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Merozoite invasion of erythrocytes:
Revealing functional characteristics of the
Plasmodium knowlesi Normocyte Binding Proteins

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Abstract

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By Amma Atei Semenya

Malaria remains as one of the most problematic global infectious diseases; annually as many as 500 million people become clinically ill with malaria. For over thirty years a malaria vaccine has been promised, but to date, none has been implemented. My research has been focused on a family of proteins, the reticulocyte binding-like (RBL) protein superfamily that have been proposed as potential vaccine candidates. It is believed that RBL proteins function in the critical initial step of invasion when merozoites attach to the host cell via their apical end. The goal of my dissertation has been to characterize the functional properties of two RBL proteins that are expressed in the simian malaria species, *Plasmodium knowlesi*. The *P. knowlesi* Normocyte Binding Proteins, PkNBPXa and PkNBPXb, have been show to bind to rhesus macaque erythrocytes in *in vitro* erythrocyte binding assays. Additionally both proteins appear to be crucial for the propagation of blood-stage parasites, as genetic disruption of either *pknbpx* gene results in the inability to recover parasites. Importantly, an N-terminal domain of PkNBPXb has been identified that binds to erythrocytes, and specifically to a receptor cleaved by chymotrypsin. The binding of this domain (PkNBPXb-II) appears dependent on disulfide bond formation. PkNBPXb-II binds to erythrocytes from Old World monkeys and gibbons (i.e. Lesser Apes). Surprisingly, this domain was unable to bind to erythrocytes from New World monkeys and humans, which are known to be susceptible to *P. knowlesi* infection, indicating that an alternative binding domain or protein (i.e. PkNBPXa) mediates the attachment to erythrocytes. This dissertation confirms that RBL proteins function by binding to erythrocytes. Interestingly, this dissertation introduces data suggesting that PkNBPXa and PkNBPXb mediate independent binding functions. Additionally, a functional binding domain of PkNBPXb was identified and affords the opportunity to test this domain in pre-clinical vaccine studies.

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CHAPTER ONE

THE MALARIA GLOBAL HEALTH PROBLEM, SIMIAN MALARIA, VACCINE CANDIDATES, AND MEROZOITE BINDING PROTEINS

Malaria: Global Health Burden

Malaria has existed with the human population for many millennia, as records dating back over 3000 years provide evidence of its existence (reviewed [1]). The term mal-aria originated in the 18th century from the Italian words for bad air and related to the belief that toxic air was the causative agent for this disease [2]. It was not until 1880 that the definitive cause of malaria was defined by the studies of Alphonse Laveran. While working at a hospital in Algeria during the Franco-Prussian war, he observed in the blood of a malaria patient several moving filaments attached to red blood cells. He later examined hundreds of other patients and observed similar results in the majority of those patients [3]. Laveran termed the observed parasite *Oscillaria malaria.* Bacteriologists Ettore Marchiafava and Angelo Celli later renamed this parasite *Plasmodium* [4].

To date approximately 120 *Plasmodium* species have been identified with four species causing malaria in humans, *P. falciparum, P. vivax, P. malariae,* and *P. ovale* [5]. Of these four species, *P. falciparum* and *P. vivax* are the most prevalent and clinically relevant [6]. Importantly malaria exists in several animal species that can be used as models for understanding the biological, molecular, and immunological characteristics of this disease. Some of the well-characterized animal models are the non-human primate species, *P. knowlesi, P. cynomolgi,* and *P. coatneyi* along with rodent malaria species *P. yoelii* and *P. berghei.*

Animal models have historically been important in understanding malaria. One of the earliest examples is the use of an avian malaria species to identify the vector (*Anopheles)* that transmits malaria. In 1898, Ronald Ross observed transmission of malaria from infected birds to mosquitoes and experimentally demonstrated that infected

mosquitoes could transmit malaria to healthy birds. Alphonse Laveran and Patrick Manson had earlier hypothesized that mosquitoes transmitted malaria [7], but the experiments conducted by Ronald Ross proved that, "it is impossible to question the fact of proteosoma being communicable by mosquitoes" [8].

Mosquito transmission of malaria in humans was illustrated by a group of Italian scientists led by Giovanni Batista Grassi. During the years of 1898-1899, *Anopheles* mosquitoes were collected and allowed to feed on patients infected with malaria; these mosquitoes were then allowed to feed on volunteers who subsequently developed malaria. These experiments demonstrated the complete sporogonic cycle of the human malarias *P. falciparum, P. vivax,* and *P. malariae* [9].

Alphonse Laveran and Ronald Ross both received the Nobel Prize (1907 and 1902 respectively) for their scientific contributions to the understanding of malaria. Although these achievements were made over 100 years ago, malaria is still a significant global health problem. There are more than 500 million cases of severe malaria every year, and forty percent of the world's population lives at risk for malaria infection. While Africa accounts for the majority of cases and deaths, malaria is widely distributed around the globe with South and Central America, Asia, the Middle East, and parts of Europe also being affected [10].

In 2002, Jeffrey Sachs and Pia Malaney analyzed the long-term effect of *P. falciparum* transmission on the economies in thirty-one African countries. The analysis controlled for growth determinants such as life expectancy, initial income, and human capital as well as geographical factors such as tropical location. The total economic loss for the thirty-one nations during the years of 1980 – 1995, was determined to be 74

billion dollars. It was also noted that the 1995 gross domestic product (GDP) from malaria endemic countries was fivefold less than the GDP of malaria free countries [11]. Malaria is not only a significant global health problem simply from the sheer number of cases reported annually, but also because it negatively impacts other societal aspects such as economic loss, loss of human capital, and loss of productivity, etc. of the greater than 90 countries where malaria is transmitted.

In the 19th century, improvements in sanitary living conditions caused significant declines in the number of malaria cases, specifically in the U.S. and Europe. While in the mid 20th century, efforts to control malaria (led by the World Health Organization's global malaria-eradication program from 1955-1969) were mainly based on using the insecticide dichlorodiphenyltrichloroethane (DDT) and chemoprophylaxis to prevent malarial transmission; this eradication program largely excluded Sub-Saharan Africa [12].

A current report by the World Health Organization recommends three primary measures for malaria control 1) diagnosis of malaria cases and treatment with effective medicines; 2) distribution of long-lasting insecticidal nets (LLINs), to achieve full coverage of populations at risk of malaria; and 3) indoor residual spraying (IRS) to reduce and eliminate malaria transmission [13]. It is also important to recognize the need for vaccine development and the identification of new targets for drug intervention.

Drug resistance has contributed to a re-emergence of malaria in South America and South East Asia, and has also contributed to the sustained high levels of malaria transmission in sub-Saharan Africa. *P. falciparum* resistance to the widely used drug chloroquine was initially documented in East Africa in 1978 [14]. Since that time

chloroquine resistance has become increasingly widespread in Africa, and is also present in Southeast Asia and South America. This has resulted in countries switching their public health policy from treatment with chloroquine to another first line drug, sulfadoxine-pyrimethamine S/P [15]. Increasing resistance to S/P, however, has resulted in the recommendation of alternative therapies (primarily using artemisinin-based drugs either alone or in combination with other anti-malarials such as S/P, mefloquine, and amodiaquine) to combat the increase in malaria infections and prevent resistance [16]. Drug resistance, high cases of malaria, the extension of malaria transmission into new geographic areas and the evolution of the parasite's biology [17] highlight the necessity of targeted programs to eliminate this disease burden.

The purpose of this dissertation is to provide biological information for understanding and defining potential vaccine candidates for possible future use in helping to combat the high incidence of malaria. The remaining sections in this first chapter provide an overview of the malaria life cycle, clinical symptoms of malaria, and the simian malaria *P. knowlesi*. This chapter also highlights and discusses the parasite merozoite structure, the invasion cascade, blood-stage vaccine candidate proteins, and the reticulocyte binding-like (RBL) superfamily of proteins.

Malaria Life Cycle

There are three stages that occur during the *Plasmodium* infection life cycle. The sporogonic stage occurs in the mosquito, the pre-erythrocytic stage occurs in the liver of the susceptible host, and the erythrocytic stage occurs in the blood of the susceptible host. Female anopheline mosquitoes establish malaria infections by injecting parasite

sporozoites into the skin. Minutes after sporozoites are injected they migrate into the bloodstream and subsequently invade the hepatocytes of the liver. In *P. vivax* and *P. ovale*, hypnozoites (dormant parasites) can develop in the liver of patients and cause a relapse of malaria infection weeks, months, or even years after the first onset of the disease [18, 19].

Once sporozoites are in the liver, they undergo a period of maturation, replication, and asexual reproduction, lasting between 2-16 days depending on the species. Sporozoites will mature to liver schizonts that release thousands of uninucleate merozoites into the bloodstream (reviewed in [20]). A recent report has demonstrated that merozoites released from the liver are transported to the bloodstream via membrane-bound vesicles that contain no host nuclei. This vesicle called the merosome enables efficient transport of liver merozoites to the bloodstream allowing the parasite to remain undetected by the immune system [21].

Once merozoites enter the bloodstream they attach to and invade uninfected erythrocytes. Once in erythrocytes, the merozoites differentiate into ring forms. The ring structures differentiate to trophozoites and then to multinucleated and then segmented schizonts. Mature schizonts release merozoites into the bloodstream that subsequently invade free erythrocytes and undergo differentiation (reviewed in [22]). This cycle is repeated every 24-72 hours depending on the *Plasmodium* species.

In the erythrocytic stage, a small number of merozoites will terminally differentiate into the sexual forms of the parasite, the male and female gametocytes. The sexual stage of the parasite can be ingested by female anopheline mosquitoes during a blood meal on an infected host. In the mosquitoes, the ingested gametocytes enter the

mosquito gut and undergo gametogenesis to form male and female gametes in a process that occurs within minutes of the blood meal. During fertilization the gametes produce zygotes and mature to form ookinetes. The ookinetes migrate to reside in between the midgut epithelium and basal lamina where they differentiate to oocysts. During oocyst differentiation, sporozoites form within two weeks and migrate to the salivary glands of the mosquito where they can be transmitted to susceptible hosts (reviewed in [23]).

Clinical Symptoms of Malaria

Malaria infections can be classified as being either complicated or uncomplicated. Uncomplicated malaria is defined as a malaria infection that does not result in dysfunction to vital organs (reviewed in [24]). Some of the clinical features that are presented by uncomplicated malaria include a combination of fever, chills, general malaise, vomiting, and headaches [25].

Complicated malaria (also known as severe malaria) is primarily observed in populations that lack immunity to malaria infections. These populations include young children living in endemic areas who have not yet gained immunity to malaria; persons traveling into malaria endemic areas with little or no previous exposure to malaria infections; persons who previously lived in malaria endemic areas (whose immunity has waned) and who have traveled back into malaria endemic areas; and pregnant women who lose their immunity to malaria during pregnancy.

The most common forms of severe or complicated malaria are cerebral malaria and severe malarial anemia. Other forms include hyperparasitemia, pulmonary edema, hemoglobinuria, metabolic acidosis, and cardiovascular collapse [26]. Although all four

human malarias present with the symptoms associated with uncomplicated malaria, only rarely does *P. malariae* and *P. ovale* lead to cases of severe malaria (reviewed in [27]). *P. falciparum* causes the majority of severe malaria cases and it has been recently shown that *P. vivax* can also lead to severe forms of malaria infections [28].

Plasmodium knowlesi: **Simian Malaria Parasite**

P. knowlesi is a simian malaria parasite that is phylogenetically closely related to the human malaria *P. vivax* [29]. *P. knowlesi* was named for Dr. R. Knowles who along with Dr. Das Gupta first described the erythrocytic stage of this parasite in 1932. *P. knowlesi,* in contrast to the 48-hour schizogony of *P. vivax* and *P. falciparum*, has a 24-hour schizogonic cycle (the only primate malaria with a 24 hour cycle). *P. knowlesi* merozoites are more stable in comparison to *P. falciparum* merozoites and can be isolated in an invasive state [30]. Much of the known basic biology of merozoites and the invasive steps of the parasite come from information obtained from studies using *P. knowlesi* [31, 32].

This parasite species has also been used as a model for the study of vaccines targeted against the parasite erythrocytic stage. Recently, rhesus macaques infected with *P. knowlesi* were used for the development of a malaria vaccine model using DNA prime and poxvirus boost regimens that previously showed partial protection against malaria infection. The DNA prime/poxvirus boost included two pre-erythrocytic antigens (PkCSP and PkTRAP) and two erythrocytic antigens (PkAMA1 and PkMSP1) [33].

One advantage to using *P. knowlesi* is that experimentation can be accomplished *in vitro* (using both adapted and non-adapted *P. knowlesi* parasites) and *in vivo* using

Macaca mulatta/rhesus macaque, *Saimiri/*squirrel, or *Aotus/*owl monkeys. Additionally, The Wellcome Trust Sanger Institute *P. knowlesi* sequencing project has been published [34], providing a wealth of genomic information that allows for easier identification and analysis of *P. knowlesi* genes. In addition, other *Plasmodium* genome sequences (i.e. *P. falciparum* [35]*, P. vivax* [36]), have been completed and allow comparison with *P. knowlesi*.

Experimental and natural transmission of *P. knowlesi* to humans [37] has been previously demonstrated. In 1965, a traveler to Malaysia naturally acquired a *P. knowlesi* infection. Blood from this patient (called *P. knowlesi* H strain) was subsequently passed to human volunteers and rhesus monkeys. The infections in humans were cured with antimalarial therapy while the infections in rhesus monkeys became lethal. This was the first documentation of zoonotic transfer of a simian parasite [38]. There have been recent documented cases of natural human *P. knowlesi* infections in several countries in South East Asia [39-44]. These infections were first described in 2004 and experimental studies demonstrated there were greater than 120 cases of *P. knowlesi* human infections in patients that were believed to be infected with the human malaria *P. malariae.* Microscopically these two *Plasmodium* species appear similar but by nested PCR analysis, the suspected *P. malariae* infections were determined to be *P. knowlesi* [43]. Recent studies published in 2008 have shown that these *P. knowlesi* human cases in Malaysia are more widespread than initially believed and can also become lethal infections [45]. These recent numerous cases of natural human infections necessitate understanding of this malaria parasite species both as a primate model for the human malarias as well as its role in natural human malaria infections. The stability of the *P.*

knowlesi merozoite structure, the ability to isolate merozoites in an invasive state, as well as the availability of biological, molecular, and genomic information makes this parasite-host combination the preferred model to study invasion.

Merozoite Structure

Merozoites play a critical role in the erythrocytic stage of the *Plasmodium* life cycle as they initiate invasion of erythrocytes in the blood. The surface of the merozoite is covered by filaments that are 2-3nm in length and are anchored to the merozoite plasma membrane [46]. The merozoite contains three membranes that together form the pellicle. The plasma membrane surrounds the entire merozoite whereas the inner two membranes do not cover the apical end of the merozoite [47]. The apical prominence of the merozoite contains three membranous vesicles, the rhoptry organelles, micronemes, and dense bodies.

The rhoptry organelles are pear shaped and are approximately 650nm long and 300nm wide. The micronemes are located close to the rhoptry organelles and are more elongated in shape and are approximately 120nm long and 40nm wide [47]. These two organelles contain various proteins that are involved in merozoite invasion of the erythrocyte. The dense bodies lie within the cytoplasm at the apical end of the merozoite and are approximately 80nm in length [48]. Similar to the rhoptries and micronemes, the dense bodies release their contents upon merozoite invasion of the erythrocyte and these contents are presumed to aid the merozoite in development to the ring stage.

The merozoite also contains organelles that are typically found in eukaryotic organisms, such as mitochondria, a nucleus, microtubules, endoplasmic reticulum, and

ribosomes. The merozoite nucleus is enclosed by a nuclear envelope that contains nuclear pores. Free ribosomes are located between the rhoptry organelles and the nucleus. The merozoite also contains three polar rings which are composed of cytoskeletal matrix-like material and are located at the apical prominence of the merozoite [47].

A recent paper has described the presence of a new secretory organelle in *P. falciparum* called a mononeme [49]. This organelle was determined to be present in *P. falciparum* merozoites by comparative localization studies using antigens known to localize to specific apical organelles and another antigen (*P. falciparum* rhomboid-1, PfROM1) whose specific apical organelle location was unknown. Comparative studies using micronemal proteins, Apical Membrane Antigen (PfAMA1) and Erythrocyte Binding Antigen-175 (PfEBA175) along with a rhoptry protein, Rhoptry-Associated-Protein-2 (PfRAP2) and a dense granule marker, Ring-Infected Erythrocyte Surface Antigen (PfRESA) demonstrates that PfROM1 localizes to a distinct organelle, the mononeme. The mononeme is described as being located from the posterior of the nucleus to the merozoite apical end [49]. Future experiments are needed to determine if these structures may be present in other *Plasmodium* merozoites.

The Invasion Cascade

Propagation of the erythrocytic stage of the *Plasmodium* life cycle is achieved by merozoite invasion of erythrocytes, making this stage and specifically merozoite invasion of erythrocytes an attractive target for vaccines. Merozoite invasion of erythrocytes can be divided into three stages: 1) initial merozoite contact with the erythrocyte surface 2) apical reorientation and tight junction formation 3) entry and formation of the

parasitophorous vacuole. These invasive steps have been determined by both video microscopy and electron microscopy [31, 32, 50]. Before invasion can occur merozoites must be released from erythrocytes. There are various hypotheses on the precise method of merozoite release; but it is agreed that parasites inside the erythrocytes cause extensive modifications of the erythrocyte which culminates with the release of merozoites from the erythrocyte membrane as well as the parasitophorous vacuole membrane, PVM [50]. The PVM is a membrane that surrounds the infected erythrocyte and is created when the merozoite invades the erythrocyte. Once merozoites are freed from both membranes they are released into the bloodstream and the invasive process is initiated.

Initial Merozoite Contact with the Erythrocyte Surface

Free merozoites are unstable in blood and must adhere to and invade erythrocytes within minutes after they are released. Initial contact with the erythrocyte surface occurs via merozoite surface coat filaments and can occur anywhere on the merozoite surface. The merozoite contact with the erythrocyte membrane causes distortion of the uninfected erythrocyte [50]. These initial contacts appear to be made through specific molecular interactions as *P. knowlesi* merozoites do not attach to guinea pig erythrocytes, whose hosts are resistant to *P. knowlesi* infection. However, *P. knowlesi* merozoites attach to erythrocytes from the non-resistant human and rhesus monkey hosts [51]. During this phase of invasion, the initial interaction between the merozoites and the erythrocyte surface are reversible contacts. Merozoites can detach from the erythrocyte surface and re-attach to the same erythrocytes or different erythrocytes through other areas on its surface (reviewed in [52]).

Apical Reorientation and Tight Junction Formation

For invasion to occur, merozoites must make contact with the erythrocyte surface through its apical end [50]. Initial contact with the erythrocyte surface can occur on any part of the merozoite surface as discussed above. If the initial contact does not occur with the merozoite's apical end, the merozoite must reorient its position so that its apical end is juxtaposed to the erythrocyte membrane. The initial contact of the apical end of the merozoite and the erythrocyte membrane occurs with the erythrocyte membrane raised. After continued contact, the merozoite forms a depression in the erythrocyte membrane. At this stage, a tight junction is formed between the erythrocyte membrane and the apical end of the merozoite that is approximately 4nm in length; this is an irreversible contact with the host cell [31]. Contact at this stage is mediated by specific adhesive interactions between the host and parasite.

Entry and Formation of Parasitophorous Vacuole

Parasites that belong to the phylum Apicomplexa (of which *Plasmodium* is a member) enter their respective hosts without modification of the host cytoskeleton. After tight junction formation, the junction zone moves over the merozoite surface. This movement is proposed to be mediated by signaling from the merozoite through host cell receptors (reviewed in [53]). At this stage, the merozoite secretes proteins and lipids from the rhoptry organelles [54]. Since the erythrocyte is unable to undergo phagocytosis or receptor-mediated endocytosis, the active process of merozoite entry into the erythrocyte is accomplished by the merozoite (reviewed in [52]).

Parasite proteases are a critical component of erythrocyte invasion. There are two theories as to the location of the precise function of these enzymes in invasion: 1)

proteases function at the host cell surface and/or 2) proteases function at the surface or in the apical organelles of the merozoite. The implication for the first theory is that parasite proteases modify the surface of the erythrocyte and allow for parasitophorous vacuole (PV) formation (reviewed in [55]). The implication for the second theory is that the proteases modify the surface of the parasite as it enters into the erythrocyte.

As the merozoite continues to invade the erythrocyte, the erythrocyte membrane will continue to surround the merozoite. Invasion is complete when the merozoite is completely enveloped inside the erythrocyte. The merozoite invasive steps are known, but the molecular interactions and proteins involved in invasion are less characterized. The following sections describe known merozoite proteins that are involved in or are proposed to be involved in invasion.

Plasmodium Merozoite Proteins: Vaccine Candidates

Malaria vaccines can be classified as pre-erythrocytic, erythrocytic stage, or transmission blocking vaccines. Pre-erythrocytic stage vaccines target the liver stage of *Plasmodium,* erythrocytic stage vaccines target the blood stage, and transmission blocking vaccines target the sexual stages of the parasite and prevent transmission of *Plasmodium* from the infected host to the mosquito.

Several decades ago the promise of a malaria vaccine was spurred on by evidence that protective immunity could be achieved by immunization with live-attenuated sporozoites in both mice and humans [56, 57]. At the time, the idea of an irradiated sporozoite vaccine was thought to be impractical and so the alternative was to identify sporozoite antigens that could be potential vaccine targets. This lead to the identification

of the circumsporozoite protein, CSP [58] and thrombrospondin-related adhesion protein, TRAP [59]. Since that time most vaccine development projects against the liver stage of malaria have primarily been focused on CSP and TRAP, essentially ignoring the many thousand other antigens expressed during the liver stage.

Currently, the RTS,S vaccine is considered to be the most promising malaria vaccine. This vaccine is composed of a recombinant protein against the C-terminus of PfCSP fused to a hepatitis B virus surface antigen. In a study conducted in children (age 1-4 years) from Mozambique, a clinical phase IIb trial demonstrated that individuals immunized with the RTS,S vaccine displayed a reduction of clinical malaria by 35% and the incidence of severe malaria was decreased by 50%; the partial protection induced against the clinical symptoms of malaria lasted for approximately eighteen months [60]. Another clinical phase IIb trial was conducted using the same vaccine in Tanzania. The vaccine was administered to infants at 8, 12, and 16 weeks of age and demonstrated reduction of clinical disease by 65% in these infants [61]. There is currently a debate within the malaria research community concerning RTS,S as a promising malaria vaccine. Some researchers have questioned whether the location of the trials has affected the rates of malaria infections, as the studies have been conducted in areas of low disease burden. Performing the same trials in areas of high disease burden may affect the incidence of clinical malaria observed after vaccination [62]. More research needs to be performed to address this and other questions concerning this malaria vaccine. However, these recent vaccine trials give renewed hope that an effective malaria vaccine can and will be discovered.

Blood-stage erythrocytic vaccines have primarily focused on targeting merozoite proteins because the merozoite becomes extracellular and is exposed to the immune mechanisms of the host (reviewed in [63, 64]). Table I highlights the current status of several blood-stage vaccines in *P. falciparum* and *P. vivax*. With the publishing of the genomes for each of these species, there must be a greater impetus to identify and characterize other antigens that are expressed in the liver and blood stages; and to define their potential as vaccine targets. Few merozoite proteins have been identified as vaccine candidates and extensively characterized; some are described in greater detail below.

