254	
PY00649	205	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	254	
PY06018	1	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	1	
PY02033	1	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	1	
PY03432	1	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	1	
PY03184	150	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	208	
PY06381	202	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	260	
PY02104	1	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	1	
PY04930	150	ATTAATGATGAAAT	TTTATGACACG	CAATTCATTTACT	208	
PY01185	231	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	286	
PY05995	249	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	304	
PY01365	258	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	313	
PY03534	294	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	353	
PY05054	264	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	319	
PY04630	255	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	310	
PY00649	255	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	310	
PY06018	1	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	1	
PY02033	1	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	1	
PY03432	1	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	1	
PY03184	209	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	264	
PY06381	261	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	307	
PY02104	1	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	1	
PY04930	209	AAAGACA	CTCGAATGAC	AAATACATTTATACATGACTACCAATATAAAGC	264	
PY01185	287	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	343	
PY05995	305	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	361	
PY01365	314	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	370	
PY03534	354	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	409	
PY05054	320	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	376	
PY04630	311	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	367	
PY00649	311	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	367	
PY06018	1	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	1	
PY02033	1	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	1	
PY03432	1	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	1	
PY03184	265	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	321	
PY06381	308	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	364	
PY02104	1	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	1	
PY04930	265	ACTTGAATCTTTTAAAGT	TTTAAATGATGAAATGACCAATATAAAGC	AAATACATTTATACATGACTACCAATATAAAGC	321	
PY01185	344	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	397	
PY05995	362	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	415	
PY01365	371	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	424	
PY03534	410	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	469	
PY05054	377	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	430	
PY04630	368	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	421	
PY00649	368	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	421	
PY06018	1	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	1	
PY02033	1	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	1	
PY03432	1	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	1	
PY03184	322	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	375	
PY06381	365	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	418	
PY02104	1	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	28	
PY04930	322	CTAT	AGTTTAAAGTCTTTTATTC	CAAACTTCTTCTGACCCGATTCGATGCT	375	