Merozoite surface protein-1 (MSP-1) was first identified in the rodent malaria species, *P. yoelii,* as a high molecular weight protein (230,000 MW) that was recognized and immunoprecipitated by a *Plasmodium yoelii yoelii* specific monoclonal antibody, 25.1. MSP-1 was defined as localized to the surface of the merozoite because of the reactivity of antibody 25.1 with free merozoites. MSP-1 is also immunologically significant as immunization with *P. yoelii* MSP-1 and its proteolytic fragments enabled immunized mice to clear a lethal *P. yoelii* infection [65]. MSP-1 has subsequently been identified in all analyzed *Plasmodium* species [66], most notably, *P. falciparum* [67]*, P. vivax* [68, 69], and *P. knowlesi* [70]. Further studies have revealed that MSP-1 is part of a much larger and more complex protein family appropriately named merozoite surface proteins. *P. falciparum* contains at least 10 MSPs (PfMSP-1 through PfMSP-10) while other species (i.e. *P. vivax* and *P. knowlesi*) also contain several MSPs (reviewed in [52]). Protein family members differ in their protein structure as some contain glycosylphosphatidylinositol (GPI) membrane anchors (PfMSP-1, 2, 4, 5, 8 and 10) and

Species	Antigen	Stages
P. falciparum	MSP-1	Clinical Ia; IIb
	AMA-1	Clinical Ia; Ib; IIb
	MSP-2	Clinical Ia
	MSP-3	Clinical IIb
	EBA-175	Clinical Ia
	MSP-1	Pre-Clinical
	MSP-2	Pre-Clinical
	MSP-4	Pre-Clinical
	AMA I	Pre-Clinical
	MSP1/EBA-175	Pre-Clinical
P. vivax	MSP-1	Pre-Clinical
	MSP-9	Pre-Clinical
	DBP-RII	Pre-Clinical
	AMA-I	Pre-Clinical

Table 1.1. Current status of malaria vaccines.

A simplified summary of the various malaria vaccines are listed in the above table. The specific *Plasmodium* species is listed along with the target antigen and the current clinical stage of the specific vaccine. Data compiled from WHO vaccine development tables (http://www.who.int/vaccine_research/documents/en/) and [71].

others are soluble proteins (PfMSP-3, 6, 7, and 9). However, most members in the family contain at their carboxy-terminus at least one epidermal growth factor (EGF)-like domain (the exception being PfMSP-2) (reviewed in [72]).

It has been postulated that MSPs function in the initial contact of the merozoite with the host erythrocyte because of the even distribution of MSP-1 over the surface of the merozoite (reviewed in [72]). Although there has been extensive research into this family of proteins, the specific function(s) of the MSPs are still unknown. However, it has been reported that MSP-1 binds the protein band 3 on the surface of erythrocytes [73]. Despite the lack of comprehensive understanding concerning the role of MSPs in invasion, several protein family members (PfMSP-1, MSP-2, MSP-3, and MSP-4) have been developed as potential malaria blood-stage vaccine candidates.

Apical membrane antigen 1 (AMA-1) is another merozoite protein that has been developed as a blood-stage vaccine candidate. AMA-1 has been characterized in all *Plasmodium* species analyzed [74-77] and in *Toxoplasma gondii* [78], which is also a member of the phylum *Apicomplexa*. The conservation of this protein suggests that AMA-1 plays a crucial role in parasite invasion. The cytoplasmic domain on the C-terminal end of AMA-1 is highly conserved in *Plasmodium* and it has been proposed that the protein functioned either in signaling or in the moving junction of the merozoite (reviewed in [72]). A study using electron microscopy and an inhibitory monoclonal antibody against an invasion epitope of AMA-1 demonstrated that this protein appears to be involved in apical reorientation of the merozoite [79]. The significance of this protein is further evidenced by PfAMA-1 [80] and PkAMA-1 [81] specific antibodies that block *in vitro* invasion of the respective species. Experimental studies that deleted AMA-1 in *P.*

falciparum, also resulted in the inability to recover parasites suggesting that AMA-1 is essential for parasite invasion [80].

AMA1 in *P. falciparum* exists in two different forms, a full-length 83-kDa protein and a proteolytically cleaved 62-kDa protein [82, 83]. The unprocessed form of AMA1 localizes to the microneme organelles in the merozoite [82]. Once the full-length protein is made, processing of this protein occurs within fifteen minutes to create the mature 62-kDa protein. The proteolytically cleaved protein translocates to the rhoptry organelles and then to the merozoite surface [83].

An important discovery in understanding the functional role of AMA-1 was the identification of the binding domain. The disulfide bond pattern of AMA-1 defines three discrete domains [84]. Domains of the *pfama-1* gene were cloned into a plasmid vector (T8 plasmid), transfected into mammalian cells (CHO-K1), and expressed on the surface of CHO-K1 cells. Erythrocyte adhesion assays were performed on transfected mammalian cells and it was observed that domain III of PfAMA-1 bound to trypsin-resistant erythrocytes. Although the receptor for AMA-1 is unknown, the identification of the binding domain provides insight into the type of receptor to which AMA-1 binds.

The binding domain of another family of proteins, the Duffy Binding-Like/Erythrocyte Binding-Like (DBL/EBL) family of proteins, has also been defined. This domain was determined by cloning the various protein domains of the Duffy binding proteins of *P. knowlesi* (PkDBPα, β, γ) and *P. vivax* (PvDBP) into a plasmid vector (pRE4) that allows for protein expression on the surface of mammalian cells. Erythrocyte adhesion assays were subsequently performed to determine the binding capability of each

domain; the N-terminal cysteine-rich domain termed region II was defined as the binding domain [85].

DBL/EBL members have similar gene and protein structures; *dbl/ebl* genes have one small 5' intron, three small 3' introns, and a primary, centrally located exon, and the proteins contain two cysteine-rich regions (reviewed in [52]). The first protein member identified was a 175-kDa soluble, merozoite protein in *P. falciparum* that bound to human erythrocytes and was named erythrocyte-binding antigen 175 or EBA-175 [86]. *P. falciparum* has since been found to contain a total of six *dbl/ebl* genes [87]. The binding domain of EBA-175 was also identified as being the N-terminal cysteine-rich region II by performing similar erythrocyte binding assays used to identify the PkDBP and PvDBP binding domains. The receptor of EBA-175 was also defined as glycophorin A by the addition of soluble glycophorin A and B into the erythrocyte binding assays. Glycophorin B was unable to inhibit the binding activity of EBA-175 region II to target erythrocytes while glycophorin A exhibited 80% inhibition [88].

PkDBPs and PvDBP are termed Duffy-binding proteins because PkDBPα [89] and PvDBP [90] were found to adhere specifically to human red blood cells expressing the Duffy blood group antigen. Duffy negative human erythrocytes are believed to be resistant to invasion by *P. knowlesi* and *P. vivax* parasites [89], leading to the hypothesis that DBP proteins are necessary for invasion. However, recently published data suggests that *P. vivax* transmission may occur in Duffy negative populations [91, 92].

Experimental studies deleting the *pkdbpα* gene have also demonstrated that PkDBPα is necessary for invasion of *P. knowlesi* parasites into Duffy positive human erythrocytes. These studies have confirmed through electron microscopy using the

transgenic parasites, that PkDBPα plays a crucial role in mediating tight junction formation during invasion [93]. Truncation of *P. falciparum* EBA-175 (with regions III-V deleted) demonstrates that the full length gene is not necessary for proper protein localization and expression. Additionally, truncated PfEBA-175 causes a switch from a sialic-acid dependent invasion pathway to a sialic-acid independent invasion pathway [94]. Future studies will determine the specific roles of other DBL/EBL proteins.

Reticulocyte Binding-Like (RBL) Protein Superfamily

The RBL protein superfamily has been localized to the apical prominence of the merozoite, and it is believed that these proteins mediate molecular interactions with the erythrocyte that lead to merozoite invasion (reviewed in [52]). The RBL proteins are large, typically between 230-350 kDa. Protein members of the RBL family have a conserved gene structure with two exons; the first exon encoding a signal peptide and the second exon encoding the hydrophilic protein [95]. They are defined as Type I integral membrane proteins (reviewed in [52]).

The first two members of the family were identified in *P. vivax* [96]. These two *P. vivax* proteins adhere specifically to reticulocytes and have been implicated as the proteins responsible for preferential invasion of reticulocytes; for this reason they have been termed *P. vivax* Reticulocyte Binding Proteins 1 and 2 (PvRBP1 and PvRBP2). Erythrocyte binding assays using reticulocyte-enriched preparations demonstrate increased binding of both PvRBP1 and PvRBP2 as compared to reticulocyte-depleted preparations. The PvRBPs are α-helical in shape and co-expressed at the apical end of the merozoite [96]. They are proposed to form a heterotetrameric complex and function in

reticulocyte recognition as well as in subsequent signaling that leads to merozoite junction formation and entry [97].

Because of the proposed critical function of PvRBPs in merozoite invasion, it was proposed that homologous proteins existed in all species of *Plasmodium*. In the publication describing the initial identification of the *P. vivax* RBPs, hybridization between *pvrbp* DNA probes and *P. cynomolgi* (a simian malaria that is closely related to *P. vivax* [98]) genomic DNA was demonstrated, suggesting that homologous antigens existed in that *Plasmodium* species [96]. A subsequent paper outlined the gene and protein structure of the two *P. cynomolgi* RBP proteins termed *P. cynomolgi* Reticulocyte Binding Proteins 1 and 2, PcyRBP1 and PcyRBP2 [99]. These proteins are called reticulocyte binding proteins because similar to *P. vivax*, *P. cynomolgi* preferentially invades reticulocytes [100]. Similarities between *P. vivax* and *P. cynomolgi* RBL proteins have been shown through cross-reactivity of *P. vivax* rabbit antiserum with *P. cynomolgi* parasite extracts; high levels of protein and genetic sequence identity are also observed between the RBL proteins of these two species [99].

Homologous RBL proteins have also been identified in the rodent malaria species, *P. yoelii*. The first RBL protein defined was a 235-kDa protein identified by immunoprecipitation of *P. yoelii* parasites with a monoclonal antibody raised against blood stage parasites [65]. The RBL proteins of *P. yoelii* are a multi-gene family and together are referred to as Py235. This group of proteins contain fifteen identified gene members [101, 102]. In contrast to the RBL proteins of *P. vivax* and *P. cynomolgi*, it has been shown that the Py235 proteins bind to all erythrocytes. *P. yoelii* proteins are similar in structure to other RBL proteins being α-helical in structure and containing a

transmembrane domain along with a short cytoplasmic tail. The Py235 proteins have also been localized to the rhoptry organelles; and passive and active immunization with a monoclonal antibody raised against the originally defined Py235 protein demonstrates protection against challenge with a lethal strain of *P. yoelii* [65, 103].

The human malaria, *P. falciparum*, also contains homologous RBL proteins that are capable of binding to all erythrocytes and so are termed normocyte binding proteins, PfNBPs [104], or RBL protein homologues, PfRh [105]. There are five known genes that encode normocyte binding proteins in *P. falciparum (pfnbp1, pfnbp2a, pfnbp2b, pfnbp4, pfnbp5)* and one psuedogene *(ψpfnbp3)* (reviewed in [52]). PfNBP1/PfRH1 is an ortholog of PvRBP1 that has been localized to the apical pole of the merozoite. PfNBP2a/PfRh2a and PfNBP2b/PfRh2b are orthologs of PvRBP2 [104, 105]. The PfNBPs share structural features with the PvRBPs, containing two exons that encode a signal peptide along with a hydrophilic protein containing a transmembrane and cytoplasmic domain. Antisera against PfNBP1, PfNBP2a, and PfNBP2b partially inhibits invasion of *P. falciparum* 3D7 clone parasites [104]. Targeted disruption of *pfnbp1* demonstrates it is required for sialic-acid dependent invasion [106]; targeted disruption of *pfnbp2b* also results in parasites that show a dramatic decrease in the ability to invade neuraminidase- and trypsin-treated erythrocytes [107].

Interestingly, *P. reichenowi*, a chimpanzee parasite closely related to *P. falciparum,* has a repertoire of RBL proteins similar to that observed in *P. falciparum.* In *P. reichenowi*, there are four genes that encode for RBL proteins (*prnbp2a, prnbp2b, prnbp3*, and *prnbp4*) and one pseudogene (ψ*prnbp1*). The amino acid similarity between the *P. falciparum* and *P. reichenowi* RBLs is between 91% and 97% [95].

The initial identification of the *P. vivax* Reticulocyte Binding Proteins demonstrated no hybridization between the *pvrbp* DNA probes and *P. knowlesi* genomic DNA suggesting that *P. knowlesi* contained no homologous proteins [96]. The following chapters detail the experimental procedures that identified homologous RBL proteins in *P. knowlesi* and define the functional role(s) of these proteins in invasion.

Overview of *Plasmodium knowlesi* Normocyte Binding Protein Studies

The RBL proteins are proposed to function by adhering to erythrocytes and mediating subsequent events in merozoite invasion; they are believed to play a necessary role in the propagation of the blood-stage in the parasite life cycle. The specific function(s) of the RBL proteins are not comprehensively understood. The purpose of this dissertation is to contribute to the advancement of biological and molecular understanding of *Plasmodium* merozoite invasion by utilizing the simian malaria parasite, *P. knowlesi*. These studies characterize the two recently identified RBL proteins in *P. knowlesi,* PkNBPXa and PkNBPXb. The studies leading to the identification of these two proteins is detailed along with functional studies that have defined a binding domain of PkNBPXb. Additional studies related to understanding the function of PkNBPs in merozoite invasion are also described. The information garnered from these studies can be used to better understand the functions of other *Plasmodium* RBL proteins and their interaction with host cells.

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CHAPTER TWO

THE RETICULOCYTE BINDING-LIKE PROTEINS OF *P. KNOWLESI* LOCATE TO THE MICRONEMES OF MEROZOITES AND DEFINE TWO NEW MEMBERS OF THIS INVASION LIGAND FAMILY

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Abstract

Members of the Reticulocyte Binding Protein-like (RBL) family are merozoite-expressed proteins hypothesized to be essential for effective invasion of host erythrocytes. Proteins of the RBL family were first defined as merozoite invasion ligands in *Plasmodium vivax*, and subsequently in *P. falciparum* and other malaria parasites species. Comparative studies are providing insights regarding the complexity and evolution of this family and the existence of possible functionally alternative members. Here, we report the experimental and bioinformatic characterization of two new *rbl* genes in the simian malaria parasite species *P. knowlesi.* Experimental analyses confirm that a *P. knowlesi* gene fragment orthologous to *P. vivax reticulocyte binding protein-1 (pvrbp1)* represents a highly degenerated pseudogene in the H strain as well as two other *P. knowlesi* strains. Our data also confirms that a gene orthologous to *pvrbp2* is not present in the *P. knowlesi* genome. However, two very diverse but related functional *rbl* genes are present and are reported here as *P. knowlesi normocyte binding protein Xa* and *Xb* (*pknbpxa* and *pknbpxb*). Analysis of these two *rbl* genes in Southern hybridizations and BLAST searches established their relationship to newly identified members of the RBL family in *P. vivax* and other species of simian malaria. Rabbit antisera specific for recombinant PkNBPXa and PkNBPXb confirmed expression of the prospective high molecular weight proteins and localized these proteins to the apical end of merozoites. Their precise location, as determined by immuno-electron microscopy (IEM), was found to be within the microneme organelles. Importantly, PkNBPXa and PkNBPXb are shown here to bind to host erythrocytes, and discussion is centered on the importance of these proteins in host cell invasion.

Introduction

Malarial merozoites gain entry into red blood cells through specific receptor-ligand interactions and a cascade of molecular interactions that are largely still undefined (reviewed in [1]). Moreover, merozoites can be characterized by their ability to invade erythrocytes of either all stages of maturation or by an evident restriction, only reticulocytes. The reticulocyte host cell specificity of human malaria *P. vivax* merozoites has been well known and attributed to the action of the Reticulocyte Binding Protein (PvRBP1 and PvRBP2) complex located at the invasive apical end of the merozoite [2-4]. Homologs of the *P. vivax* RBPs have since been characterized in the human malaria *P. falciparum* [5-9], non-human primate [10, 11] and rodent malaria species [12]. This family of invasion ligand proteins is now known as the Reticulocyte Binding-Like (RBL) family. *Rbl* genes have a characteristic small exon encoding a signal peptide, a short intron, and a second exon encoding a large (230 - 350 kDa) predominantly hydrophilic protein with a transmembrane domain and short cytoplasmic tail (reviewed in [1]).

P. cynomolgi, which is a simian malaria parasite closely related to *P. vivax,* also invades predominantly reticulocytes and the orthologous genes for *pvrbp1* and *pvrbp2* have been reported [2, 10]. In the rodent parasite *P. yoelii*, the Py235 gene family was shown to be most related to *pvrbp2* [4, 13]. In *P. falciparum*, genomic and experimental studies revealed homolog genes that have been termed normocyte binding proteins (*nbp1, nbp2a, nbp2b*) [5, 6], also referred to as reticulocyte-binding homologs 1, 2a and 2b (*rh1, rh2a* and *rh2b*) [5-7]. Other similar *P. falciparum* genes were subsequently characterized as *rh3/nbp3*, *rh4/nbp4*, and *rh5/nbp5*, with *rh3/nbp3* confirmed to be a pseudogene [8, 9, 14, 15]. Interestingly, the chimpanzee parasite, *P. reichenowi*, has an identical composite

of family members orthologous to the *P. falciparum* genes. However in *P. reichenowi, nbp1* is clearly a pseudogene without an open reading frame (ORF), while *nbp3* has an ORF and appears to be functional [16]. The RBL proteins known to date, though quite divergent in sequence, have shared structural and biological features that are presumed to be essential for the targeting of host cells and effective invasion. The initial identification and characterization of the *P. vivax* RBPs supported the hypothesis that merozoites require molecules to identify the appropriate target cells and then may signal the activation of subsequent receptor-ligand interactions involved in invasion [2-4]. It is now known that several other more distantly related *rbl* genes are present in *P. vivax* [17] and it remains to be determined whether the encoded proteins serve as alternative ligands, or if some are in fact pseudogenes, as suggested by the current gene sequences reported in the *P. vivax* genome database. The characterization of *P. falciparum* RBLs has provided additional information regarding the potential complexity of the RBL family and their role in merozoite invasion. *In vitro* experiments focused on generating *P. falciparum rbl* knock out parasites have begun to clarify the role of *rbls* in the cascading events of merozoite invasion. PfRh1/NBP1 is an important player in invasion through a sialic acid-dependent interaction [5, 18]; however, this gene can be disrupted with continued survival of the parasites *in vitro* [18]. Disruption of *pfrh2a/pfnbp2a* and *pfrh2b/pfnbp2b* genes is also possible with the retrieval of live parasites, indicating that in some *P. falciparum* strains merozoite entry is mediated through a novel, alternative invasion pathway [19]. Recent studies have also reported regions of *P. falciparum* RBLs implicated in binding to erythrocytes [20-22].

Phylogenetic analysis based on the known *rbl* genes and encoded proteins show that they form subgroups that reflect specific relationships and a degree of similarity to the PvRBP1 or PvRBP2 prototype molecules ([10] and unpublished data). Though the overall identity between ortholog molecules in widely divergent species such as *P. vivax*, *P. falciparum* and *P. yoelii* is very low (23% - 30%), within a genetic clade such as between *P. vivax* and the Asian simian malarias or between *P. falciparum* and *P. reichenowi,* the degree of identity between orthologs within the same RBL subgroup increases dramatically to high levels of 75% or greater.

To better understand the functional role(s) and fine interactions of the RBL proteins in host cell selection and entry, we sought to identify and undertake a thorough investigation of the *rbl* genes and encoded proteins expressed in a simian malaria species, *P. knowlesi,* known to also naturally infect humans [23-27]. *P. knowlesi* is closely related to *P. vivax* and its sister parasite *P. cynomolgi* [28]. It is also very closely related to the simian malaria parasite *P. coatneyi*, which in many respects is phenotypically similar to *P. falciparum* [29]. As such, and given the stability of its merozoites [30], *P. knowlesi* has traditionally provided an exceptional instrument for the comparative study of the erythrocyte invasion mechanisms pertinent to both *P. vivax* and *P. falciparum.*

The *P. knowlesi* genome has recently been completed with 8-fold sequence coverage, yet the *P. knowlesi rbl* gene repertoire in this species remained in question, with a number of partial sequences annotated as fragments of putative *rbps* on chromosome 14, and no other genes recognized as members of this family [31]. Here, using a combination of experimental investigation and bioinformatics we definitively report the presence of three *rbl* genes in the *P. knowlesi* genome, and confirm that one is

a pseudogene. The two expressed RBL members, PkNBPXa and PkNBPXb, have been investigated with regards to their structures and functions and localized by IEM to the microneme organelles of mature merozoites. The functional importance of these proteins in the context of the RBL superfamily, and the importance of *P. knowlesi* as a model for further investigations of the RBLs are discussed.

Materials and Methods

Parasite propagation and acquisition

P. knowlesi (H strain) infected erythrocytes were obtained fresh from *Macaca mulatta* monkeys or grown *in vitro* from cryopreserved samples and processed as described previously [32]. *P. vivax* Belem and Salvador I strain parasites were acquired from blood-stage infections in *Saimiri boliviensis* monkeys and processed to remove leukocytes and platelets prior to purification of infected erythrocytes. *P. coatneyi* originating from a 1961 isolation [33], *P. cynomolgi* (Berok strain) and *P. fragile* (Nilgiri strain), were expanded in *M. mulatta* monkeys for DNA isolation after removal of host cellular elements. All non-human primate experimental studies, infections and associated protocols were performed with the official approval of Emory University or CDC's Institutional Animal Care and Use Committee.

Nucleic acid isolation

Genomic DNA (gDNA) was prepared from blood-stage parasites using the QIAamp DNA Blood Extraction kit (Qiagen, Valencia CA), as described previously [2]. Total RNA was prepared from mature schizonts using TRIzol (Invitrogen, Carlsbad CA) following the manufacturer's protocol and treated with RNAase-free DNAase (Roche, Indianapolis IN).

Polymerase Chain Reaction (PCR) amplification and cloning of *rbl* genes in *P. knowlesi*

PCR amplification using the Expand High Fidelity System (Roche, Indianapolis IN) was performed with gene-specific primers on gDNA from *P. knowlesi* following the manufacturer's instruction. Fragments were cleaned using the Qiaquick purification system (Qiagen, Valencia CA), cloned into the pCR2.1 vector (Invitrogen, Carlsbad CA) and sequenced using the ABI Prism BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems, Foster City CA). DNA fragments for *pvrbp* and *pknbp* radiolabeled probes were generated by PCR amplification using primers based on the published *pvrbp1* (M88097) and *pvrbp2* (AF184623) gene sequences, data retrieved from database BLAST searches and new sequencing data. *Pknbp1* pseudogene (ψ*pknbp1)* amplicons were generated using three *P. knowlesi* strains as templates and primers were designed according to the sequence of a 1.5 kb DNA fragment identified from the H strain genome (F 5' GGT CGA AAC ATA ATA CGG TG 3' and R 5' GGA ATT CGA TGG AGT TGA TT 3'). The complete *pknbpxa* and *pknbpxb* gene structures were constructed and finalized through a combination of genome database BLAST searches of *P. knowlesi* contigs and shotgun sequences and new sequence data generated from amplified DNA fragments corresponding to the *pknbp* genes.

Confirmation of *pknbpxa* and *pknbpxb* intron/exon junctions

The 5' ends and the exon/intron junctions of the *pknbpxa* and *pknbpxb* genes were confirmed using a 5'RACE system and reverse transcriptase PCR, RT-PCR (Invitrogen, Carlsbad CA) following the manufacturer's recommendations. Two gene-specific

primers were designed to produce cDNA, Nxa1R 5' ATT CTG TCT ATC GTA GGA GC 3' and Nxb2R 5' TTG CTT CAC GGA TTT GCT 3', followed by nested amplifications using the 5' RACE Abridged Anchor Primer provided in the kit and gene-specific reverse primers, Nxa3R 5' CCA ATA ATA ATT AAC AGA AG 3' and Nxb4R 5' TGG AGA TAG CCT CAA AT 3'.

Southern and northern blot analysis and library screening

1μg to 2μg aliquots of gDNA, digested overnight with designated restriction enzymes, were separated on 0.8% agarose gel by standard electrophoresis, essentially as described [2]. Fixation of DNA to the membranes was performed by either UV cross linking (Stratagene, La Jolla CA) or baking *in vacuo* at 80°C for 2 h. DNA fragments were labeled for hybridization reactions using the Prime-It II DNA labeling system (Stratagene, La Jolla CA) according to the manufacturer's instructions. Hybridization was performed as described previously [2]. Northern blots were prepared as described previously [34]. Blots were hybridized with radiolabeled probes in 7% SDS/0.5 M NaH_2PO_4, pH 7.2/2% dextran sulphate at 65°C overnight. The membrane was washed three times in 6XSSC/0.1% SDS, 2XSSC/0.1%SDS and 0.2XSSC/0.1%SDS for 15 min each time at 60°C. The signals were visualized by exposure to Kodak BioMax MS film (Kodak). *P. knowlesi Eco*RI-digested gDNA λ Zap II libraries were constructed and screened as described previously [2] and screened with a radiolabeled probe representing the 2.5 kb from the 5' region of the *pvrbp*1 gene. Three positive clones containing a 1.5 kb *Eco*RI fragment were identified and sequenced, and subsequently compared and

shown to be identical to sequence generated from the *P. knowlesi* genome sequencing project.

Pulse Field Gel Electrophoresis

P. knowlesi chromosomes were size fractionated by pulse field gel electrophoresis (PFGE) and sequential Southern blot analyses were performed using probes representing the central region of the *pknbpxa* and *pknbpxb* genes. Briefly, *P. knowlesi* chromosomes blocks were separated by PFGE in the CHEF-DR III system (BioRad, Hercules CA). Electrophoresis was performed in 0.8% chromosomal grade agarose using 1X TAE at 14^{o}C. Chromosomes of *Hansenula wingei* were used as molecular mass standards. After electrophoresis the gel was denatured and transferred to a nylon support as described previously [2]. The *pknbpxa* and *pknbpxb* probes labeled by random priming were hybridized to the membranes following standard protocols and washed under high stringency conditions (2X SSC/0.1%SDS at 60^{o}C).

Production of fusion proteins and rabbit antisera

Two recombinant His-tagged fusion proteins, rPkNBPXa and rPkNBPXb, were produced in the Gateway (Invitrogen, Carlsbad CA) and pET (Novagen, Madison WI) systems respectively. rPkNBPXa was produced as a 1.2 kb fragment by PCR amplification using primers rNBPXaF (5' GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAC GTT GTT GAA AAC TGA A 3') and rNBPXaR (5' GGG GACCAC TTT GTA CAA GAA AGC TGG GTT GTT CAA TTT TCC TTG CAA ATC 3'). A positive recombinant clone was expressed by addition of 0.3 M NaCl for 3 h at 37°C. The

inclusion bodies were purified as described previously [35], separated by SDS-PAGE and electro-eluted for 16 h. rPkNBPXb was produced as a 1.14 kb fragment by PCR amplification using primers rNBPXbF (5'ctcgagAGCTTACGCAACATATTAAAC3') and rNBPXbR (5'ggatccGTCATC ATCATCATTATCGTG3'). A plasmid with the expected sequence was expressed using 1mM IPTG in BL21-DE3 (Novagen, Madison WI) for 3 h at 37°C. The recombinant protein was bound to Ni-NTA agarose (Qiagen, Valencia CA) and purified under native conditions using 250 mM imidazol (Sigma, St. Louis MO). The purity of the eluted proteins was assessed by SDS-PAGE. The recombinant proteins were dialyzed against PBS before inoculation in New Zealand White Rabbits (Covance, Denver PA) for production of polyclonal antisera (Rab-anti-rPkNBPXa and Rab-anti-rPkNBPXb).

SDS-PAGE and western blotting

Infected erythrocytes containing mature, segmented schizonts where harvested and extracted with reducing sample buffer. The protein extracts were analyzed by SDS-PAGE on 5% polyacrylamide gels and transferred to protean nitrocellulose (Schleicher & Schuell, Keene NH) as described previously [2]. Membranes were probed with rabbit polyclonal antisera diluted 1:500, which had been depleted of antibodies against *E. coli* using standard adsorption procedures. Alkaline phosphatase-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MD) was used for detection (1:5000) and bands were visualized by adding NBT/BCIP substrate (Promega, Madison WI).

Immunofluorescence assays and immuno-electron microscopy

For indirect immunofluorescence assay (IFA), air-dried, thin blood smears prepared with mature schizont-stage parasites were incubated with rab-anti-rPkNBPXa, and rab-anti-rPkNBPXb polyclonal antisera diluted 1:500. Goat anti-rabbit IgG antibodies conjugated to FITC were added and the slides were visualized using a fluorescence equipped microscope. For immuno-electronmicroscopy, merozoites were added to rhesus RBC's, in the presence of 5 μg ml^{-1} cytochalasin B (Sigma, St. Louis MO), and were fixed in 0.1% (v/v) double-distilled glutaraldehyde and 2% (w/v) paraformaldehyde prepared in culture medium (RPMI, pH 7.2), for 20 min on ice, and then washed four times in ice-cold RPMI and dehydrated through a progressively low-temperature ethanol series before being infiltrated with LR White resin (EMSCOPE, London, United Kingdom). Resin polymerization was induced by ultraviolet light at room temperature for 48 h. Sections were immune-stained with the polyclonal sera anti-rPkNBPXa and rPkNBPXb, diluted 1:50 or 1:100 in 1% BSA/PBS, followed by Protein A conjugated to 10 nm gold particles, diluted 1:70 in 1% BSA/PBS (a kind gift by Dr Pauline Bennett, King's College London). Parallel samples were treated with pre-immunization serum antibodies for control purposes. Sections were stained for 4 min with 2% (w/v) aqueous uranyl acetate. Sections were viewed and digital images were taken using a Hitachi 7600 electron microscope.

Erythrocyte Binding Assays

Erythrocytes infected with schizont stage parasites were purified by centrifugation on Percoll gradients, as previously described [36]. The purified schizont-infected

erythrocytes were placed into culture *in-vitro* and allowed to grow overnight as previously described [1, 5] until erythrocytes containing mature schizonts formed merozoites had completely ruptured. Cultures were centrifuged at 4000 rpm and the supernatants were removed and stored in liquid nitrogen. 500 mL of culture supernatants were rotated with 1 x 10^9 erythrocytes at room temperature for 4 h. The cells were washed twice by layering over and subsequent centrifugation through Dow Corning 550 silicone fluid. Bound proteins were eluted in 50 ul of 5x RPMI at room temperature and harvested by centrifugation. The resulting proteins were analyzed by SDS-PAGE on Pre-Cast 5% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules CA). Membranes were probed with rabbit polyclonal antisera diluted 1:500 for 2 h. Alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:5000 (Promega, Madison WI) was used for detection of bound antibodies. Bands were visualized by addition of NBT/BCIP substrate (Promega, Madison WI) to the nitrocellulose strips.

Bioinformatics and sequence analysis

TBlastN searches of preliminary and finalized *P. knowlesi* sequence database (8x coverage) at Sanger Center (http://www.sanger.ac.uk), the *P. vivax* sequencing project at TIGR Center (http://www.tigr.org) and PlasmoDB (www.PlasmoDB.org) database were performed by using translated sequence from *pvrbp1* and *pvrbp2* and the newly identified *pkrbl* genes. Signal peptide cleavage sites and transmembrane domains were predicted with SignalP V2.0 (www.cbs.dtu.dk/services/SignalPV2.1) and TMpred (www.ch.embnet.org /software/TMPRED_form.html) software, respectively. Multiple alignments of nucleic acid and protein sequences were generated using ClustalW 1.8.2

with all default parameters. Tree topology was generated using the Neighbor-Joining algorithm in the MEGA 2.2 software on distances calculated with a Poisson correction [37].

Results

The ortholog of *pvrbp1* is a highly degenerated pseudogene in *P. knowlesi,* but a functional full-length gene in the sister taxon *P. coatneyi.*

To identify and characterize a *pvrbp1* ortholog in *P. knowlesi*, Southern hybridization assays were initially performed under high stringency conditions, and no hybridization signals were observed, suggesting that a highly related intact orthologous gene was not present in this species [2]. However, when a 2.5 kb *pvrbp1* probe representing the 5' end of the gene was subsequently evaluated using lowered stringency conditions, clear distinct bands were detected in each gDNA sample digested with restriction enzymes (Fig. 2.1 A). In contrast, *pvrbp1* probes representing other downstream regions of the gene, also evaluated at lower stringencies, did not result in hybridization signals (data not shown).

To evaluate this putative related but divergent ortholog further, the 1.5 kb *Eco*RI fragment detected by Southern hybridization was cloned and sequenced. Instead of an expected single ORF, multiple ORFs were detected. In addition, 1200 bases of sequence present in *pvrbp1* and the orthologous genes in *P. cynomolgi, P. coatneyi* and *P. fragile* [10], though expected, were not present in the degenerate *pknbp1* sequence. In conclusion, a total of 626 bp of the 1.5 kb cloned sequence showed identities of 80.2% and 82.3% when aligned with the *pvrbp1* sequence. The remaining 900 bp of downstream sequence showed no apparent relationship (<28% identity) to *pvrbp1* (Fig. 2.1 B). DNA samples from the Hackeri and Philippine strains of *P. knowlesi* also hybridized with the 1.5 kb DNA probe (data not shown). Although some single nucleotide polymorphism was

observed, as would be expected between strains of parasites, amplified gene fragments corresponding to the 1.5 kb *Eco*RI clones were essentially identical (99% identity) and also showed no continuous ORF.

Contig sequences recently made available in the *P. knowlesi* (H strain) genome database (http://www.sanger.ac.uk/Projects/P_knowlesi/) [31] are consistent with our experimental analyses, supporting the presence of a degenerated pseudogene. Our searches of the genome database identified an additional 200 bp upstream corresponding to *pvrbp1* sequence, but no related sequences were present downstream. Thus, we conclude that the ortholog of *pvrbp1* in *P. knowlesi* (H strain, Hackeri and Philippine strains) is a highly degenerate pseudogene, with only short segments of the 5' region maintained in the genome for each strain tested.

P. coatneyi is a sister taxon of *P. knowlesi* [29], yet it displays unique biological and phenotypic characteristics distinct from *P. knowlesi* (reviewed in [38]). In contrast to *P. knowlesi* data, the Southern hybridizations suggested that a complete ortholog of *pvrbp*1 existed in the *P. coatneyi* genome (data not shown). To characterize the *P. coatneyi rbp1* (*pcrbp1)* gene, overlapping gene regions were amplified by PCR using primers designed from the *pvrbp1* sequence, cloned and sequenced (Genbank Accession number DQ973816). This sequence proved to be highly homologous to the *pvrbp1* gene, showing that *pcrbp1* is an intact, likely functionally expressed gene. The *pcrbp1* homolog exhibits a nucleotide identity of 84% with the *pvrbp1* gene, and the deduced protein sequence shows 75 % identity to PvRBP1. *P. fragile*, also a genetically close parasite in the simian malaria clade, has an intact and presumably functional nbp1 gene (Genbank Accession number DQ973815).

A *pvrbp2* ortholog is not present in the *P. knowlesi* or *P. coatneyi* genomes.

To determine if a *rbp2* ortholog was in *P. knowlesi* (and *P. coatneyi*), enzyme restricted *P. knowlesi* H strain and *P. coatneyi* DNA were analyzed by Southern blotting hybridization using 5' through 3'-end amplicons of the *pvrbp2* gene as probes. No distinct bands could be discerned using conditions of lowered stringency, which previously permitted easy detection of the ψ*pknbp1* gene or the *pvrbp1* and *pvrbp2* orthologs in *P. cynomolgi* [10] (Fig. 2.1 B). These data indicate that no highly similar counterpart or ortholog genes for *pvrbp2* exist in *P. knowlesi* or *P. coatneyi,* and are at least for *P. knowlesi*, in complete agreement with BLAST searches of the *P. knowlesi* genome database [31].

Characterization of two novel *P. knowlesi rbl* genes, denoted *pknbpxa* and *pknbpxb*

To search for possible distantly related *rbl* genes in *P. knowlesi,* which may serve as alternatives to *pvrbp2*, we used a combination of methods including library screening based on *pvrbp2* probes, PCR amplification based on *pvrbp2* gene sequences and database mining. A probe of about 1.5 kb representing the 3' end of the *pvrbp2* gene hybridized weakly to *P. knowlesi* gDNA in Southern blots using low stringency conditions (data not shown). This sequence was used to BLAST the *P. knowlesi* sequencing project database using both nucleotide and translated sequences. ORFs on two separate contigs showed significant, but not particularly strong scores by BLAST based searching. The sequences were used as templates for further probe design and PCR based amplification of gDNA. Screening *P. knowlesi Eco*RI digested-gDNA λ Zap II

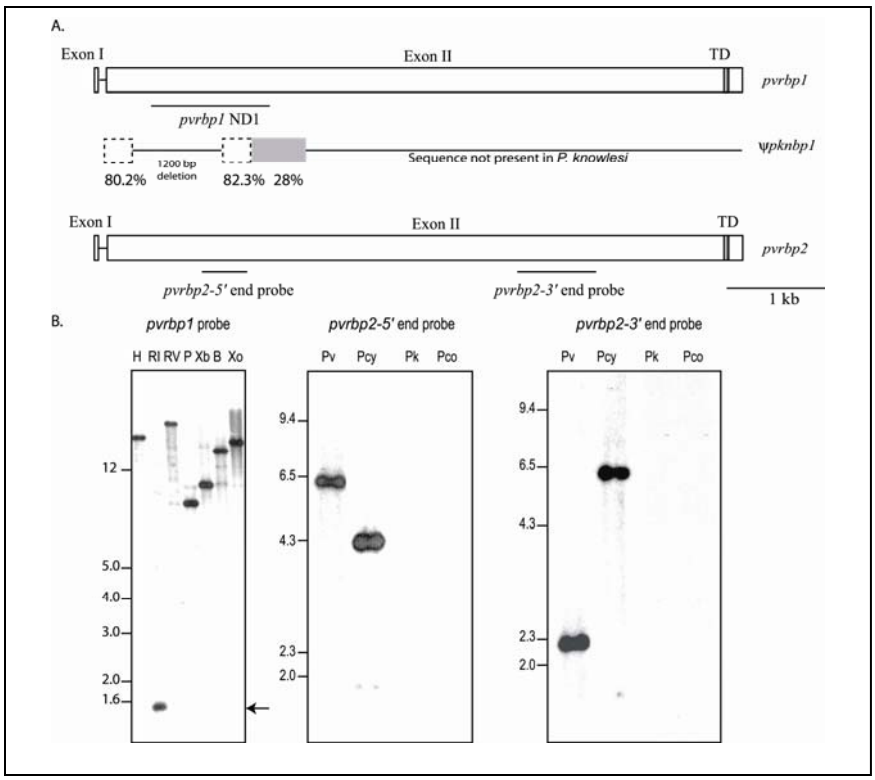

Figure 2.1. The *P. knowlesi* gene orthologous to *pvrbp1* in *P. vivax* is a pseudogene.

(A) Schematic representation of *pvrbp1* and *pvrbp2* genes and the remnant *pknbp1* locus with locations of DNA probes used in Southern blot analysis. The comparison of the 1.5 kb fragment of the *nbp1* locus in *P. knowlesi* with *P. vivax rbp1* revealed two remnant regions (dashed lines) in the *P. knowlesi* gene *ψpknbp1* comprising part of the intron and 5' end of coding region. (B) Southern blot analysis (bottom left) of *P. knowlesi* H strain gDNA digested with *Hind*III (H), *Eco*RI (RI), *Eco*RV (RV), *Pst*I (P), *Xba*I (Xb), *Bam*HI (B) and *Xho*I (Xo) hybridized with a 5'-end *pvrbp1* probe identifying a 1.5 kb fragment after *Eco*RI restriction analysis (arrow). *P. vivax (Pv), P. cynomolgi (Pcy), P. knowlesi (Pk),* and *P. coatneyi (Pc)* gDNA digested with *Hind*III restriction enzyme was analyzed by Southern blot analysis using a 5'-end (*pvrbp2*-5'end) or a 3'-end (*pvrbp2*-3'end) probe from the *pvrbp2* gene, only *P. cynomolgi* showed cross-hybridization under moderately stringent conditions.

libraries identified two clones, *pknbpxa* and *pknbpxb*. The sequences of the two cloned DNA fragments were utilized to search the *P. knowlesi* sequence database to extend the primary sequences and fill in the gaps.

The *pknbpxa* clone contains an *Eco*RI-fragment of 6641 nt with an ORF of 5742 nt but missing the expected exon I, intron, and 5' end of exon II. PCR from lambda phage and BLAST search of the *P. knowlesi* database provided enough further sequence to design gene-specific reverse primers for RT-PCR experiments and determination of the exon/intron junctions. The 9578 nt contig encodes exon I from nt 130 to nt 184, a short intron of 186 nt, and an exon II from nt 371 to nt 8679. Thus, the total coding sequence of *pknpbxa* is 8364 nt. The predicted PkNBPXa protein is 2788 amino acids, the putative signal peptide cleavage site located between S27 and E28 and the transmembrane region predicted to be 21 amino acids long (amino acid 2724 to amino acid 2745) (Fig. 2.2 A).

The *pknbpxb* clone contains 9479 nt with a single ORF of 8681 nt, but no clear typical small exon I or intron consensus splice site sequence. We therefore generated 5' RACE clones to verify the 5'UTR sequence, signal peptide-encoding exon I and the exon/intron junctions. Thus, PkNBPXb is encoded by exon I (nt 49 to nt 103) and by exon II (nt 347 to nt 8826) with an intron of 243 nt, which has a 3' AAG splice site instead of the usual TAG or CAG 3' splice motifs. Thus, the total coding sequence of *pknpbxb* is 8535 nt. The PkNBPXb protein has 2845 amino acid residues with a predicted signal peptide cleavage site between C21 and K22 and a hydrophobic transmembrane domain between amino acid 2784 and amino acid 2801 (Fig. 2.2 A).

Overall, the gross structural characteristics of PkNBPXa and PkNBPXb proteins are remarkably similar to those of PvRBP1 and PvRBP2 and the other members of the

RBL family. Similarly, these proteins are likely membrane-anchored as determined by a hydrophobic region at the carboxy-terminal ends (Fig. 2.2 A). Chromosomal mapping localized *pknbpxa* in chromosome 12 and *pknbpxb* in chromosomes 3-4 (Fig. 2.2 B). Currently, the *pknbpxa* gene is annotated in the *P. knowlesi* database as two unlinked fragments (PKH146970 and PKH146980). A contig containing the *pknbpxb* gene assembly is not currently retrieved through BLAST searches, although it is noted that shotgun sequences for this gene are present at in the Sanger database and, surprisingly, cover the complete gene.

The *pknbpxa* and *pknbpxb* genes are transcribed and expressed in mature schizonts.

Transcription of both *pknbpxa* and *pknbpxb* was verified by RT-PCR using schizont stage RNA (data not shown). Northern blot analyses were then performed and demonstrated the schizont-specific nature of the transcripts. Total RNA was extracted over the 24-hour life cycle period from synchronous parasites representing ring stages (R), late trophozoite and early schizont stages with two nuclei (T), and mature schizonts with eight or more nuclei (S). Large transcripts (> 9 kb) were detected for both genes, suggesting the presence of long untranslated regions, and they were present only in the mature schizont-stage RNA samples (Fig. 2.2 C).

Native and reduced protein was detected using rabbit anti-rNBPXa and rabbit anti-rNBPXb antisera on mature schizont extracts, and culture supernatants harvested 14 h after erythrocyte rupture by western immunoblotting. Both PkNBPXa and PkNBPXb are predicted to be high molecular weight proteins of 324 kDa and 334 kDa, respectively.