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Py01185	2145	PACCAATGAGAAATAGCAAAATGCAACTTTTGA	2204	TCCTATCAAGAGAGAGCAAAATGCAAA	2493	ATGAAA
Py05995	2163	PACCAATGAGAAATAGCAAAATGCAACTTTTGA	2222	TCCTATCAAGAGAGAGCAAAATGCAAA	2511	PACAAA
Py01365	2172	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2231	TCCTATCAAGAGAGAGCAAAATGCAAA	2520	CAGAAA
Py03534	2220	ATGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG	2276	ATGTTGTTGTTGTTGTTGTTGTTGTTGTTG	2574	ATGAAA
Py05054	2172	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2231	TCCTATCAAGAGAGAGCAAAATGCAAA	2520	ATGAAA
Py04630	2172	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2231	TCCTATCAAGAGAGAGCAAAATGCAAA	2520	ATGAAA
Py00649	2172	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2231	TCCTATCAAGAGAGAGCAAAATGCAAA	2520	ATGAAA
Py06018	72	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	128	TCCTATCAAGAGAGAGCAAAATGCAAA	417	ATGAAA
Py02033	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03432	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03184	2129	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2185	TCCTATCAAGAGAGAGCAAAATGCAAA	2474	ATGAAA
Py06381	2136	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2195	TCCTATCAAGAGAGAGCAAAATGCAAA	2484	ATGAAA
Py02104	1782	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1841	TCCTATCAAGAGAGAGCAAAATGCAAA	2130	ATGAAA
Py04930	2129	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2188	TCCTATCAAGAGAGAGCAAAATGCAAA	2477	ATGAAA
Py01185	2205	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2263	TCCTATCAAGAGAGAGCAAAATGCAAA	2546	ATGAAA
Py05995	2223	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2281	TCCTATCAAGAGAGAGCAAAATGCAAA	2564	ATGAAA
Py01365	2232	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2290	TCCTATCAAGAGAGAGCAAAATGCAAA	2573	ATGAAA
Py03534	2277	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2336	TCCTATCAAGAGAGAGCAAAATGCAAA	2634	ATGAAA
Py05054	2232	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2291	TCCTATCAAGAGAGAGCAAAATGCAAA	2573	ATGAAA
Py04630	2232	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2290	TCCTATCAAGAGAGAGCAAAATGCAAA	2573	ATGAAA
Py00649	2232	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2290	TCCTATCAAGAGAGAGCAAAATGCAAA	2573	ATGAAA
Py06018	129	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	187	TCCTATCAAGAGAGAGCAAAATGCAAA	470	ATGAAA
Py02033	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03432	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03184	2186	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2244	TCCTATCAAGAGAGAGCAAAATGCAAA	2527	ATGAAA
Py06381	2196	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2254	TCCTATCAAGAGAGAGCAAAATGCAAA	2537	ATGAAA
Py02104	1842	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1900	TCCTATCAAGAGAGAGCAAAATGCAAA	2183	ATGAAA
Py04930	2189	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2247	TCCTATCAAGAGAGAGCAAAATGCAAA	2530	ATGAAA
Py01185	2264	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2323	TCCTATCAAGAGAGAGCAAAATGCAAA	2606	ATGAAA
Py05995	2282	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2341	TCCTATCAAGAGAGAGCAAAATGCAAA	2624	ATGAAA
Py01365	2291	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2350	TCCTATCAAGAGAGAGCAAAATGCAAA	2633	ATGAAA
Py03534	2337	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2394	TCCTATCAAGAGAGAGCAAAATGCAAA	2690	ATGAAA
Py05054	2292	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2350	TCCTATCAAGAGAGAGCAAAATGCAAA	2633	ATGAAA
Py04630	2291	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2350	TCCTATCAAGAGAGAGCAAAATGCAAA	2633	ATGAAA
Py00649	2291	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2350	TCCTATCAAGAGAGAGCAAAATGCAAA	2633	ATGAAA
Py06018	188	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	247	TCCTATCAAGAGAGAGCAAAATGCAAA	530	ATGAAA
Py02033	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03432	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03184	2245	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2304	TCCTATCAAGAGAGAGCAAAATGCAAA	2587	ATGAAA
Py06381	2255	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2314	TCCTATCAAGAGAGAGCAAAATGCAAA	2597	ATGAAA
Py02104	1901	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1960	TCCTATCAAGAGAGAGCAAAATGCAAA	2243	ATGAAA
Py04930	2248	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2307	TCCTATCAAGAGAGAGCAAAATGCAAA	2590	ATGAAA
Py01185	2324	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2379	TCCTATCAAGAGAGAGCAAAATGCAAA	2665	ATGAAA
Py05995	2342	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2397	TCCTATCAAGAGAGAGCAAAATGCAAA	2683	ATGAAA
Py01365	2351	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2406	TCCTATCAAGAGAGAGCAAAATGCAAA	2692	ATGAAA
Py03534	2395	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2454	TCCTATCAAGAGAGAGCAAAATGCAAA	2748	ATGAAA
Py05054	2351	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2406	TCCTATCAAGAGAGAGCAAAATGCAAA	2692	ATGAAA
Py04630	2351	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2406	TCCTATCAAGAGAGAGCAAAATGCAAA	2692	ATGAAA
Py00649	2351	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2406	TCCTATCAAGAGAGAGCAAAATGCAAA	2692	ATGAAA
Py06018	248	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	303	TCCTATCAAGAGAGAGCAAAATGCAAA	589	ATGAAA
Py02033	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03432	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03184	2305	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2360	TCCTATCAAGAGAGAGCAAAATGCAAA	2646	ATGAAA
Py06381	2315	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2370	TCCTATCAAGAGAGAGCAAAATGCAAA	2656	ATGAAA
Py02104	1961	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2016	TCCTATCAAGAGAGAGCAAAATGCAAA	2302	ATGAAA
Py04930	2308	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2363	TCCTATCAAGAGAGAGCAAAATGCAAA	2649	ATGAAA
Py01185	2380	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2439	TCCTATCAAGAGAGAGCAAAATGCAAA	2725	ATGAAA
Py05995	2398	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2457	TCCTATCAAGAGAGAGCAAAATGCAAA	2685	ATGAAA
Py01365	2407	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2466	TCCTATCAAGAGAGAGCAAAATGCAAA	2752	ATGAAA
Py03534	2455	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2514	TCCTATCAAGAGAGAGCAAAATGCAAA	2808	ATGAAA
Py05054	2407	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2466	TCCTATCAAGAGAGAGCAAAATGCAAA	2752	ATGAAA
Py04630	2407	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2466	TCCTATCAAGAGAGAGCAAAATGCAAA	2752	ATGAAA
Py00649	2407	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2466	TCCTATCAAGAGAGAGCAAAATGCAAA	2752	ATGAAA
Py06018	304	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	363	TCCTATCAAGAGAGAGCAAAATGCAAA	649	ATGAAA
Py02033	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03432	1	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	1	TCCTATCAAGAGAGAGCAAAATGCAAA	1	ATGAAA
Py03184	2361	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2420	TCCTATCAAGAGAGAGCAAAATGCAAA	2706	ATGAAA
Py06381	2371	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2430	TCCTATCAAGAGAGAGCAAAATGCAAA	2716	ATGAAA
Py02104	2017	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2076	TCCTATCAAGAGAGAGCAAAATGCAAA	2362	ATGAAA
Py04930	2364	TCCTATCAAGAGAGAGCAAAATGCAACTTTTGA	2423	TCCTATCAAGAGAGAGCAAAATGCAAA	2709	ATGAAA