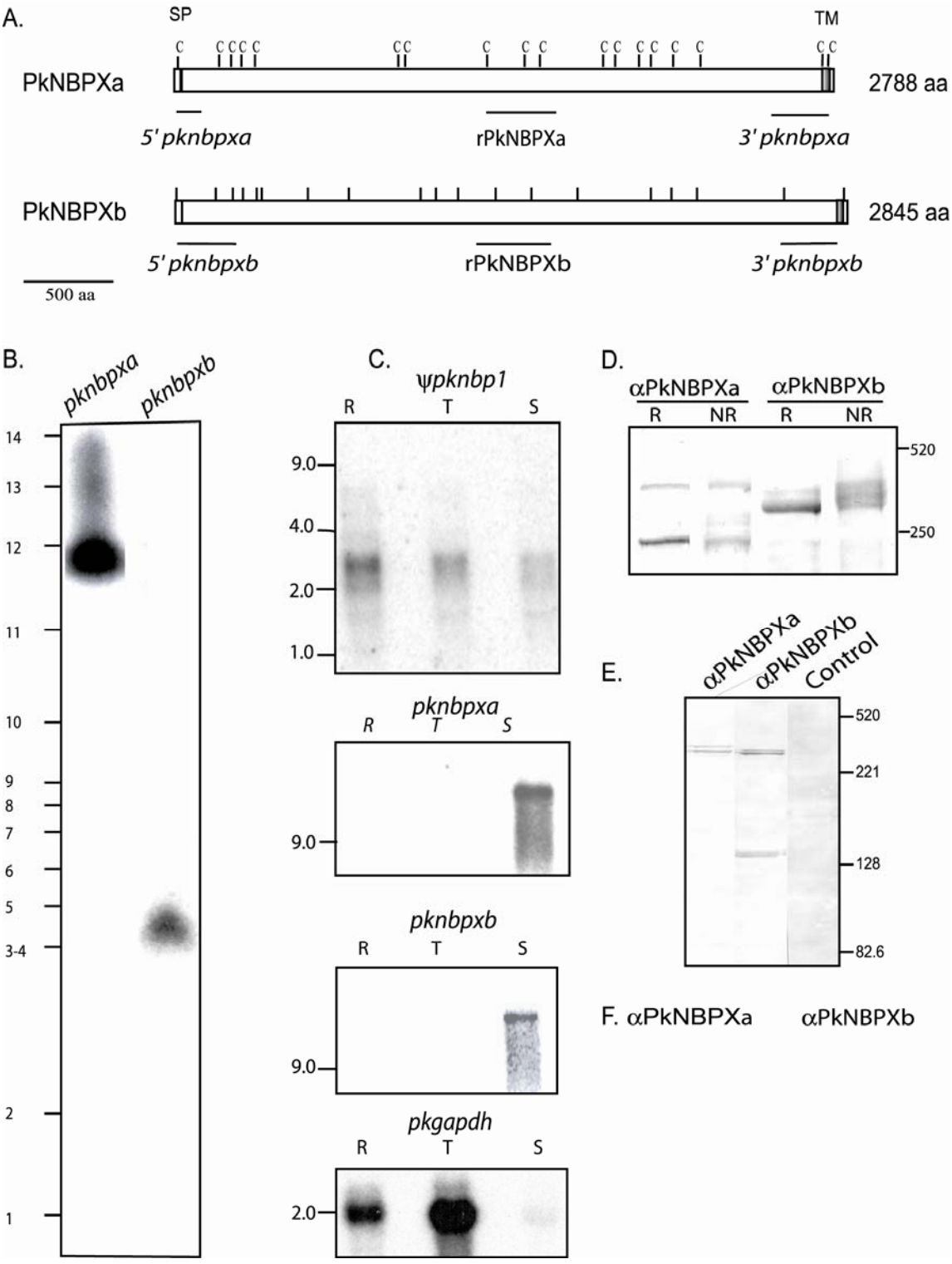
A.
SP
TM
PkNBPXa
2788 aa
5′ pknbpxa
rPkNBPXa
3′ pknbpxa
PkNBPXb
2845 aa
5′ pknbpxb
rPkNBPXb
3′ pknbpxb
500 aa
B.
pknbpxa
pknbpxb
14
13
12
11
10
9
8
7
6
5
3-4
2
1
C.
ψpknbp1
R
T
S
9.0
4.0
2.0
1.0
pknbpxa
R
T
S
9.0
pknbpxb
R
T
S
9.0
pkgapdh
R
T
S
2.0
D.
αPkNBPXa
αPkNBPXb
R
NR
R
NR
520
250
E.
αPkNBPXa
αPkNBPXb
Control
520
221
128
82.6
F. αPkNBPXa
αPkNBPXb

Figure 2.2. (A) Schematic of the *P. knowlesi* PkNBPXa and PkNBPXb RBL proteins with cysteine residues (tick marks), positions of the signal peptides (SP), a large extracellular domain and a transmembrane domain (TM) shown. Probes used for chromosomal Southern and Northern blot hybridizations are indicated in italics and the fragments expressed as recombinant proteins are indicated in bold letters. (B) The *pknbpxa* and *pknbpxb* genes were localized in chromosomes 3-4 and 12, respectively, by PFGE using the *pknbpxa* and *pknbpxb* probes. (C) Total RNA from ring stages (R), late trophozoites and early schizonts (T) and late schizonts (S) was hybridized with radiolabeled probes representing the 5' end of the *ψpknbp1* gene, and the central regions of *pknbpxa* and *pknbpxb* genes using high stringency conditions. Apparent *ψpknbp1* partial transcripts (~3 kb) were detected in ring, trophozoite and schizont stages, compared to *pknbpxa* and *pknbpxb* transcripts of >9kb, which were detected only in the matured schizonts. The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as positive control (*pkgapdh*). PkNBPXa and PkNBPXb were detected by western blot in *P. knowlesi*-schizont-infected erythrocytes solubilized with SDS-PAGE sample buffer with (R, reduced) or without (NR, non-reduced) 2-mercaptoethanol (D), and in supernatant collected after schizont rupture (E). The NBPXa1 antisera recognized specific bands of ~300 kDa and 250 kDa, and the NBPXb1 antisera recognized bands of ~300k Da and 140 kDa (D and E). PkNBPXa and PkNBPXb native proteins were identified by IFA using rabbit antisera PkNBPXa1 or PkNBPXb1, respectively. The robust single dot pattern typical for the PvRBPs at the apical pole was observed in segmented schizont-stage parasites; pre-immune sera were used as negative control (F).

The two polyclonal antisera recognized proteins of ~300 kDa as predicted by the software algorithms without noting any migration differences between the reduced and non-reduced samples. However, rabbit anti-rPkNBPXa also recognized an additional band of 220 kDa which could be a processed fragment of PkNBPXa or attributed to spurious reactivity with the erythrocyte protein, spectrin (although there is no evidence of this from immuno-fluorescence or immuno-electronmicroscopy, see below). Rabbit anti-rNBPXb recognizes an additional band of ~140 kDa in these immunoblots (Fig. 2.2 D). Soluble PkNBPXa and PkNBPXb of the expected size (~300 kDa) were also detected in culture supernatants by western blot by using rabbit anti-rNBPXa and rabbit anti-rNBPXb antisera (Fig. 2.2 E).

PkNBPXa and PkNBPXb localize to the apical microneme organelles of merozoites.

Rabbit antibodies specific for rPkNBPXa and rPkNBPXb located the native proteins at the apical end of merozoites in segmented schizont stage parasites by IFA (Fig. 2.2 F). A single robust dot of fluorescent signal sometimes seemingly capping the merozoite, which is typical for the PvRBPs at the apical pole of *P. vivax* merozoites [2], was also observed in *P. knowlesi* mature segmented schizonts with these two antisera. No reactivity was detected with pre-immune rabbit sera and with the *P. knowlesi* RBL antisera on earlier stages of parasite development. Immuno-electron microscopy of *ex-vivo* parasite preparations showed that anti-PkNBPXb was predominantly located in the micronemes (Fig. 2.3 A-D). Anti-rPkNBPXa antibodies also reacted with micronemes, but less strongly (data not shown). Significantly, in the invading merozoite (Fig. 2.3 D), very little labeling remains within the merozoite, mostly restricted to micronemes that

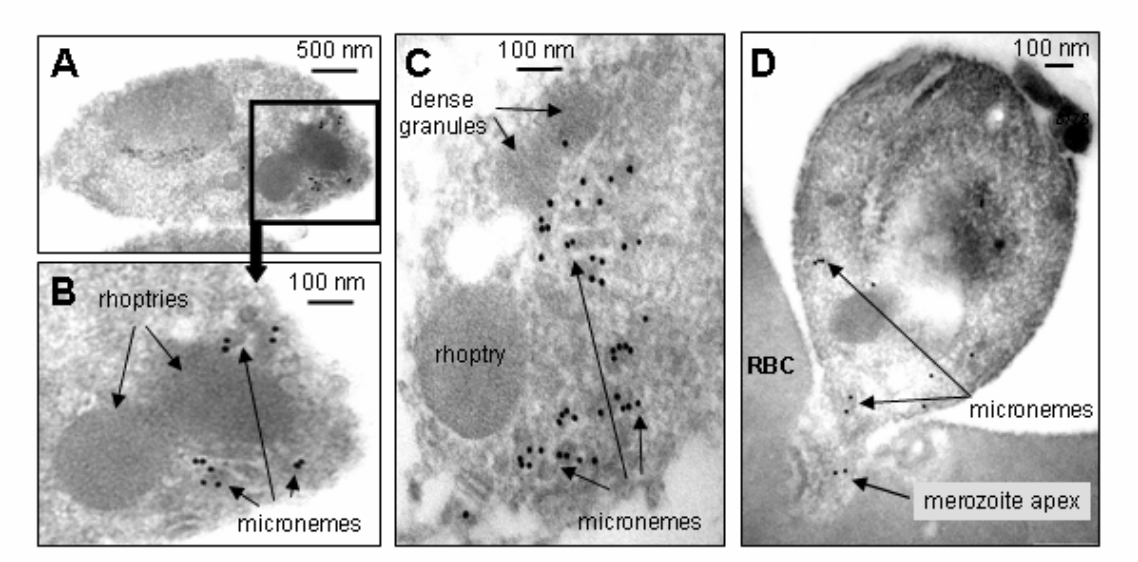

Figure 2.3. Immuno-electron microscopic localization of the PkRBL protein, PkNBPXb. In A – C, PkNBPXb is shown to be strongly localized to the apical micronemes of free *P. knowlesi* merozoites, using rabbit anti- NBPXb anti-serum (1:50) as primary antibody, and Protein A -10 nm gold for detection. . In an invading merozoite (D), very little labelling remains (rabbit anti-NBPXb anti-serum 1:100 dilution used), and is mostly detected on micronemes that have apparently failed to locate apically, suggesting that the RBL had already been mostly secreted and lost, as expected if the protein is important in red cell adhesion. Note the absence of general labelling around the merozoite perimeters in all examples. RBC – red blood cell.

have apparently failed to locate apically. This suggests that PkNBPXb had already been secreted, as would be expected if the protein is important in early red cell adhesion.

PkNBPXa and PkNBPXb bind to host erythrocytes

The capacity of the two expressed RBL proteins to bind to rhesus macaque erythrocytes was determined by traditional erythrocyte binding assays. Intense bands were detected in immunoblots probed with rabbit anti-rNBPXa and rabbit anti-rNBPXb antisera (Fig. 2.4 A and 2.4 B). No bands were detected with erythrocytes that were incubated without supernatant and subsequently washed and eluted under the same conditions as the samples incubated with culture supernatants. There were no differences detected in the migration of PkNBPXa and PkNBPXb proteins between reduced and non-reduced samples (data not shown). To ensure that the binding detected was not due to carryover contamination, immunoblots were also probed with antiserum against the 140kDa PkMSP3 [39]. PkMSP3 is known to be present in culture supernatants but does not bind to the surface of erythrocytes. As expected, antibody reactivity with PkMSP3 was detected when testing culture supernatants (~140kD) but not the erythrocyte binding assay eluates (Fig. 2.4 C and unpublished data). This indicates the binding of expressed *P. knowlesi* RBLs to rhesus macaque erythrocytes is specific.

Identification of a *rbl* gene in *P. vivax* that is an ortholog to *pknbpxa*.

P. vivax gDNA was examined by Southern hybridization with fragments of the 5' and 3' portions of the *P. knowlesi rbl* genes as probes. Weak bands of approximately 7.0 kb were noted on *P. vivax* digested gDNA when probed with a *pknbpxa* 5' DNA

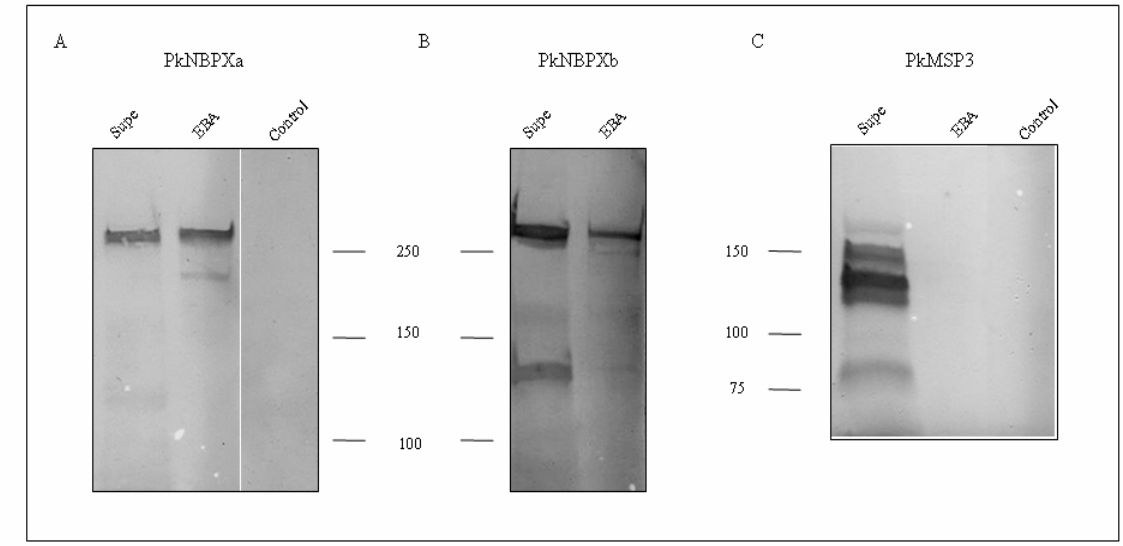

Figure 2.4. PkNBPXa and PkNBPXb bind to host erythrocytes.

Culture supernatants (Supe) containing soluble proteins released from free merozoites were electrophoresed by SDS-PAGE and transferred to nitrocellulose membranes as were protein samples eluted from rhesus macaque erythrocytes after incubation with supernatants in binding assays (EBA). Western blots using NBPXa1 or NBPXb1 antisera demonstrate that PkNBPXa and PkNBPXb bind to rhesus erythrocytes. PkMSP3 140 was abundantly detected in the culture supernatants by PkMSP3 140 rabbit antisera but was not detected in the eluted protein samples. Rhesus monkey erythrocytes were incubated within culture medium (Control) and eluted under the same conditions as erythrocytes incubated with culture supernatants. No protein bands were detected by any of the antisera (data not shown for PkNBPXb).

fragment (Fig. 2.5 A) or approximately 9.0 kb, when probed with a *pknbpxa* 3' fragment (Fig. 2.5 B). These data suggested the presence of at least one more *rbl* gene more closely related to *pknbpxa* in this species. No hybridization was observed when *pknbpxb* probes were used at low stringency on the *P. vivax* DNA. Database search of the *P. vivax* genome sequencing project **(www.plasmodb.org/P.vivax)** using *pknbpxa* sequences provided a contig of 11726 bp containing several large ORFs totaling about 8366 bp. Though the gene predicted for the protein has a potential consensus start methionine (TATAATG), the expected intron/exon 5' and 3' consensus splicing sequences were not readily observed, and two stop codons at positions 5503 and 7071 interrupt a potential 8366 bp ORF [17]. Although *pknbpxb* was not detected in *P. vivax*, homologous sequences to both *pknbpxa* and *pknbpxb* were detected by Southern hybridization in the simian malaria parasites, *P. cynomolgi* and *P. coatneyi* (Fig. 2.5 A and 2.5 B).

Phylogenetic relationship of *pknbpxa* and *pknbpxb* within the RBL family of proteins

To achieve a comprehensive updated phylogenetic analysis of known *rbl* genes, the *rbl* sequences from human, simian, chimpanzee, and rodent malaria parasites were aligned and analyzed. These alignments included orthologs of *pvrbp1* from *P. coatneyi* (GenBank accession number DQ973816) and *P. fragile* (GenBank accession number DQ973815). The analysis of the RBL proteins demonstrates that these proteins comprise a large family of two distinct groups, namely, PvRBP1-like and PvRBP2-like homologs. Functional *pvrbp1*-like genes have been identified in *P. falciparum, P. fragile, P. cynomolgi* and *P. coatneyi*, but are degenerated pseudogenes in *P. knowlesi* and *P.*

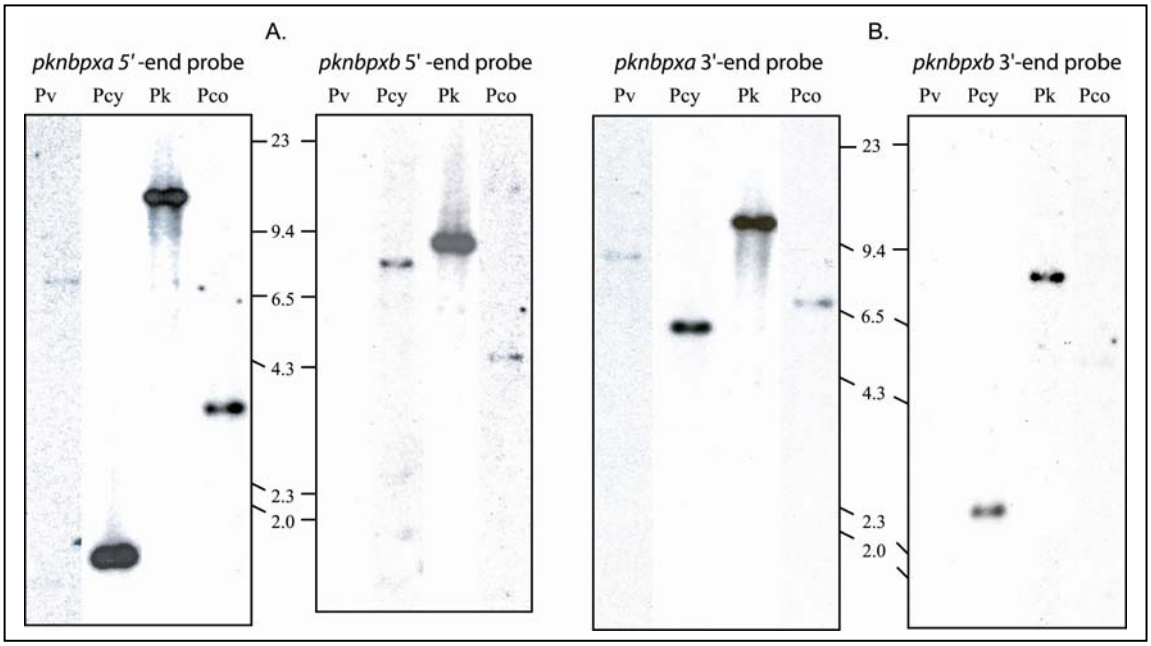

Figure 2.5. Genes homologous to *pknbpxa* and *pknbpxb* are in *P. vivax* and other related simian malaria species. *P. vivax* (Pv), *P. cynomolgi* (Pcy), *P. knowlesi* (Pk), and *P. coatneyi* (Pco) gDNAs were digested with the restriction enzyme *Hind*III, blotted and probed with the 5' (Panel A) and 3' (Panel B) probes from *pknbpxa* and *pknbpxb* genes as indicated in Fig. 2.2. Sizes are in kilobases. The hybridization temperature was 60^{o}C followed by low stringency washes as described in the materials and methods. The 5' and 3' probes from *pknbpxa* hybridized strongly with *P. vivax* and *P. cynomolgi,* and with less intensity to *P. coatneyi* gDNA. The 5' and 3' probes from *pknbpxb* hybridized with *P. cynomolgi* and *P. coatneyi* gDNA but not with *P. vivax* gDNA.

reichenowi. In contrast, various paralogs and orthologs of *pvrbp2* have been identified in *P. vivax, P. falciparum, P. reichenowi, P. cynomolgi, P. knowlesi,* and *P. yoelii* (Fig. 2.6). Importantly, the genes designated here as *pknbpxa* and *pvrbpxb* share a paralogous relationship with *pvrbp2*, and *pknbpxa* may constitute a new *rbl2* subgroup sharing greater affinity with *pfnbp3/rh3* and *pfnbp2a-b/rh2a-b.* Though quite divergent, *pknbpxb* is clearly more related to *rbp2* of *P. vivax* and *P. cynomolgi* than is *pknbpxa.*

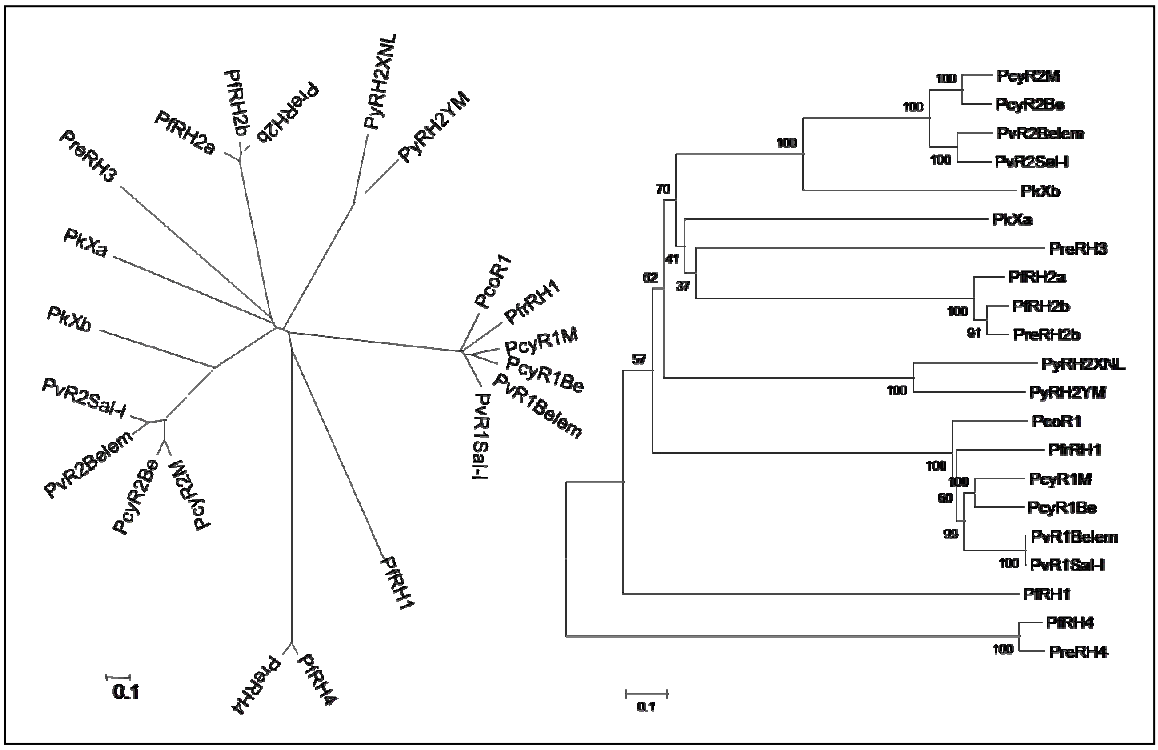

Figure 2.6. Phylogenetic relationships among members of the RBL invasion ligand family. Unrooted neighbor-joining tree obtained from the alignment of RBL protein sequences from *P. vivax*, *P. cynomolgi*, *P. coatneyi*, *P. knowlesi*, *P. fragile, P. yoelii, P. falciparum*, and *P. reichenowi*. The cladogram (right) was generated using the Neighbor-Joining method following a Poisson model with pairwise deletion from MEGA 4 software. Bootstrap values for 1000 replicas are indicated in the tree. Distinct groups can definitely be identified for RBL1, RBL2, RBL3 and RBL4.

Discussion

Here we report the experimental characterization of the *rbl* family in *P. knowlesi* and show it is comprised of one pseudogene related to *pvrbp1* and two functional intact members, which we have called *pknbpxa* and *pknbpxb*. These two functional genes encode large RNA transcripts and high molecular weight proteins as predicted, and we have confirmed that they become expressed in the late schizont stage. Their products localize to the microneme organelles of mature merozoites and bind specifically to rhesus monkey erythrocytes.

Our initial identification of these genes was through hybridization of *pvrbp* gene fragments to restriction enzyme digests of *P. knowlesi* gDNA (and other species), followed by sequencing and RT-PCR to fully characterize the genes. These experimental findings have been important alongside the development of the *P. knowlesi* genome project [31], since this genome-wide sequencing and annotation effort alone failed to recognize the *ψpknbp1* pseudogene and the *pknbpxb* gene reported here, and annotate them accordingly. On the other hand, two fragments of the *pknbpxa* gene were annotated by the genome project, but not recognized as segments of one gene. Our experimental and bioinformatic work was required to identify unannotated members of the *rbl* family (ψ*pknbp1* and *pknbpxb*), correctly link annotated fragments of the *pknbpxa* gene, and confirm all the sequences, structures, and importantly, their expression and functional properties.

The *P. knowlesi* ortholog of *pvrbp1* is only a relic pseudogene (*ψpknbp1*) in the H, Hackeri, and Philippine strains of *P. knowlesi*. What is left of this pseudogene has a high level of identity with ~0.8 kb of the 5' end of *pvrbp1* (a gene of ~8.6 kb) with no

ORF. The *ψpknbp1* sequence downstream has been entirely lost in relationship with *rbp1* gene structure, presumably through an ancient recombination event, or extensive drift over time through mutations. Interestingly, this pseudogene is differentially transcribed as a small fragment that is consistent with the size of the gene remnant. This truncated transcript is primarily produced during ring-stage parasite growth, as opposed to schizont stage development for *bona fide* full-length *rbl* genes. The presence of *rbl* pseudogenes has been recognized before in *P. falciparum* and *P. reichenowi,* and different levels of degeneracy have been observed. For example, the *P. falciparum rbl3* (*pfnbp3*/*rh3*) gene transcript is present, yet not translated into protein in the 3D7 strain due to two frameshift mutations [8]. The *P. reichenowi rbl1* (*prnbp1/rh1)* gene, however, is much more degenerated containing numerous frameshifts leading to termination codons throughout the sequence [11].

Regardless of the mechanism by which *rbl* pseudogenes are generated and maintained, it has now become evident that *P. knowlesi* parasites can survive without an *rbl1* ortholog. Furthermore, this apparently extends also to a *pvrbp2* ortholog as our data shows that the *P. knowlesi* genome lacks completely a direct counterpart of *pvrbp2,* although an ortholog is in *P. cynomolgi*. Initial hybridization experiments were surprising, since the closely related simian malaria parasite *P. cynomolgi* [10], and the sister taxon *P. coatneyi* (unpublished), clearly had orthologs of *rbp1* and *rbp2* in their genomes. Thus, only two *rbl* genes function in *P. knowlesi, pknbpxa,* reported in the genome database as PKH_146970 and PKH_146980, and *pknbpxb* as reported here but remains unannotated. Clearly, the loss of the *rbp1* and *rbp2* orthologs in *P. knowlesi* is a

recent evolutionary event occurring sometime after the divergence of *P. coatneyi* and *P. knowlesi*, perhaps two to three million years ago [40, 41].

Our data indicate *P. knowlesi* has evolved to maintain just two functioning members of the *rbl* gene family (*pknbpxa* and *pknbpxb*), while losing the *rbp1* and *rbp2* orthologs and perhaps others present in the other members of the vivax-simian malaria clade of *Plasmodium* species. In contrast, most other species of *Plasmodium* seem to have a larger repertoire of expressed *rbl* genes, including *P. vivax*. *P. yoelii* has at least a dozen *rbl* genes that may be functional; all falling within the *rbp2*-like grouping of *rbl* genes. *P. falciparum* has a group of six genes of which five are deemed functional and likely serve to interact with alternative receptors in whatever role they play in erythrocyte invasion. *P. vivax* also apparently has more than two functional *rbl* genes.

We have already noted here that the *P. vivax* (Sal I) genome has an *nbpxa* ortholog gene, although it seems to be a non-functional pseudogene. Hypothetical translation of the long ORF, across the stop codons and without frameshifts gives an amino acid identity of 63% between PkNBPXa and the *P. vivax* homologue. It remains to be demonstrated if the *pvrbpxa* gene is transcribed, as is the *nbp3/rh3* gene in *P. falciparum* [8]. The two frameshift mutations and lack of evident intron splice sites indicate this is likely a pseudogene and probably does not produce a functional protein. This gene has been designated as *pvrbp3* [16]. At least four other gene sequences in the *P. vivax* (Sal I) genome [17] are annotated as *rbp genes*, besides those discussed above (*pvrbp1, pvrbp2,* and *pvrbp3*), but none are orthologs of *pknbpxb.* Importantly, of these four additional genes designated as *pvrbp1b*, *pvrbp2a*, *pvrbp2b* and *pvrbp2d*, two, *pvrbp1b* and *pvrbp2d* are likely non-functional genes with mutations that disrupt the

reading frame. Thus, *P. vivax* may have a larger set of functional *rbl* genes than previously realized, but less than recently reported [16], whereas *P. knowlesi* has reduced its *rbl* allotment to only two ligands. Nevertheless, *P. knowlesi* has compensated by retaining its invasion ligand diversity through three *dbl* genes, while *P. vivax* only has one.

The *pknbpxa* and *pknbpxb* genes have the signature structure of *rbl* genes but they do not exhibit a high degree of homology between them, as is also the case for the *rbp1* and *rbp2* genes in *P. vivax* or *P. cynomolgi* [2, 4, 10]. The absence of the *rbp1* and *rbp2* family members in *P. knowlesi* may simply be a reflection of the adaptation of *P. knowlesi* to invade all red blood cells and not be restricted to reticulocytes like *P. vivax* and *P. cynomolgi.* However, *P. coatneyi* does not preferentially invade reticulocytes and it has a complete *rbl1* gene structure that is functional from the perspective that there is a complete ORF. The presence of distant members in *P. knowlesi* may also simply be indicative of a dynamic evolutionary process and the parasite's strategies to maintain a minimum number of functional members of this gene family. Experiments aiming to disrupt *pknbpxa* and *pknbpxb* genes support the hypothesis that the encoded proteins are essential for *P. knowlesi* survival, as no parasites were retrieved after selection for disrupted parasite phenotypes in *in vivo* and *in vitro* experiments, although disruptions of other genes performed at the same time were successful in each case (unpublished data).

Phylogenetically, these two *rbl* genes have helped to define new ortholog genes in *P. vivax*, *P. cynomolgi*, *P. coatneyi* and *P. fragile*, which complement the *pvrbp2* subgroup of the RBL family (Fig. 2.6). The data presented here and contained in genome databases suggest that the *rbl* gene family in *Plasmodium* is dynamic, in that gene

duplications, recombination events and mutations have been common occurrences; yet all species investigated to date seem to minimally maintain at least two *bona fide* family members.

A micronemal location is evident for PkNBPXb by IEM analysis, and a similar pattern is observed for PkNBPXa, though the antibody reactivity was most clear for PkNBPXb. The micronemal location of these proteins contrasts with the rhoptry localization reported for PfNBP2a/b [19] and Py235 [42]. IFA patterns in merozoites for RBP1 and 2, and for RH1 and RH4 that colocalize with micronemal invasion ligands such as DBP, EBA-175 and AMA1 suggest a microneme location for these *rbl* gene products. Further studies are necessary to thoroughly evaluate the locations of different RBLs in and between different species of *Plasmodium* to reliably locate them by IEM. If RBLs are stored in different locations as seems to be the case (i.e. in the micronemes versus rhoptries), or potentially in different microneme organelles, this may reflect different functional roles in invasion attributable to specific family members. More so than rhoptries, the micronemes appear to store invasion ligands that interact with specific RBC receptors to initiate actions during the early stages of recognition and entry. Precise localization data could be helpful for formulating and testing hypotheses relating to the function of the RBLs in the cascade of events that dictate erythrocyte invasion.

RBL proteins have been hypothesized to be critical components in host cell identification and invasion, and accumulating evidence with counterpart molecules present in each malaria species examined supports this premise. As their names denote, reticulocyte or normocyte binding proteins are predicted to function by binding to (as yet undefined) receptors on erythrocytes. The specific adhesion of RBL proteins to host

erythrocytes in *in vitro* erythrocyte binding assays has been demonstrated for *P. vivax* (with reticulocyte specificity) [2], *P. falciparum* [5, 20-22], and *P. yoelii* [12], and here we demonstrate that the expressed RBLs of *P. knowlesi* bind to erythrocytes from the rhesus macaque model.

P. knowlesi is the traditional model for investigating *Plasmodium* merozoite invasion of erythrocytes [1, 43, 44]. *Ex-vivo P. knowlesi* merozoites have a biological half-life of about 20 minutes and remain viable in the extracellular milieu much longer than other species [43]. This trait allows for the unique experimental capture and analysis of merozoites during different stages of attachment and entry of erythrocyte host cells [1, 43, 44] (and see Fig. 2.3 D). Of interest, our IEM data shown here, depicting an invading parasite suggests that the RBLs are released from microneme storage at a time before the merozoite begins to enter the host red blood cell (Fig. 2.3 D). It is also worth noting that labeling was absent from the general surface of free merozoites, consistent with the notion that the RBLs are likely to function in apically-related events rather than initial red blood cell adhesion. Considering the likelihood that binding of the RBLs may be critical for invasion of erythrocytes, further investigation to define the binding properties and characteristics of these molecules is warranted.

With the goal of using the *P. knowlesi* model to improve our understanding of the invasion of red blood cells, we set out to define the *rbl* genes and proteins present in this species. Moreover, the importance of the RBL family as potential vaccine candidates is emphasized by the parasite's maintenance of functional members, ability to bind erythrocytes, proposed role in signaling release of other merozoite proteins early during invasion [1-4], and evidence suggesting their critical role in parasite survival. *P. knowlesi*

has become a public health concern since a series of recent reports have confirmed the zoonotic transmission in human populations throughout South East Asia, and severe disease and four deaths have so far been attributed to these infections [25-27]. It is possible that expressed RBLs characterized in this report are the ligands used to gain entry into human erythrocytes, as well as the erythrocytes of simian hosts, be they the experimental rhesus monkey host (*Macaca mulatta*) or the natural host (*M. fascicularis* or *M. nemestrina*) in South East Asia. Further studies are in progress to define the host cell binding specificities of the *P. knowlesi* RBLs, identify the *P. knowlesi* RBL red blood cell binding domain(s), and maximize the use of this model parasite to pave the way for the possible inclusion of RBL molecules in future malaria vaccines.

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CHAPTER THREE

AN N-TERMINAL DOMAIN OF THE *PLASMODIUM KNOWLESI* NORMOCYTE BINDING PROTEINS ADHERES TO ERYTHROCYTES AND IS DEPENDENT ON CYSTEINE BOND CONFORMATION

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Abstract

Invasion of erythrocytes by *Plasmodium* merozoites entails a cascade of molecular steps, including receptor-ligand interactions, which are poorly understood. Two Normocyte Binding Proteins have been defined in the simian malaria parasite, *P. knowlesi* (PkNBPXa and PkNBPXb), as members of the reticulocyte binding-like (RBL) protein superfamily. Here we demonstrate using recombinant constructs expressed at the surface of COS7 cells and erythrocyte adhesion (rosetting) assays that an N-terminal region of PkNBPXb binds to rhesus macaque monkey erythrocytes. This binding domain, called PkNBPXb-II, was also tested with a panel of erythrocytes from a variety of primates and binding was similarly observed with erythrocytes from other Old World monkeys and gibbons, a Lesser Ape. The binding of PkNBPXb-II was abolished when the erythrocytes were pretreated with chymotrypsin, but not trypsin or neuraminidase; thus the adhesion of this domain to its erythrocyte receptor is sensitive to treatment with chymotrypsin. However, the native protein binds to chymotrypsin treated cells in standard erythrocyte binding assays, suggesting that this protein may have more than one ligand capable of binding red blood cells. Here, we also show that disulfide bond formation appears to be necessary to achieve the functional conformation of this protein and adhesion to target erythrocytes.

Introduction

The obligate, intracellular parasite *Plasmodium* utilizes various strategies to gain entry into host cells and evade the host immune response; thereby achieving the ability to replicate and survive. The erythrocytic stage of the *Plasmodium* life cycle and specifically merozoite invasion is of particular importance for the parasite's maintenance of a malaria infection. Invasion of erythrocytes occurs in distinct steps, each of which is critical to successful entry of *Plasmodium* parasites into target erythrocytes and involves specific molecular interactions that are still largely undefined. Members of the reticulocyte-binding like (RBL) protein superfamily are merozoite expressed proteins proposed to be involved in the initial apical irreversible attachment of the merozoite with the host erythrocyte. RBL proteins from the human malarias *P. falciparum* and *P. vivax,* the rodent malaria *P. yoelii,* and the simian malaria *P. knowlesi* have been shown to bind to host cells [1-6], but the specific ligand-receptor interactions that mediate the attachment of these merozoite proteins to the host cells are unknown.

During merozoite invasion, after the initial attachment and reorientation phase, the merozoite forms an irreversible tight junction with its target erythrocyte [7]. The Duffy Binding-Like/Erythrocyte Binding-Like (DBL/EBL) family of proteins has been shown to play a role in the formation of this tight junction [8], and RBL proteins may mediate signaling events that lead to the timely release of the DBL/EBL proteins from their location in the micronemes [6, 9]. The molecular interactions of the DBL/EBL proteins at this critical step of merozoite invasion are more comprehensively understood than are the functions of the RBL proteins. For example, the Duffy binding proteins of *P. knowlesi* and *P. vivax* are known to bind to the Duffy antigen on the surface of target

erythrocytes [10-13] and the *P. falciparum* erythrocyte binding antigen-175 binds to glycophorin A as a receptor [14]. The functional binding domain of these proteins, known as Region II, was identified by hypothetically defining a series of domains based upon cysteine residue clustering and then testing them for possible binding functions in erythrocyte adhesion assays [15, 16].

Identifying binding domains in RBL proteins has been somewhat hampered by the lack of easily identifiable cysteine residue clusters and inherent challenges expressing segments as functional recombinant proteins. Nonetheless, erythrocyte binding domains of two *P. falciparum* RBL proteins, PfRH4 and PfRH1, have been described recently. The reported binding domain of PfRH4 was predicted through an analysis of clustal alignments of PvRBP1 and PfRH4. An N-terminally localized domain from PfRH4 was defined, and a recombinant protein based on this region bound to erythrocytes in a specific and competitive manner [3]. In contrast, the presumptive binding domain of PfRH1 was identified by cloning and expressing fragments of the *pfrh1* gene into the pRE4 vector and conducting *in vitro* erythrocyte adhesion assays [15, 16]. An N-terminal portion of this protein was identified as an erythrocyte binding domain when expressed at the surface of COS7 cells [2].

Here we report the identification of an RBL binding domain for the first time from *P. knowlesi,* which is known to contain two intact *rbl* genes as well as one *rbl* pseudogene [17]. The two *bona fide rbl* genes express proteins called the normocyte binding proteins, PkNBPXa and PkNBPXb, at the apical end of the merozoite, in the micronemes, and these proteins bind erythrocytes in traditional erythrocyte binding assays [17]. The PkNBP erythrocyte binding domain described here begins with the 20th

amino acid downstream of the N-terminus of PkNBPXb. This binding region, called PkNBPXb-II, consists of 347 amino acids, and when expressed at the surface of COS7 cells it binds to rhesus macaque erythrocytes, forming rosettes. The functional properties and insights regarding this and other presumptive RBL binding domains in *P. knowlesi* are discussed in relationship to understanding merozoite invasion in *P. knowlesi*, other experimental model species, and in the human malaria parasites, *P. falciparum* and *P. vivax*. These insights are pertinent to the continued consideration of incorporating RBL binding domains as part of future malaria vaccines.

Materials and Methods

Polymerase Chain Reaction (PCR) and cloning

Regions of PkNBPXb were amplified using gene-specific primers under standard PCR conditions (Supplementary Table 3.1). The amplified regions were inserted into the *Bgl*II and *Sal*I restriction sites in the pDisplay vector (Invitrogen, Carlsbad, CA). This vector contains an immunoglobulin κ chain leader sequence for targeting of the recombinant protein into the secretory pathway and a platelet derived growth factor receptor transmembrane domain for anchoring of the recombinant protein on the surface of mammalian cells. Positive clones were identified and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing v3.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA). Resulting sequences were analyzed using MacVector^{TM} 7.2.2 to ensure the fidelity of the sequence.

Maintenance of mammalian culture cell line and transfections

African green monkey kidney fibroblast (COS7) cells were cultured in DMEM containing 10% Fetal Bovine Serum, HEPES buffer, and antibiotics at 37ºC in 5% CO_2. For transfections, COS7 cells were plated in 6-well plates at a concentration of $1x10^5$ cells per well using culture medium without antibiotics. Cells were grown for 24 hours at 37ºC in 5% CO_2 and 1μg of vector DNA was subsequently transfected into COS7 cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol.

Lysis of COS7 cells and western blots

Twenty four hours after transfections, cells were lysed using CellLytic M (Sigma-Aldrich, St. Louis, MO) according to manufacterur's protocol. Lysed cells containing expressed recombinant protein were electrophoresed on Pre-Cast 10% polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were probed with a mouse monoclonal antibody (Millipore, Billerica, MA) diluted 1:1000 for one hour and alkaline phosphatase conjugated anti-rabbit IgG (Promega, Madison, WI) diluted 1:5000 for 1 hour. NBT/BCIP substrate (Promega, Madison, WI) was added to the nitrocellulose membranes to detect the protein bands.

Erythrocytes

Whole blood from rhesus macaques (Indian and Chinese), pigtail macaques, sooty mangabeys, and mice was collected in blood collection tubes containing ACD or CPDA anticoagulant; and whole blood from *Aotus* and *Saimiri* monkeys was collected in heparinized blood collection tubes. All procedures were in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committees. Rabbit whole blood collected in ACD was obtained from Covance (Denver, PA). Whole blood was washed with RPMI media and centrifuged to collect erythrocytes. Cryopreserved blood (marmoset, tamarin, capuchin monkey, chimpanzee, and gibbon) was thawed and washed with RPMI media using standard laboratory procedures.

Erythrocyte Adhesion Assay

At 24-36 hours after transfection, COS7 cells were washed with complete culture media and incubated for at least two hours at room temperature with 0.2% erythrocytes in complete culture media. Cells were washed thrice with DPBS and incubated with 1% formaldehyde for 10 minutes at room temperature to stabilize rosettes. Cells were then incubated with 0.1ug/ml of Hoescht dye (Invitrogen, Carlsbad, CA) for 5 minutes to stain the nucleus. Erythrocyte adhesion was analyzed by observing cells using a Nikon ECLIPSE TE300 inverted microscope.

The percent of cells expressing protein on the surface was determined by counting the number of nuclei and the number of cells expressing protein by microscopic analysis. COS7 cells were stained for surface expression at 24-36 hours after transfection. Briefly, COS7 cells were incubated with mouse anti-HA antibody (Millipore, Billerica, MA) diluted 1:250 for 1hour and then washed twice in DPBS. Cells were incubated with an Alexa Fluor 488 conjugated anti-mouse IgG antibody (Invitrogen, Carlsbad, CA) diluted 1:200 for 1 hour and then washed twice in DPBS. COS7 cells were fixed using 1% formaldehyde for 10 minutes at room temperature.

Treatment of erythrocytes

50 µl of rhesus erythrocytes were incubated in 1ml RPMI with 1mg/ml trypsin (Calbiochem, San Diego, CA), 1mg/ml chymotrypsin (Calbiochem, San Diego, CA), or 0.025U/ml neuraminidase (Roche, Indianapolis, IN) for 1 hour at 37ºC. Cells were washed twice with RPMI and incubated with 0.5mg/ml soybean trypsin inhibitor or 1mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO) for 15 minutes at room

temperature. Cells were washed twice with RPMI and subsequently used in erythrocyte adhesion assays.

Erythrocyte Binding Assay

Schizont stage parasites were purified by centrifugation on Percoll gradients, as previously described [18]. Purified schizonts, at a concentration of 2.5×10^7 parasites/ml, were cultured in tissue culture flasks and grown overnight until schizonts ruptured. Cells were centrifuged and supernatants were stored in liquid nitrogen. Culture supernatants were rotated with $1x10^9$ erythrocytes at room temperature for 4 hours. Cells were washed twice by centrifugation through a Dow Corning 550 fluid cushion. Bound proteins were eluted in 50ul of 5x RPMI at room temperature and harvested by centrifugation at 4°C. The eluted proteins were electrophoresed on Pre-Cast 5% polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were probed with rabbit polyclonal antisera diluted 1:500 for 2 hours and alkaline phosphatase conjugated anti-rabbit IgG (Promega, Madison, WI) diluted 1:1000 for 1 hour. Bands were visualized by adding NBT/BCIP substrate (Promega, Madison, WI) to the nitrocellulose membranes.

Reduction and Alkylation

COS7 cells were transfected as previously described and incubated at 37°C for 24 hours to allow for protein surface expression. Cells were rinsed with DPBS and incubated with 10mM dithiothreitol, DTT (Sigma-Aldrich, St. Louis, MO) or 10mM beta-mercaptoethanol (Bio-Rad, Hercules, CA) for 30 minutes at 37°C. Cells were then rinsed

with DPBS and incubated with 10mM iodoacetamide, IAA (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C. Erythrocyte adhesion assays were subsequently performed as previously described above.

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using QuickChange Multi Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primers were designed according to manufacturer's suggestion (Supplementary Table 3.2). DNA of pDisplay-PkNBPXb-II was used a template for the mutagenesis PCR reactions. Thermocycling was performed as follows: 95ºC for 1 minute, followed by 30 cycles of 95ºC for 1 minute, 55 ºC for 1 minute, and 65ºC for 14 minutes. PCR products were digested for 1 hour with *Dpn*I to cleave non-mutant parental template strands. Positive clones were identified and sequenced using a BigDye Terminator Cycle Sequencing v3.1 Ready Reaction Kit on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Resulting sequences were analyzed using MacVector^{TM} 7.2.2 to ensure the presence of mutated sites.

Statistical Analysis

Statistical analysis was done using Student *t* test for paired samples.

Results

An N-terminal region of PkNBPXb binds to rhesus macaque monkey erythrocytes

Experiments were designed to determine which regions of *P. knowlesi* NBPXa or NBPXb [17] may be responsible for adhesion of these proteins to the rhesus macaque monkey host erythrocytes. To identify a potential erythrocyte binding domain(s) within the structure of the *P. knowlesi* NBPXa or NBPXb, three gene fragments starting at the 5' end of the second exon of the *pknbpxa* gene and eight fragments encompassing the 5' half of the *pknbpxb* gene were cloned into the pDisplay vector (Figure 3.1). Each of these gene fragments is approximately 1 kb, encoding about 350 amino acids, and the contiguous protein fragments overlap by about 165 amino acids. All pDisplay vector constructs were examined for their ability to express the relevant protein intracellularly and at the surface of COS7 cells (Table 3.1). Total protein expression quantities as judged by immunoblot analysis varied for each construct (Figure 3.2). Protein surface expression, tested by indirect immunofluorescence assays (IFAs) on intact COS7 cells, indicated that all three cloned fragments of *pknbpxa* did not display protein on the surface. Seven of the *pknbpxb* constructs, on the other hand, were expressed at the surface, albeit at different levels. The one exception was construct #3, which despite repeated attempts was not expressed at the surface of the COS7 cells. The expression levels of the seven *pknbpxb* constructs were optimized by determining differences in protein expression at various times and then the corresponding expressed PkNBPXb regions were evaluated for the ability to adhere to erythrocytes. The differences in surface expression for each construct correlated with the differences observed when comparing the total protein expression profiles (Table 3.1; Figure 3.2).