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9.5 Recipes

Name of Solution, Buffer or Media	Component	Concentration/amount
Transfer buffer	Trizma base	3g/L
	Glycine	0.192M
	SDS	0.1% w/v
SDS PAGE Running buffer	MOPS	50mM
	Trizma base	50mM
	SDS	0.10%
	EDTA	1mM
2X SDS PAGE sample buffer	Tris HCl pH 6.8	100mM
	SDS	4% w/v
	Bromophenol blue	0.2% w/v
	Glycerol	20% v/v
	DTT	200mM
Western blocking buffer	PBS	1X
	Tween20	0.1% v/v
	Milk powder Marvel	5% w/v

Name of Solution, Buffer or Media	Component	Concentration/amount
Staining reagent	Coomassie Brilliant Blue	1.25g
	Methanol (Merck)	225ml
	Glacial Acetic Acid (Merck)	50ml
	H2O	to 500ml
Destaining reagent	Methanol (Merck)	225ml
	Glacial Acetic Acid (Merck)	50ml
	H2O	to 500ml
HiTrap Protein G Sepharose Binding Buffer: 20mM Sodium Phosphate pH 7	Na2HPO4.7H2O	1.64g
	NaH2PO4	0.47g
	NaCl	8.77g
	H2O	to 500 ml
HiTrap Protein G Sepharose Elution Buffer: 0.1M Glycine pH 2.7	Glycine	3.75g
	H2O	to 500ml
HiTrap Protein G Sepharose column reconstitution buffer: 20%	Ethanol	20ml
	H2O	to 100ml

Name of Solution, Buffer or Media	Component	Concentration/amount
Kreb's Henseleit buffer pH 7.2	NaCl	124mM
	KCl	5mM
	KH2PO4	1.2mM
	MgSO4	1.2mM
	NaHCO3	26mM
	Glucose	5mM
	CaCl2	1.2mM
Tris Buffered Medium	Tris HCl pH 7.5	5mM
	KCl	3mM
	Na2EDTA	0.3mM
Nycodenz working solution (NWS)	Nycodenz powder (Sigma)	27.6ml
	Tris buffered medium	to 100ml
	Autoclave and store at 4C	
Saline	NaCl	0.9% w/v
	Glucose	0.2% w/v

Name of Solution, Buffer or Media	Component	Concentration/amount
Parasite Lysis Buffer	NP40	1%
	EGTA	5mM
	NaCl	150mM
	TrisHCl pH 8.2	50mM
	EDTA	5mM
	Protease inhibitor (Roche)	1X
MACs IP Wash Buffer 1	Tris HCl pH 6.8	50mM
	NaCl	0.5M
	EDTA	5mM
	NP40	0.50%
	BSA	1mg/ml
MACs IP Wash Buffer 2	Tris HCl pH 6.8	50mM
	EDTA	5mM
	NP40	0.50%
MACs IP SDS loading buffer	Tris HCl pH 6.8	100mM
	Bromophenol Blue	0.20%
	SDS	4%
	Glycerol	20%
	DTT	200mM

Name of Solution, Buffer or Media	Component	Concentration/amount
Schizont maturation medium	RPMI 1640	50ml
	Albumax	0.50%
	Brefeldin A (if required)	5ug/ml
Rodent <i>Plasmodium</i> Freezing Medium	Glycerol	40%
	NaAcetate	0.14M
	KCl	0.05M
10X PBS pH 7.4	NaCl	80g
	KCl	2g
	Na ₂ HPO ₄	14.4g
	KH ₂ PO ₄	2.4g
	H ₂ O	to 1L
10X TBE Buffer pH 8	Trizma base	108g
	Boric Acid	55g
	EDTA	9.3g
	H ₂ O	to 1L
Agarose	Agarose	1%
	Ethidium Bromide	1ug/ml
	1X TBE	to 100ml

Name of Solution, Buffer or Media	Component	Concentration/amount
Metaphor agarose	Metaphor Agarose	2.50%
	Ethidium Bromide	1ug/ml
	1X TBE	to 100ml
Luria-Bertani (LB) Media pH 7	Tryptone	1%
	Yeast extract	0.50%
	NaCl	1%
Luria-Bertani (LB) plates pH7	Tryptone	1%
	Yeast extract	0.50%
	NaCl	1%
	Agar	15g/L
SOC medium	Tryptone	2%
	Yeast extract	0.50%
	NaCl	10mM
	KCl	2.5mM
	MgCl ₂	10mM
	Glucose	20mM
Hybridoma culture medium	RPMI1640	75%
	Fetal Bovine Serum	25%

Name of Solution, Buffer or Media	Component	Concentration/amount
Agarose Gel Loading buffer	Bromophenol Blue	0.25%
	Xylene Cyanol FF	0.25%
	Glycerol	30% v/v
	EDTA	3mM
Southern Blot Wash Solution: Low stringency	SSC	2X
	SDS	0.10%
moderate stringency	SSC	0.5X
	SDS	0.10%
High stringency	SSC	0.1X
	SDS	0.10%
Sodium chloride/Sodium citrate (SSC) 20X pH 7	NaCl	3M
	Sodium Citrate	0.5M
Depurination solution	HCl	0.25M
Southern Transfer Buffer	NaOH	0.4M

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