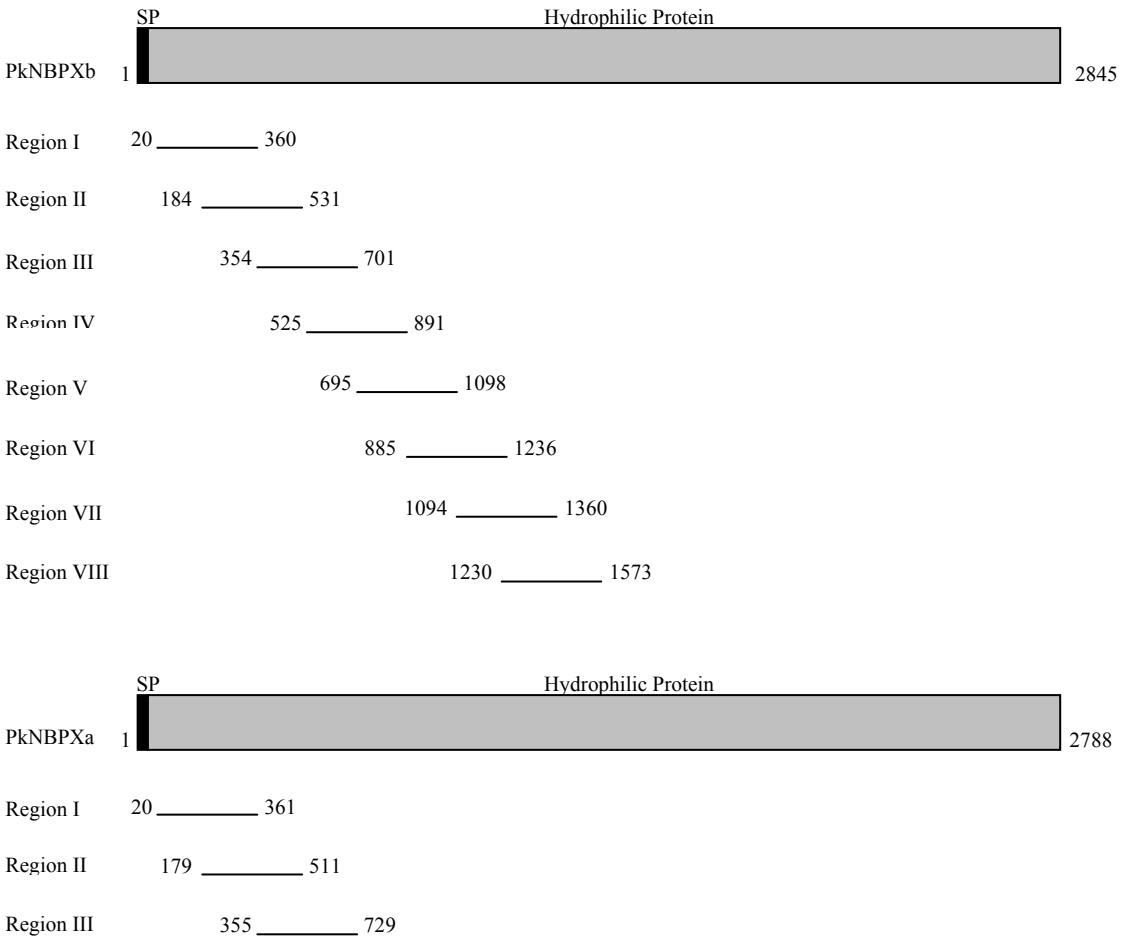

Figure 3.1. Schematic of PkNBPXa and PkNBPXb regions tested for binding to erythrocytes.

Eight regions of PkNBPXb were expressed in pDisplay vector that are approximately 350 amino acids in length and overlap each other by approximately 165 amino acids. The first expressed region starts at the beginning of the second exon. The first exon is the signal peptide (SP) that is cleaved during protein trafficking and is indicated on the full length PkNBPXb schematic. The numbers listed are the amino acid locations for each specific region. The regions cover the first half of the PkNBPXb protein and the total protein size as indicated is 2845 amino acids in length.

Table 3.1. Binding of specific regions of PkNBPXb to rhesus erythrocytes

Region of PkNBPXb	Binding of rhesus erythrocytes	% of cells with surface protein expression (IFA – HA antibody)
I	-	1.1% +/- 0.6%
II*	+	3.8% +/- 1.3%
III**	N/A	0.0%
IV	-	0.6% +/- 0.4%
V	-	1.8% +/- 0.9%
VI	-	1.2% +/- 0.6%
VII	-	7.3% +/- 3.7%
VIII	-	11.5% +/- 2.2%

* Average number of rosettes for region II observed in fifty fields = 93

** Surface expression was not observed in three separate experiments for this region.

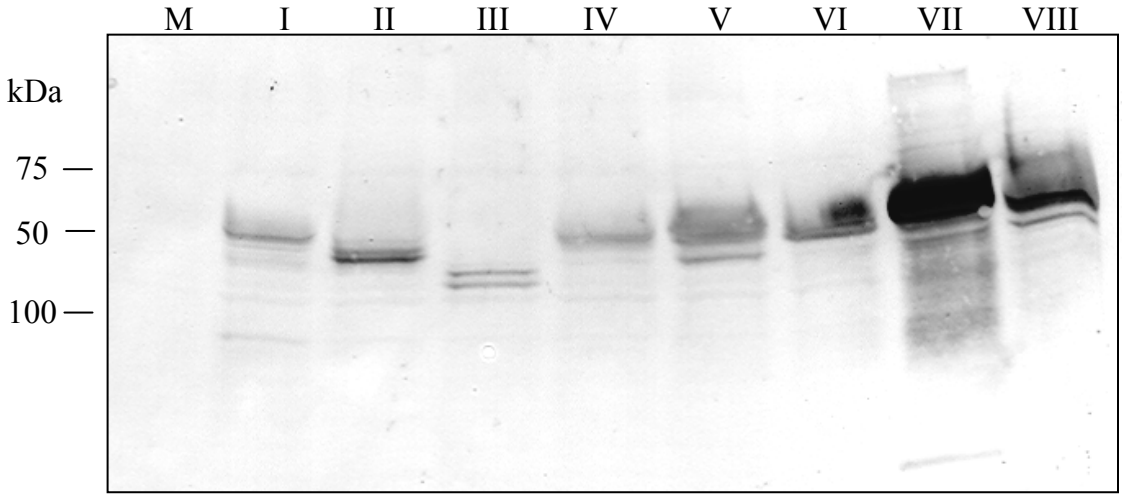

Figure 3.2. Internal expression of PkNBPXb segments varies for each region.

A cmyc specific antibody that binds to an epitope tage located at the C-terminus of the pDisplay vector was used in western blot analysis to determine the amounts of protein expressed by the different PkNBPXb constructs. Differing amounts of protein were expressed for each region (I-VIII) when COS7 cells transfected with each separate construct were examined for internal protein expression 24 hours after transfection. COS7 cells that were mock transfected (i.e. no DNA added) did not display any protein bands in the membranes demonstrating that protein expression for the eight constructs was specific for the recombinant protein and were not proteins expressed by the COS7 cells.

Erythrocyte adhesion (rosetting) assays were then performed to test the ability of the seven distinct regions of PkNBPXb, expressed at the surface of COS7 cells, to bind rhesus macaque monkey erythrocytes (of Indian origin). The data generated from five separate experiments consistently demonstrated binding for Region II (termed here as PkNBPXb-II), but was not demonstrated for other regions (Table 3.1). The observed rosettes were a clear indication of binding, with the adhesion of numerous erythrocytes recorded for the COS7 cells transfected with the *pknbpxb*-II construct. The specificity of this observation was also examined by formally demonstrating that rhesus monkey erythrocytes adhered only to COS7 cells that specifically exhibited surface expression of the PkNBPXb-II protein (Figure 3.3A). Other segments of PkNBPXb that displayed relatively high amounts of surface expression were unable to bind to the rhesus monkey erythrocytes (Figure 3.3B), also an indication that the observed adherence of the rhesus macaque erythrocytes to PkNBPXb-II was specific.

PkNBPXb-II demonstrates binding to erythrocytes from several species, particularly from Old World monkeys and Lesser Apes of South East Asia

Erythrocytes from mice, rabbits and various primate species (Table 3.2) were tested for the ability to bind to PkNBPXb-II (Figure 3.4). Erythrocytes from the Old World monkey species tested, from the family cercophithidae, consistently bound to COS7 cells expressing PkNBPXb-II at the surface. Binding of rhesus macaque erythrocytes of Indian origin (experiments to date) was compared to binding with rhesus macaque erythrocytes of Chinese origin, and no significant difference was observed. PkNBPXb-II also bound to erythrocytes from another macaque species, *M. nemestrina* the pigtail macaque and from

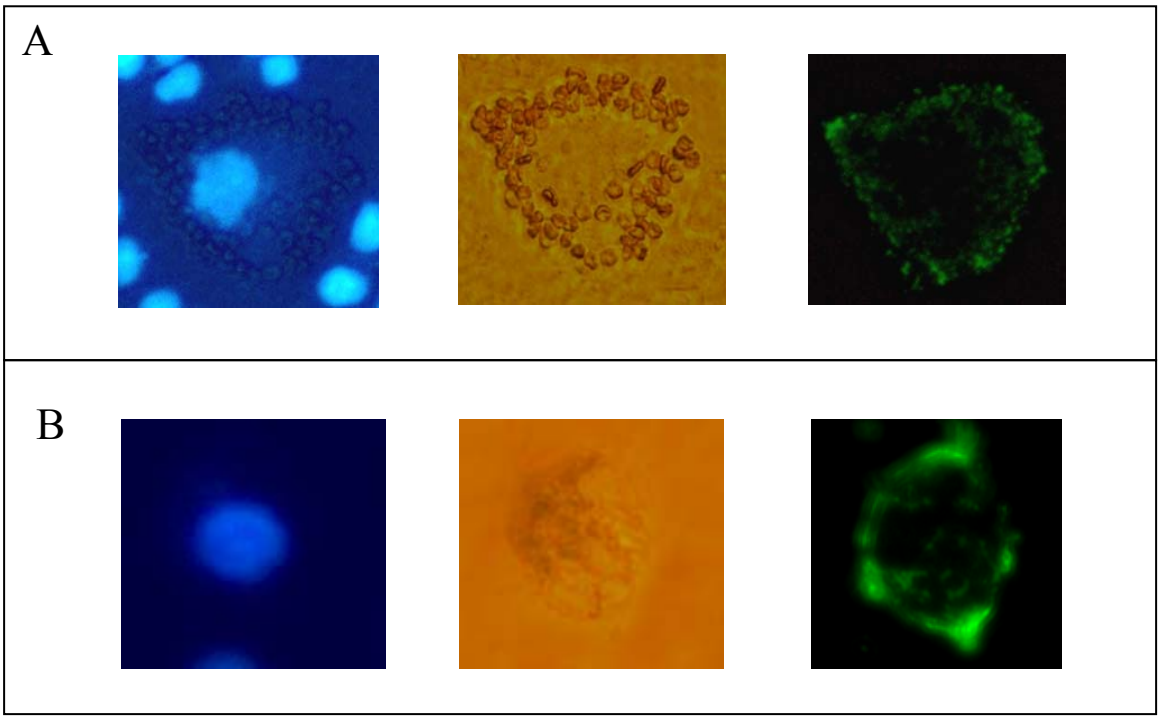

Figure 3.3. Binding of PkNBPXb-II to erythrocytes is specific for COS7 cells that express recombinant protein on their surface.

(A) First panel, rhesus monkey erythrocytes outline the nucleus (Hoechst stained) and cover the surface of a COS7 cell examined for binding and surface expression. The second panel demonstrates binding of rhesus erythrocytes to the COS7 cell examined under the light microscope. The expression of the recombinant PkNBPXb-II protein on the surface of the COS7 cell (third panel) was examined by FITC-labeled anti-mouse IgG. The anti-HA antibody used to identify surface expression binds to an epitope tag that is located on the N-terminal portion of the recombinant protein. (B) The protein surface expression of region VIII is displayed as an example of regions that express higher amounts of protein than PkNBPXb-II (third panel) but do not demonstrate binding to rhesus erythrocytes when examined under the light microscope (second panel).

Table 3.2. Classification of erythrocytes tested for binding to PkNBPXb-II

Common Name	Scientific Name	Classification	Geographical Location	Binds to PkNBPXb-II
Rhesus Macaque (Indian)*	*Macaca mulatta*	Old World Monkey	India	Yes
Rhesus Macaque (Chinese) *	*Macaca mulatta*	Old World Monkey	China	Yes
Pigtail Macaque*	*Macaca nemestrina*	Old World Monkey	Southeast Asia	Yes
Sooty Mangabey *	*Cercocebus atys*	Old World Monkey	West Africa	Yes
Marmoset *	*Callithrix jaccus*	New World Monkey	South America	No
Squirrel Monkey *	*Saimiri boliviensis*	New World Monkey	South America	No
Tamarin *	*Saguinus midas*	New World Monkey	South America	Yes
Tamarin *	*Saguinus niger*	New World Monkey	South America	Yes
Owl Monkey *	*Aotus nancymaae*	New World Monkey	South America	No
Capuchin Monkey	*Cebus apella*	New World Monkey	South America	No
Capuchin Monkey	*Cebus albifrons*	New World Monkey	South America	No
Chimpanzee	*Pan troglodytes*	Great Apes	West and Central Africa	No
Human *	*Homo sapiens*	Great Apes	Global	No
Gibbon *	*Hylobates lars*	Lesser Apes	Southeast Asia; China	Yes
Mouse	*Mus musculus*	N/A	N/A	No
Rabbit	*Oryctolagus cuniculus*	N/A	N/A	Yes

* These species are capable of being infected with *P. knowlesi*

sooty mangabey monkeys, an African primate species related to the macaques of South East Asia. Surprisingly, COS7 cells expressing PkNBPXb-II also demonstrated particularly strong rosette formation with erythrocytes from gibbons, a Lesser Ape from South East Asia. However, erythrocytes from humans and chimpanzees (Great Apes) did not interact with PkNBPXb-II. New World monkeys (owl and squirrel monkeys) can be experimentally infected with *P. knowlesi* [19-21] but erythrocytes from these two monkey hosts did not adhere to surface-expressed PkNBPXb-II. However, erythrocytes from two tamarin species, *S. midas* and *S. niger*, demonstrated weak rosetting with PkNBPXb-II expressing cells, while erythrocytes from marmosets and capuchin monkeys did not bind.

Previously, it was demonstrated that the *P. vivax* RBL proteins, PvRBP1 and PvRBP2, could adhere to rabbit erythrocytes [6]. PkNBPXb-II was therefore also tested for any possible binding to rabbit erythrocytes and only relatively weak binding was observed (Figure 3.4).

PkNBPXb-II adhesion to erythrocytes is abrogated with chymotryspin treatment

The specific erythrocyte receptor(s) for any of the RBL proteins as well as for PkNBPXb are unknown. To begin to investigate what receptors may be involved in the interaction of PkNBPXb-II, rhesus erythrocytes of Indian origin were treated with neuraminidase, trypsin, or chymotrypsin and then tested in erythrocyte adhesion assays. Unexpectedly, PkNBPXb-II bound more strongly to trypsin and neuraminidase treated erythrocytes than to erythrocytes that were not enzymatically treated. However, binding

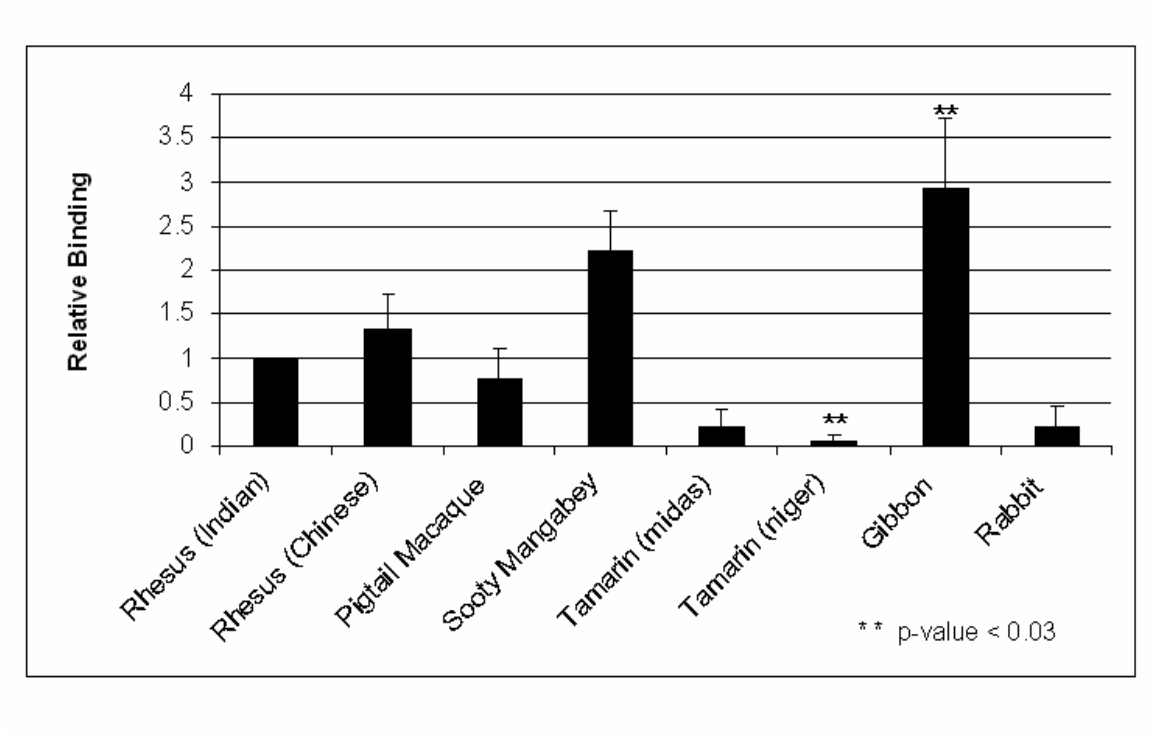

Figure 3.4. Erythrocytes from other primate species and rabbits are capable of binding to PkNBPXb-II.

Binding of erythrocytes from various primate species were compared relative to the binding of rhesus erythrocytes of Indian origin. The binding of erythrocytes from other species is expressed as a ratio of the binding of rhesus erythrocytes to expressed PkNBPXb-II. The binding of rhesus erythrocytes of Chinese origin, pigtail macaque and sooty mangabey erythrocytes are comparable to the binding of rhesus erythrocytes of Indian origin. Tamarin and rabbit erythrocytes showed minimal or marginal binding. The increase in binding of gibbon erythrocytes is statistically significant ($p<0.03$) when compared to the binding of rhesus erythrocytes.

of PkNBPXb-II was significantly almost entirely abrogated when erythrocytes were treated with chymotrypsin (Figure 3.5A).

This binding profile was also assessed for native PkNBPXb adhesion to rhesus erythrocytes by conducting standard erythrocyte binding assays with *P. knowlesi* culture supernatants. Erythrocytes were treated with chymotrypsin, trypsin, or neuraminidase and when the EBA eluates were probed in an immunoblot with a PkNBPXb specific antibody, intense bands were observed in all the sample lanes corresponding to the full length of the predicted protein (Figure 3.5B). No bands were detected in the lane where non-treated erythrocytes had been incubated in the absence of culture supernatants (R). Thus, in contrast to the binding of PkNBPXb-II expressed on the surface of COS7 cells to enzymatically treated erythrocytes, the native PkNBPXb protein found in the culture supernatant binds with equal avidity to all enzymatically treated and untreated erythrocytes (Figure 3.5B).

To ensure binding specificity of PkNBPXb to enzymatically treated erythrocytes, the same samples probed with a PkNBPXb specific antibody were also probed with an antibody against a merozoite surface protein (PkMSP3). This protein does not adhere to the erythrocyte surface but is known to be present in the culture supernatants [17]. When the samples were probed with the PkMSP3 antibody, only the samples containing supernatant alone recognized the PkMSP3 protein (Figure 3.5C).

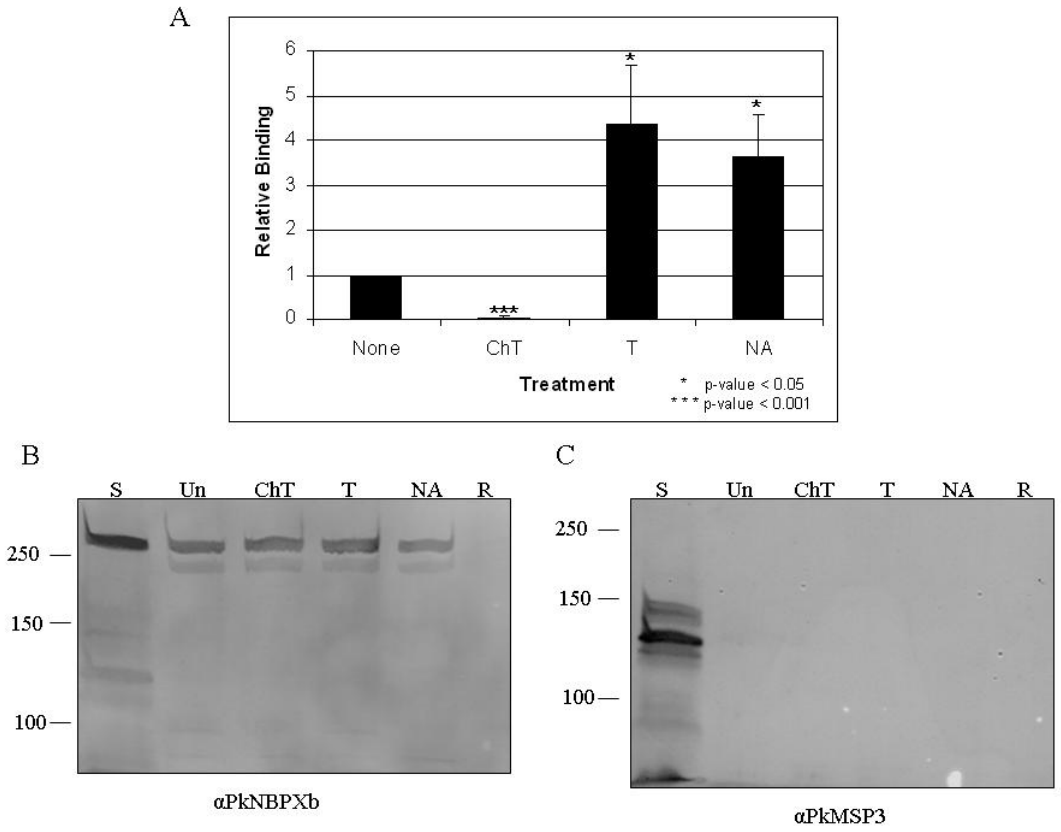

Figure 3.5. Binding of PkNBPXb-II and native protein PkNBPXb to enzymatically treated erythrocytes.

(A) PkNBPXb-II expressed in COS7 cells was tested for binding to enzymatically treated erythrocytes. Rhesus erythrocytes were either untreated (None), treated with chymotrypsin (ChT), treated with trypsin (T), or treated with neuraminidase (NA). The increase in binding of trypsin and neuraminidase treated erythrocytes is statistically significant ($p<0.05$) when compared to the binding of untreated erythrocytes. The decrease in binding of chymotrypsin treated erythrocytes is statistically significant ($p<0.001$) when compared to the binding of none treated erythrocytes. (B) Native protein (PkNBPXb) binding to target erythrocytes was tested by erythrocyte binding assays.

Erythrocytes were also untreated (Un) or treated similarly as above with chymotrypsin (ChT), trypsin (T), or neuraminidase (NA). Total supernatants (S) were also electrophoresed and transferred to nitrocellulose membranes. Target untreated erythrocytes were also incubated without supernatants (R) to ensure that the bands observed were specific for PkNBPXb. The nitrocellulose membranes were probed with an anti-PkNBPXb antibody [17]. Bands were observed in the supernatant (S) lane, lanes for untreated (Un), and the lanes for all enzymatically treated erythrocytes (ChT, T, and NA). No bands were observed in the lane with erythrocytes incubated without supernatant (R). (C) To ensure that the samples eluted from the erythrocytes were specific and not due to contamination from residual supernatant, the same samples used in immunoblots with PkNBPXb antisera were also tested with an anti-PkMSP3 140 antibody. PkMSP3 140 is found in culture supernatants but does not bind to target erythrocytes**.** PkMSP3 is found only in the supernatant lane (S).

Four cysteine residues expressed in PkNBPXb-II are critical for protein surface expression and binding to target erythrocytes

The sequence of PkNBPXb-II contains five cysteine residues (Cys 193, Cys 254, Cys 298, Cys 326, Cys 332), which we hypothesized may be critical in the formation of the correct tertiary conformation of the protein, necessary for binding to erythrocytes. Potential disulfide bonds formed when PkNBPXb-II is expressed on the surface of COS7 cells were reduced and then alkylated to ensure that broken disulfide bonds would not reform. Subsequent performance of erythrocyte adhesion assays under these conditions indicated that there was a significant reduction in erythrocyte binding to PkNBPXb-II whether dithiothreitol or 2-mercaptoethanol was used (Figure 3.6).

To determine which of these cysteine residues were important for the formation of disulfide bonds and structural characteristics conducive for the adhesion of erythrocytes, site-directed mutagenesis was performed to replace each of the five cysteine residues present in the PkNBPXb-II region with glycine residues, and then erythrocyte adhesion assays were repeated. The five mutated *pknbpxb*-II pDisplay constructs, confirmed by DNA sequencing to encode the glycine residues, were tested for protein surface expression every three hours from 18-36 hours after transfection. When the first cysteine was mutated (C1), protein surface expression was observed, although at reduced levels, and this region was capable of binding to erythrocytes (Figure 3.7). On the other hand, protein surface expression was not observed for four of the mutated *pknbpxb*-II constructs (C2-C5), and as a consequence, rosettes were not observed with cells transfected with these constructs (Figure 3.7). These clones (C2-C5) are able to express protein

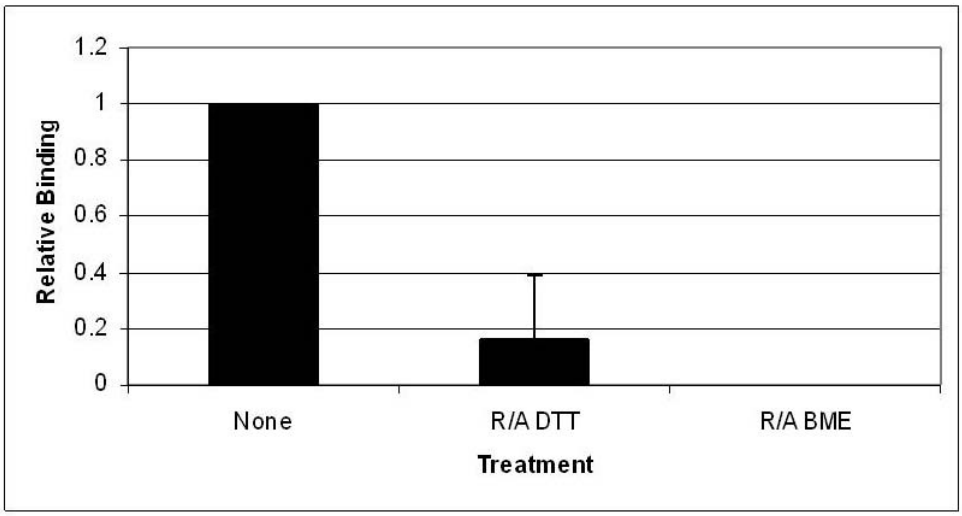

Figure 3.6. Disulfide bond formation appears to be critical for adhesion of PkNBPXb-II expressed on COS7 cells to target erythrocytes.

COS7 cells expressing PkNBPXb-II on their surface were reduced to break formed disulfide bridges by the addition of either dithiothreitol (DTT) or beta-mercaptoethanol (BME). The cells were subsequently alklyated to ensure that disulfide bridges remained broken by the addition of iodacetamide (IAA). The COS7 cells that were reduced with DTT and alkylated with IAA (R/A DTT) were unable to bind to rhesus erythrocytes in subsequent erythrocyte adhesion assays when compared to control COS7 cells that were neither reduced nor alkylated (None). Similarly COS7 cells that were reduced with BME and alkylated with IAA (R/A BME) were also unable to bind to rhesus erythrocytes in subsequent adhesion assays.

intracellularly (data not shown). However, when these mutated constructs were genetically manipulated again to encode for the cysteine residues in place of the glycine residues, protein surface expression was restored (Figure 3.7).

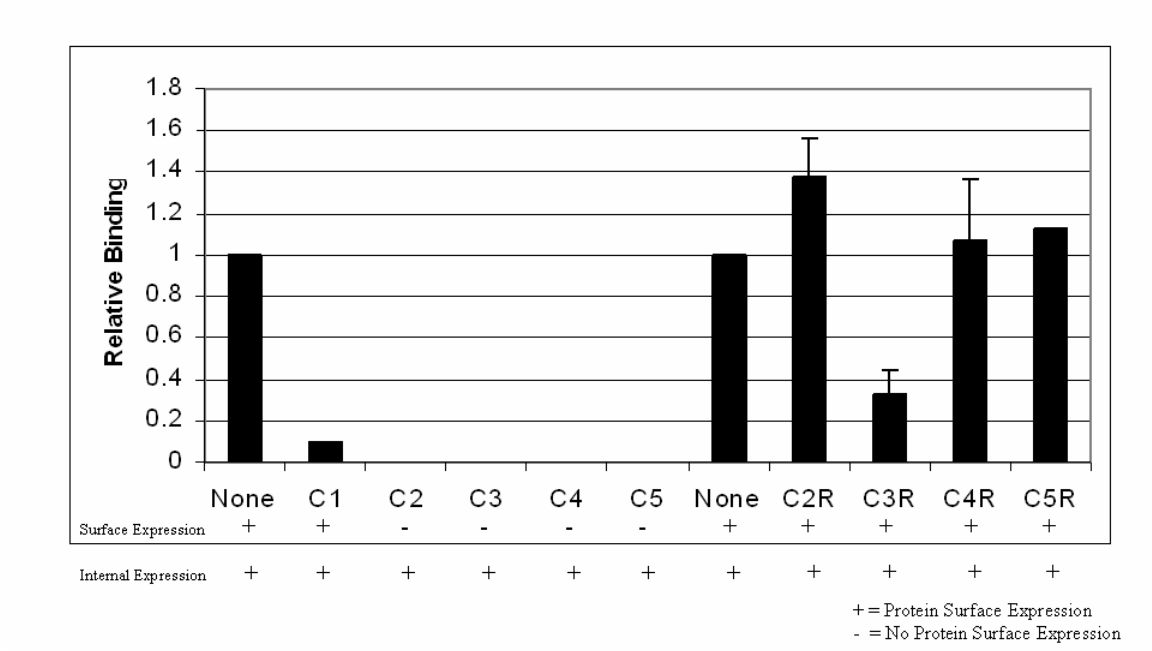

Figure 3.7. Mutagenesis of cysteine residues in *pknbpxb*-II DNA constructs.

The five cysteine residues present in the PkNBPXb-II region were mutated to glycine residues (C1, C2, C3, C4, C5). No binding to rhesus erythrocytes was observed with constructs C2-C5 because these constructs display no protein on the surface of COS7 cells. The construct containing the first cysteine residue mutated to a glycine residue (C1) displayed surface expression and was capable of binding to erythrocytes. The glycine residues present in constructs C2-C5 were mutated back to cysteine residues (C2R, C3R, C4R, C5R); these constructs were able to express protein on the surface. Subsequent testing of these clones determined that they were able to bind erythrocytes.

Supplemental Table 3.1. Primer sequences for PkNBPXb regions cloned into pDisplay

Gene	Region	Primer Sequence
pknbpxb	I (pDisplay)	Sense: 5'-ccccagatctTCATGTAAGGACAATAATAG-3' Antisense: 5'-ccccgtcgacTAGAAATGACGTAATATCTC-3'
	II (pDisplay)	Sense: 5'-ccccagatctACACAAGTGAATAATTTGGA-3' Antisense: 5'-ccccgtcgacATTGGAATAAAGAATATGTTC-3'
	III (pDisplay)	Sense: 5'-ccccagatctAGAGATATTACGTCATTTCT-3' Antisense: 5'-ccccgtcgacGTATTCTTTTATCAACGTGT-3'
	IV (pDisplay)	Sense: 5'-ccccagatctGAACATATTCTTTATTCCAAT-3' Antisense: 5'-ccccgtcgacGGTACTTTCCTTATTAACAA-3'
	V (pDisplay)	Sense: 5'-ccccagatctGACACGTTGATAAAAGAATA-3' Antisense: 5'-ccccgtcgacGTCCACAAGGGATGATTT-3'
	VI (pDisplay)	Sense: 5'-ccccagatctGTTGTTAATAAGGAAAGTAC-3' Antisense: 5'-ccccgtcgacGAACATTTCCATATACGAAT-3'
	VII (pDisplay)	Sense: 5'-ccccagatctTCATCCCTTGTGGACATG-3' Antisense: 5'-ccccgtcgacGTAGACATCTTCTGTTCC-3'
	VIII (pDisplay)	Sense: 5'-ccccagatctGATTCGTATATGGAAATGTT-3' Antisense: 5'-ccccgtcgacGTCCACATTATTAGAATTATT-3'

Supplemental Table 3.2. Primer sequences for site-directed mutagenesis of PkNBPXb-II

Gene	Region	Primer Sequence
pknbpxb-II	First Cysteine	MPC1: 5' GGATTATGTTGGTGTCATGAATAATGATGATAATATAATTTCTTTATTCCG 3'
	Second Cysteine	MPC2: 5' TCGGAGTATGAAGGAAGGCTCCAATCAGACTTCTG 3'
	Third Cysteine	MPC3: 5' GTATTTTACATGAAGGCATGAAAAGAAATTATAACAAACATAAAGAGG 3'
	Fourth Cysteine	MPC4: 5' ATAGATCATATGGATGGCAGCTGGACTAACTACTGTCC 3'
	Fifth Cysteine	MPC5: 5' TGCAGCTGGACTAACTACGGTCCAACAGGTACATATTAC 3'
	Second Cysteine Reversion Mutation	MPRC2: 5' TCGGAGTATGAAGGAATGCTCCAATCAGACTTCTG 3'
	Third Cysteine Reversion Mutation	MPRC3: 5' GTATTTTACATGAATGCATGAAAAGAAATTATAACAAACATAAAGAGG 3'
	Fourth Cysteine Reversion Mutation	MPRC4: 5' ATAGATCATATGGATTGCAGCTGGACTAACTACTGTCC 3'
	Fifth Cysteine Reversion Mutation	MPRC5: 5' TGCAGCTGGACTAACTACTGTCCAACAGGTACATATTAC 3'

Discussion

We have identified an erythrocyte binding domain from one of two *P. knowlesi* RBL proteins, namely PkNBPXb [17]**,** and have developed a body of research suggesting that additional RBL domains, yet to be defined, are also relevant for entry of *P. knowlesi* merozoites into erythrocytes. Investigations to reveal the boundaries and binding specificities of the RBL proteins in *P. knowlesi* is key to determining the role of these proteins in the cascade of molecular events that define merozoite invasion of erythrocytes, and to advance research on these proteins as vaccine candidates.

Seven overlapping regions from the N-terminal half of PkNBPXb, each being approximately 335 amino acids, were expressed in the pDisplay vector and tested for their ability to bind to erythrocytes when expressed at the surface of COS7 cells. This research was focused on the N-terminal half of this protein given the intuitive considerations that the N-terminal regions may be better exposed structurally to bind host target cells and previous data based on studies to identify a binding domain of homologous RBL proteins in *P. vivax,* which supported this view [22]. An N-terminal domain from the *P. vivax* RBL protein known as the reticulocyte binding protein-1 (PvRBP-1) expressed at the surface of mammalian cells was shown to exhibit binding specificity for erythrocytes, although without a clear specificity for reticulocytes. These data support the prediction of a PvRBP-1 N-terminal adhesive receptor ligand interaction with erythrocytes; yet also indicated that inherent fine specificities may be involved. This is, in fact, what is being revealed regarding the *P. knowlesi* RBLs, as the erythrocytes binding specificities are being investigated for PkNBPXa and PkNBPXb.

While the N-terminal series of fragments of PkNBPXb were designed to overlap by about 165 amino acids, only one region (termed Region II, or PkNBPXb-II) demonstrated any binding to erythrocytes when expressed at the surface of COS7 cells. This binding was strong and consistent with large rosette formation and the complete covering of the surface of the COS7 cells. PkNBPXb-II begins about 160 amino after the predicted signal peptide cleavage site and is 347 amino acids in length (Figure 3.1). Three N-terminal fragments of PkNBPXa were also expressed in the pDisplay vector with internal protein expression observed in transfected COS7 cells; however, surface expression was never observed for any of these constructs. Current studies are underway to achieve surface expression for alternative PkNBPXa constructs. The future focus on PkNBPXa adhesion domains is important because, unlike PkNBPXb, this RBL protein binds both to rhesus macaque and human red blood cells in standard erythrocyte binding assays ([17] and data not shown). Expanded studies involving PkNBPXa adhesion to human and other non-human primate erythrocytes will hopefully shed light on the tentative role of this protein as an RBL ligand used by *P. knowlesi* to naturally infect humans in Southeast Asia [23].

Further evaluation of other PkNBPXb constructs is also warranted to identify additional binding domains within this large protein, particularly since the data presented here shows that the native protein binds to erythrocytes pre-treated with chymotrypsin, while chymotrypsin treatment abrogates the binding of the PkNBPXb-II domain. This is a strong indication that this protein has more than one means to adhere to target red blood cells: one, by using a receptor that is cleaved by chymotrypsin and another with different characteristics yet to be described.

Interestingly, PkNBPXb-II binds strongly to erythrocytes from Old World monkeys (rhesus and pigtail macaques), sooty mangabees (African monkeys with a South East Asian origin) and to Lesser Apes (gibbons) that are located in South East Asia. Thus, the host origin correlates to some degree with the binding specificities observed, regardless of the actual ability of *P. knowlesi* to invade the particular non-human primate cell type. This observation is consistent with the necessity of multiple receptor-ligand interactions being critical in a cascade of molecular events to achieve successful invasion of red blood cells. Rosetting of macaque erythrocytes was expected because rhesus macaques are utilized as an experimental model studying *P. knowlesi* infections, and pigtail macaques are one of the natural hosts for this species. Less expected was the strong binding observed to gibbon cells. While gibbons are neither natural nor experimental hosts for *P. knowlesi*, like the Old World macaque monkeys, they are found in South East Asia and China. In contrast, squirrel and owl monkeys can be experimentally infected with *P. knowlesi,* and marmosets are known to be susceptible to *P. knowlesi* infection [24]; yet erythrocytes from these species did not form rosettes with the PkNBPXb-II expressed at the surface of COS7 cells. This fact supports the view that more than one RBL ligand is capable of performing the biological functional role of this family of proteins.

Different enzymatic treatments of erythrocyte target cells can be useful to generate specific receptor profiles and allow for some preliminary characterization of potential receptors for particular binding proteins. For instance, neuraminidase cleaves sialic acid from the surface of cells, while trypsin cleaves receptors glycophorin A and glycophorin C, and chymotrypsin cleaves glycophorin B and Band 3. The *P. falciparum*

RBL homologue, known as PfNBP2b or PfRH2b, has been shown to bind erythrocytes with a chymotrypsin sensitive and neuraminidase and trypsin-resistant pattern, denoted as Receptor Z characteristics [25]. PkNBPXb-II binds to a receptor that is cleaved by chymotrypsin and its binding is resistant to treatment with neuraminidase and trypsin. It is therefore possible that PkNBPXb-II binds to Receptor Z or Band 3, and further research is needed to explore these possibilities.

The native PkNBPXb adheres to all enzymatically treated erythrocytes tested in contrast to the binding profile of PkNBPXb-II. Thus, it can be concluded that the binding domain identified here for PkNBPXb may not include the boundaries of the entire ligand that functions in nature, or an entirely different region of PkNBPXb may also serve as a binding domain. To address these options, studies are currently ongoing to include sequences N-terminal or C-terminal to the PkNBPXb-II region to better define the boundaries of this binding domain. Future experiments may also include the design of a new series of constructs that span the whole molecule.

PkNBPXb-II contains five cysteine residues that were believed to be important for mediating disulfide bridges leading to the correct conformation and allowing for binding to target erythrocytes. Alignments of PkNBPXb-II with the N-terminus of homologous RBL proteins in *P. vivax* and *P. falciparum* demonstrated conservation amongst three cysteine residues. Rosette formation was significantly abrogated when the suspected disulfide bonds of PkNBPXb-II were broken with DTT or beta-mercaptoethanol. Site-directed mutagenesis experiments also demonstrated that a lack of expression of four of the five cysteine residues eliminated the trafficking of the PkNBPXb-II protein to the surface of COS7 cells. These experiments suggested that the

ability of the protein to be displayed on the surface was dependent on expression of those four cysteine residues and that erythrocyte adhesion was ultimately dependent on formation of the disulfide bonds.

The identification of a binding domain in a *P. knowlesi* RBL protein affords the opportunity to test this specific binding domain in pre-clinical vaccine trials as a proof of principle for targeting an RBL binding domain(s) through vaccine interventions. *P. knowlesi* infection in rhesus macaques is a lethal infection unless treated with anti-malarial drugs. Future studies are needed to determine if immunization against the binding domain, PkNBPXb-II, can cause a normally lethal *P. knowlesi* infection to become a non-lethal infection in rhesus macaque models.

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CHAPTER FOUR

DISCUSSION AND CONCLUSION

DISCUSSION AND CONCLUSION

Since the initial discovery of the *P. vivax* reticulocyte binding proteins, PvRBP1 and PvRBP2 [1], homologous proteins have been identified in several other *Plasmodium* species [2-5], defining a complex family of proteins whose precise function in merozoite invasion is unknown. *Plasmodium* species such as *P. falciparum, P. reichnowi*, *P. yoelii,* and *P. vivax* contain several reticulocyte binding-like proteins that are most likely retained because they serve important functional roles during merozoite invasion. It has been estimated that 1-2% of the entire *P. yoelii* genome is dedicated to encoding its *rbl* gene repertoire [6]; *P. yoelii* contains fifteen *rbl* gene members [7, 8]. With the evidence that *P. yoelii* dedicates a large amount of its genome to this one group of proteins, from an evolutionary standpoint, it appears the RBL proteins are essential to the survival of the parasite. Additionally, *Plasmodium* species that contain pseudogenes also maintain functional RBL protein members demonstrating the importance of functional RBL proteins in the *Plasmodium* protein repertoire [9, 10].

The preceding chapters in this dissertation have described the *rbl* genes and corresponding proteins that are present in the simian malaria parasite *P. knowlesi*, and the identification of one RBL domain that binds erythrocytes. An initial primary aim of this dissertation was to determine whether the *P. knowlesi* normocyte binding proteins (PkNBPXa and PkNBPXb) were essential for the survival of the blood-stage parasite by performing transfection/knock-out studies using both the *in vivo* and *in vitro P. knowlesi* model systems (Appendix Figure 1). The results of these studies were briefly stated in the discussion of Chapter Two. In both the *in vivo* and *in vitro* model systems, no blood-stage parasites were retrieved when either *pknbxa* or *pknbpxb* was disrupted by knock-

out/transfection experimentation (Appendix Figure 6). When control genes were similarly disrupted, *P. knowlesi* parasites were able to survive and propagate (Appendix Figures 2-5). Specifically, the circumsporozoite protein, *pkcsp,* and merozoite surface protein 3, *pkmsp3,* genes were used as controls because their expression is not needed for propagation of the blood-stage of *Plasmodium.* These experiments showed that PkNBPXa/Xb appear to be essential for merozoite invasion. The optimal transfection experiments would be conditional knock-out experimentation that would allow for control of the expression of target proteins at specific time points. One question that arises with transfection experimentation where the genes are disrupted in the parasite genetic repertoire is whether the parasites are unable to propagate due to the experimental manipulation of the parasites (i.e. electroporation). The parasites may be unable to survive and grow because of this experimental manipulation and not because of the critical nature in *Plasmodium* invasion of the target gene that is disrupted. Our control experimentation performed alongside the PkNBPXa/Xb transfection experiments demonstrated that the experimental procedure itself was not harmful to the parasites. Molecular follow-up experiments (i.e. PCR experimentation, Appendix Figure 3, and western blot analysis, Appendix Figure 5) showed that our target control genes were indeed disrupted. Thus, we have confidence that the inability of *P. knowlesi* parasites to survive both *in vivo* and *in vitro* upon disruption of the *pknbpx* genes is due to the need for the encoded proteins. Conditional knock-out technologies have not yet been put to practice in the context of experiments using the *P. knowlesi* model; to date, successful conditional knock-out experiments have been demonstrated only in the human malaria *P.*

falciparum [11] and such experiments have yet to become widely used for the study of various *Plasmodium* antigens of interest.

RBL proteins are proposed to function by binding to target erythrocytes mediating subsequent signaling events that lead to downstream functions during merozoite invasion. RBL proteins are believed to function early in invasion when the merozoite irreversibly attaches its apical end to the erythrocyte surface. It is hypothesized that these binding events lead to downstream signals that cause the release of Duffy Binding-Like/Erythrocyte Binding-Like (DBL/EBL) proteins; these proteins then form tight bonds between the erythrocyte and the invading merozoite that lead to subsequent completion of invasion. In the second chapter of this dissertation, it was shown that PkNBPs bind to target (rhesus macaque) erythrocytes demonstrating that these RBL proteins function by binding to their host erythrocytes.

Interestingly, PkNBPXa binds to both Duffy blood group negative and Duffy blood group positive human erythrocytes (Appendix, Figure 7), although PkNBPXb was never shown to bind to human erythrocytes. However, previous reports have demonstrated that *P. knowlesi* is unable to invade Duffy blood group negative erythrocytes [12]. PkNBPXa is able to bind to Duffy negative erythrocytes but presumably subsequent invasion does not occur because of the absence of the receptor for the PkDBPs on Duffy negative erythrocytes. Thus, invasion of *P. knowlesi* into human Duffy negative erythrocytes does not progress naturally past the stage of PkNBPXa binding to human erythrocytes. It is important to note that PkNBPXa and PkNBPXb are able to bind to different target erythrocytes, suggesting this relates to functional differences between these two proteins; this will be discussed later in this chapter.

By immuno-electron microscopy, it was shown that PkNBPXb locates to the micronemes of free merozoites; preliminary immuno-electron microscopy using PkNBPXa antibodies also demonstrates that this antigen was found in the micronemes. It was also determined by immuno-electron microscopy that PkNBPXb functions early during invasion. A merozoite captured in the process of invasion displayed significantly less staining of PkNBPXb, unlike the ample staining that was observed on free merozoites. The invading merozoite had progressed beyond the stage in invasion of tight junction formation, and PkNBPXb may have completed its function before this stage of invasion. Such images are consistent with RBL proteins functioning prior to entry of the merozoite into the host cell.

The micronemal location of PkNBPs noted in Chapter Two is in contrast to published reports of a rhoptry organelle localization of homologous RBL proteins in *P. yoelii* and *P. falciparum* [13, 14]. Concerns can be raised about the lack of concentrated staining in these studies as well as the weak specificity of staining in the rhoptries, as these organelles are know to exhibit non-specific staining in IEMs. However, it is also possible that the location of the RBL proteins may differ among species, and original localization data should be re-examined and expanded in the future with all possibilities in mind.

The next major aim of this dissertation was to define the binding domain of PkNBPXa and/or PkNBPXb. After demonstrating that PkNBPs were capable of binding to host erythrocytes, it was hypothesized that PkNBPs contained a specific domain that mediated its binding to target erythrocytes. Unlike the binding proteins of the Duffy Binding-Like/Erythrocyte Binding-Like (DBL/EBL) family of proteins (PvDBP,

PkDBPs, and PfEBA-175) and Apical Membrane Antigen (PfAMA1) that contain discrete domains defined by cysteine residue clusters [15-17], the RBL proteins have many cysteine residues dispersed throughout the protein. Despite this hindrance in identifying binding domains in the RBL proteins, in Chapter Three of this dissertation a binding domain of PkNBPXb (PkNBPXb-II) is described. The binding domain of PkNBPXa was not defined due to the inability to produce protein on the surface of mammalian cells although internal protein expression was demonstrated. Current experiments are underway to optimize the surface expression of these PkNBPXa regions and subsequently define a binding domain in this RBL protein to better understand the role this protein plays in merozoite invasion.

Plasmodium species may maintain more than one functional RBL protein because these individual proteins mediate independent binding specificities. In identifying the binding domain PkNBPXb-II, we demonstrated that this domain preferentially bound to erythrocytes from Old World monkeys. *P. knowlesi* is capable of invading several New World monkey species and recent publications demonstrate increasing numbers of zoonotic transmission of *P. knowlesi* to humans [18-21]. The inability of PkNBPXb-II to bind to erythrocytes from New World monkeys and humans was a surprising result and suggested that there was an alternative binding protein that mediated invasion into these hosts. The most likely protein to mediate this binding is PkNBPXa, primarily because it is the only other functional RBL protein that has been identified in *P. knowlesi.* Other evidence for PkNBPXa as the protein that is responsible for this adhesion is demonstrated by the ability of PkNBPXa to bind to human cells in contrast to PkNBPXb that does not bind. We have demonstrated that PkNBPXa and PkNBPXb serve both redundant

functional roles (i.e. both bind to rhesus macaque erythrocytes), and independent functional roles during invasion (i.e. PkNBPXa binds to human cells and PkNBPXb does not bind). Future studies, primarily with PkNBPXa, are needed to accurately state the complete independent and redundant functional roles of the PkNBPs. The observed difference in the functional roles of the PkNBPs also suggests that this same difference exists in other *Plasmodium* species; the maintenance in other *Plasmodium* species of several functional RBL proteins suggests that different functions are mediated by specific RBL proteins. It is also possible that there exists another binding domain in PkNBPXb that functions in invasion of New World monkeys or humans. Future experiments are needed to explore this possibility.

The receptor(s) to which the PkNBPs bind on rhesus erythrocytes is unknown. We determined that PkNBPXb-II binds to an erythrocyte receptor that was cleaved by the enzyme chymotrypsin. The erythrocyte receptor that PkNBPXb-II binds is proposed to be either Receptor Z or Band 3 that have been identified as receptors for other *Plasmodium* merozoite proteins [13, 22]. Receptor Z and Band 3 are cleaved by chymotrypsin and are resistant to cleavage by trypsin and neuraminidase, similar to the PkNBPXb-II erythrocyte receptor. Future studies are needed to determine the specific receptor that PkNBPXb-II binds on the surface of erythrocytes.

In examining the binding of the native protein PkNBPXb to rhesus erythrocytes, it was demonstrated that PkNBPXb binds to all treated erythrocytes. This data appears to contradict the observation that PkNBPXb-II does not bind to chymotrypsin-treated erythrocytes. However, it is possible that the binding domain we have identified is not the full binding domain that functions in nature. The overlapping contiguous regions of

PkNBPXa and PkNBPXb tested for adhesion to erythrocytes were chosen by starting from the N-terminal portion of the protein immediately downstream of the signal peptide. Although these regions appear to be chosen arbitrarily, the selection of these domains was determined as efficiently as possible since there are no discrete domains identified in these proteins unlike the other binding proteins discussed above. It is possible there are upstream and/or downstream sequences that encode part of the binding domain in PkNBPXb; i.e. a construct that encompasses PkNBPXb-II and additional contiguous sequence may better represent the binding domain. Current studies are underway to determine the exact spatial location of this binding domain.

A stable tertiary confirmation of recombinant proteins expressed by pDisplay is essential for protein surface expression. Alignments of PkNBPXb-II protein sequence with homologous RBL proteins in *P. vivax* and *P. falciparum* displays conservation amongst three cysteine residues present in the N-terminal domain. It was hypothesized that expression of the cysteine residues in PkNBPXb-II was necessary for protein surface expression and subsequent adhesion to erythrocytes. We have demonstrated that four of the five cysteine residues expressed in PkNBPXb-II are required for protein surface expression. The importance of the expression of these residues is believed to be in the formation of disulfide bridges. However, it is unclear which cysteine residues pair to form the two predicted disulfide bonds; it is also possible the two predicted disulfide bonds pair with each other. The exact tertiary structure of this domain remains to be determined.

Inhibition of invasion experiments have also been conducted using purified IgG from antisera against a central domain of PkNBPXb and against PkNBPXb-II to address

the role that blocking of each domain may play in the ability of *P. knowlesi* to invade erythrocytes, *in vitro,* and potentially *in vivo*. The target rhesus erythrocytes used in these assays were either untreated, or treated with the enzymes chymotrypsin, trypsin, or neuraminidase (Appendix Table 2). These results demonstrate that inhibition can be achieved into untreated erythrocytes with antibodies directed against the central domain of PkNBPXb, although the inhibition into untreated cells was low. A greater amount of inhibition of *P. knowlesi* invasion was achieved into neuraminidase treated erythrocytes with antibodies against both domains of PkNBPXb; the same antibodies demonstrated very little ability to inhibit invasion of *P. knowlesi* into trypsin treated erythrocytes. There was also a difference in the ability of these different antibodies to inhibit invasion into chymotrypsin treated erythrocytes; however this difference in inhibition is not what would be expected for a domain that binds to a chymotrypsin sensitive receptor.

One conclusion that can be drawn from these studies is that antibodies targeted against the PkNBPXb protein can inhibit invasion. However, further studies are needed to better understand the role that blocking of each domain plays in the ability of *P. knowlesi* to invade different enzyme treated erythrocytes *in vitro.* The antisera made against the PkNBPXb-II domain did not identify the native PkNBPXb protein when used to probe mature *P. knowlesi* schizonts in western blot assays; however, the antisera did recognize the native protein in immunofluoresence assays although non-specific antibody reaction was also observed. Future inhibition of invasion studies should use an antisera directed against PkNBPXb-II that has higher antibody titers, recognizes the native protein in western blots assays, and does not exhibit non-specific reactions in immunofluorescence assays. Merozoite invasion of erythrocytes is a dynamic and complex process, and a more

comprehensive understanding of the functional role of the RBL proteins in merozoite invasion would aid in understanding how this invasion data may fit into the overall function of PkNBPXb in merozoite invasion.

The RBL proteins have been identified as potential vaccine candidates but have not yet been tested in pre-clinical studies. Determination of a functional binding domain(s) is an important step in evaluating an antigen's potential as a vaccine candidate. The identification of the erythrocyte binding domains of PvDBP and *P. falciparum* EBA-175 has accelerated these proteins as malaria vaccine candidates, with pre-clinical and clinical trial testing. Future studies are to perform pre-clinical testing of the PkNBPXb binding domain. The domain tested should optimally represent the exact domain that functions in nature and further experimental studies are needed to define the exact PkNBPXb binding domain.

P. knowlesi infections in rhesus macaques become lethal infections within a few days of inoculation unless treated with anti-malarial drugs. Studies will test the ability of antibodies directed against the PkNBPXb binding domain to prevent the lethal rise in parasitemia in rhesus macaques by immunization with a recombinant protein representing this erythrocyte binding domain. These studies will quickly assess the value of the RBL proteins as malaria vaccine immunogens.

The data included in this dissertation has contributed information on the functional roles of RBL proteins in merozoite invasion. Importantly, the identification of a binding domain in PkNBPXb affords the opportunity to test the RBL class of proteins as malaria vaccine candidates. The advancement of the malaria vaccine field will occur with identification of novel *Plasmodium* antigens, basic science research to understand

the role(s) of identified antigens, and further analysis to determine the potential of these antigens as vaccine candidates. The information provided in this dissertation will hopefully aid in the advancement of the malaria vaccine research field.

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APPENDIX

ADDITIONAL EXPERIMENTAL STUDIES ON THE *P. KNOWLESI* NORMOCYTE BINDING PROTEINS

Introduction

The second chapter briefly stated the results obtained from the *in vitro* and *in vivo* *Plasmodium knowlesi* normocyte binding protein knockout studies. Here we describe in greater detail the methods and results related to the *in vivo* transfection studies. Experimental studies not included in the preceding chapters are also described here. In this appendix *P. knowlesi in vitro* inhibition of invasion assays using PkNBPXb antisera and PkNBPXa binding studies are detailed. These additional studies are discussed in the context of the functional role of the *P. knowlesi* normocyte binding proteins during merozoite invasion in the concluding chapter.

Materials and Methods

Transfection constructs

PkNBPXa and PkNBPXb knockout transfection constructs were created using a pBluescript KS plasmid backbone (pBS/DHFR #19) containing a mutated *Toxoplasma gondii* dihydrofolate reductase gene (TgDHFR) that confers resistance to pyrimethamine. The TgDHFR construct is flanked by 5' and 3' *P. berghei* untranslated (UTR) regions that serve as promoters for the TgDHFR construct. 5' and 3' sequences for PkNBPXa and PkNBPXb used in the transfection constructs were amplified using primers listed in Appendix Table 1. Genomic *P. knowlesi* DNA was amplified using Roche Expand High Fidelity PCR system under the following conditions: initial denaturation of 94°C for 2 min.; 35 cycles of 94°C for 10s, appropriate annealing temperature for 30s, 68°C for 3 min; then final polymerization of 68°C for 7 min. PCR products and vector pBS/DHFR #19 were restricted with the appropriate enzymes and ligated using Roche Rapid DNA ligation kit (Roche Applied Sciences; Indianapolis, IN). Ligation products were transformed into MRF competent cells and plated on Luria-Bertani (LB) with ampicillin (100ug/ml) plates. Clones were grown overnight (280 rpm, 37°C) in LB/ampicillin (100ug/ml). DNA was extracted using laboratory DNA extraction miniprep protocol or Fast Plasmid Mini Kit (Eppendorf; Hamburg, Germany) according to manufacturer's protocol. Positive clones were identified by restriction enzyme digest (double digest with *Hind III* and *Sca I*) and sequenced using BigDye Terminator Cycle Sequencing v3.1 Ready Reaction Kit (Applied Biosystems; Foster City, CA). Clones were created by first ligating either the 5' or 3' PkNBP insert onto the pBS/DHFR #19 vector, identifying

positive clones and then performing ligation of the second PkNBP insert and again identifying positive clones.

Parasite reactivation and maintenaince

P. knowlesi was reactivated from cyropreserved stocks and inoculated into *Saimiri* or *Macaca mulatta* monkeys as previously described [1]. Parasitized blood was collected for transfections as previously described [2]. *P. knowlesi* culture-adapted parasites were maintained with daily refreshing of complete RPMI (RPMI with 20% Human AB+ serum) and addition of rhesus macaque erythrocytes as needed to maintain 5% hematocrit and parasitemia <5%.

***In vivo* transfections**

Transfection constructs were linearized by restriction enzyme digest using *Kpn I* and *Not I* or *BssHII*. Plasmids were purified by DNA ethanol precipitation. DNA was resuspended in H_2O or Tris-HCL buffer. Transfections were performed with either AMAXA or Biorad transfection systems. For transfections using the AMAXA system purified schizonts were resuspended in 90 µl of Nucleofector T solution (AMAXA) and 10 µl of plasmid DNA (15 ug). Parasites and DNA were transfected using program U33 (AMAXA transfection system; Gaithersburg, MD) and 400 µl of complete RPMI was immediately added to the parasites. Transfected parasites were intravenously injected into *Saimiri* or *Macaca mulatta* monkeys (modified from [3]). For transfections using the Biorad system, purified schizonts were resuspended in cytomix solution to a total volume of 200 µl. Parasites and DNA were transfected using Biorad Gene Pulser at 2.5 kV/cm

and 25 μF and complete RPMI was immediately added to the parasites. Transfected parasites were intravenously injected into *Saimiri* or *Macaca mulatta* monkeys. Parasitemia was subsequently monitored by blood smears and selection of transfected parasites was performed by administering 1mg/kg to1.5mg/kg of pyrimethamine intramuscularly.

SDS-PAGE and western blotting

Parasite samples were diluted 1:1 in 2X Reduced Sample Buffer (Biorad; Hercules, CA). 17-18 μl of samples were run on 5% polyacrylamide gels and transferred to nitrocellulose membranes (Biorad; Hercules, CA). The membrane was incubated with antibody using 1:500 dilution (45 minutes) of appropriate primary antisera and 1:5000 dilution (35 minutes) of goat anti-rabbit IgG alkaline phosphatase conjugated antibody (Promega; Madison, WI), (Kirkegaard & Perry Laboratories; Gaithersburg, MD). Blots were developed with alkaline phosphatase substrate (Promega; Madison, WI).

Erythrocyte Binding Assays

Erythrocytes infected with schizont-stage parasites were purified by centrifugation on Percoll gradients. The purified schizont-infected erythrocytes were placed into culture *in vitro* and allowed to grow overnight until erythrocytes containing mature schizonts with formed merozoites (i.e., segmenters) had completely ruptured. Cultures were centrifuged at 4000 rpm and the supernatants were removed and stored in liquid nitrogen. 500 mL of culture supernatants were rotated with 1 x 10^9 erythrocytes at room temperature for 4 h. The cells were washed twice by layering over and subsequent

centrifugation through Dow Corning 550 silicone fluid. Bound proteins were eluted in 50 ul of 5x RPMI at room temperature and harvested by centrifugation. The resulting proteins were analyzed by SDS-PAGE on Pre-Cast 5% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules CA). The membranes were probed with rabbit polyclonal antisera diluted 1:500 for 2 hours. Alkaline phosphatase-conjugated goat anti-rabbit IgG diluted 1:5000 (Promega, Madison WI) was used for detection of bound antibodies. Bands were visualized by addition of NBT/BCIP substrate (Promega, Madison WI) to the nitrocellulose strips.

Inhibition of Invasion Assays

P. knowlesi H strain *in vitro* adapted parasites [4] were cultured to the schizont stage and purified by percoll gradients as described above. Purified parasites were placed into a 96-well plate along with the appropriate target erythrocytes. IgG was purified using the Affi-Gel Protein MAPS II purification kit (Bio-Rad; Hercules, CA); IgG was subsequently dialyzed against RPMI and added at a concentration of 1mg/ml into the appropriate samples. Invasion assays were performed in duplicate and at least 15000 erythrocytes were counted for each sample.

Polymerase Chain Reaction (PCR) and cloning

Approximately 1kb segments of *pknbpxa,* with 500 bp overlaps, were amplified using gene-specific primers under standard PCR conditions (Appendix Table 3). The amplified regions were inserted into the *Bgl*II and *Sal*I restriction sites in the pDisplay vector (Invitrogen; Carlsbad, CA). This vector contains an immunoglobulin κ chain

leader sequence for targeting of the recombinant protein into the secretory pathway and a platelet derived growth factor receptor transmembrane domain for anchoring of the recombinant protein on the surface of mammalian cells. Positive clones were identified and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing v3.1 Ready Reaction Kit (Applied Biosystems; Foster City, CA). Resulting sequences were analyzed using MacVector™ 7.2.2 to ensure the fidelity of the sequence.

Table A.1. Primers used for PCR amplification of 5' and 3' *pknbpxa/b* regions

Gene	Location	Enzyme	Primer Sequence
pknbpxa	5' Forward	Sac II	5' ataccgcggGAATGGATTGTGGGGAG 3'
pknbpxa	5' Reverse	Xba I	5' gctctagaGTAGGAACTTTATAGAACT 3'
pknbpxa	3' Forward	Kpn I	5' caggtaccATCGCATTGAGGAGAATAAAT 3'
pknbpxa	3' Reverse	Kpn I	5' ctggtaccCCTTACTCATATATATTCGTT 3'
pknbpxb	5' Forward	Xba I	5' gctctagcCTGCCATCTCCTGTTTATT 3'
pknbpxb	5' Reverse	Bam HI	5' cgggatccaGAATTTCATATCTTTCGGGA 3'
pknbpxb	3' Forward	Kpn I	5' gtggtaccTACGGATAGCACATTTAC 3'
pknbpxb	3' Reverse	Kpn I	5' gtggtaccGCAAAACGAGTTCTTCC 3'

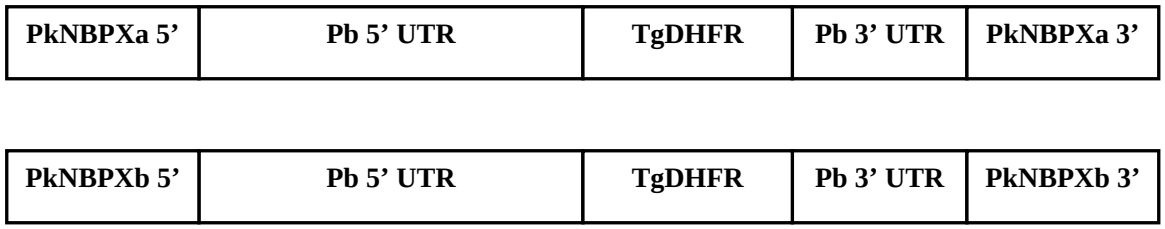

Figure A.1. Schematic of the *P. knowlesi* Normocyte Binding P/rotein knock-out constructs.

The backbone of the constructs used for knock-out experimentation includes *P. berghei* untranslated regions (UTR) that serve as promoters for the *Toxoplasma gondii* dihydrofolate reductase gene that when mutated provides resistance to the anti-malaria drug pyrimethamine. Homologous regions of *pknbpxa/b* genes are located adjacent to the UTR regions; the control regions (PkMSP3-merozoite surface protein, PkCSP-circumsporozoite protein, data not shown) are similarly positioned.

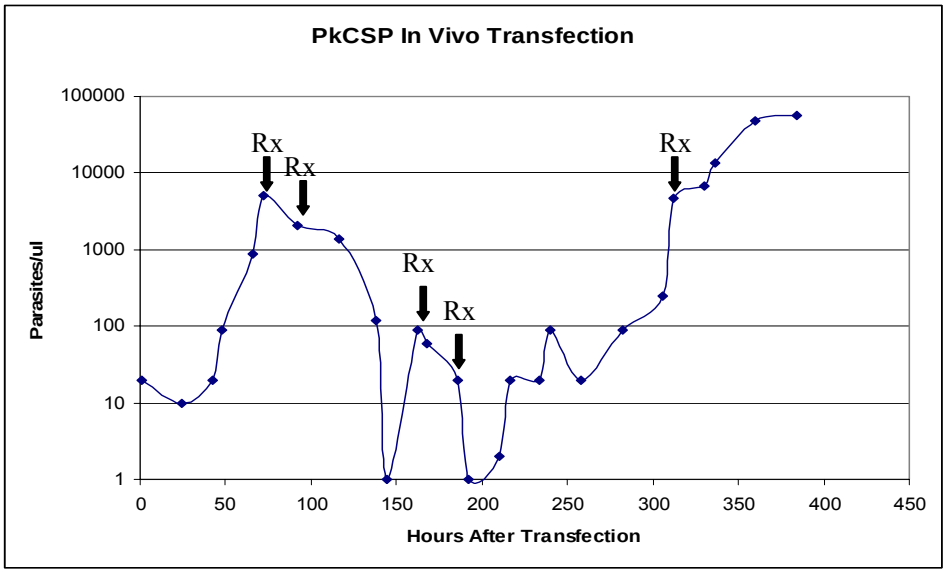

Figure A.2. PkCSP control *in vivo* transfection/knock-out experimentation.

Circumsporozoite protein (CSP) is a pre-erythrocytic antigen whose expression has previously been determined to be unnecessary for propagation of the blood stage. Control transfection experiments were performed to disrupt the expression of this antigen. Pyrimethamine was administered at the time points indicated (Rx) to select for parasites that had incorporated the transgene. As expected, parasitemia continued to increase in the control experiments even after pyrimethamine was administered at several time points.

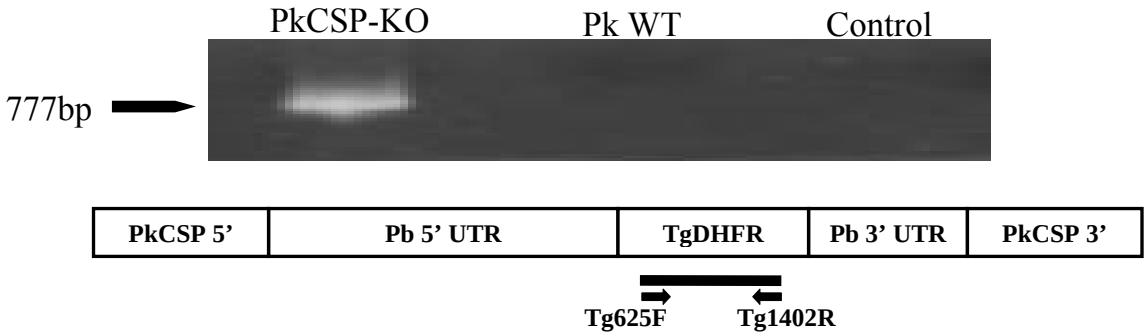

Figure A.3. PCR amplification of transfected *P. knowlesi* parasites.

Two primers (Tg625F: 5' GTGTTCGTTCCCTTTTGTCCG 3' and Tg1402R: 5' CTGTTGGATTCGTTCTCAGCATC 3') that bind within the TgDHFR cassette were used to amplify this selectable marker using standard PCR conditions. DNA was extracted from transfected *P. knowlesi* parasites (PkCSP-KO) and wild-type *P. knowlesi* parasites (PkWT). DNA extraction was performed using the QiAMP DNA blood mini kit (Qiagen). As a control, no DNA was added to the last sample (Control) to ensure specific amplification of the parasite DNA. The primers are 777 base pair apart. PCR amplification only occurs with PkCSP-KO DNA and demonstrates that TgDHFR is present only in the transfected parasites.

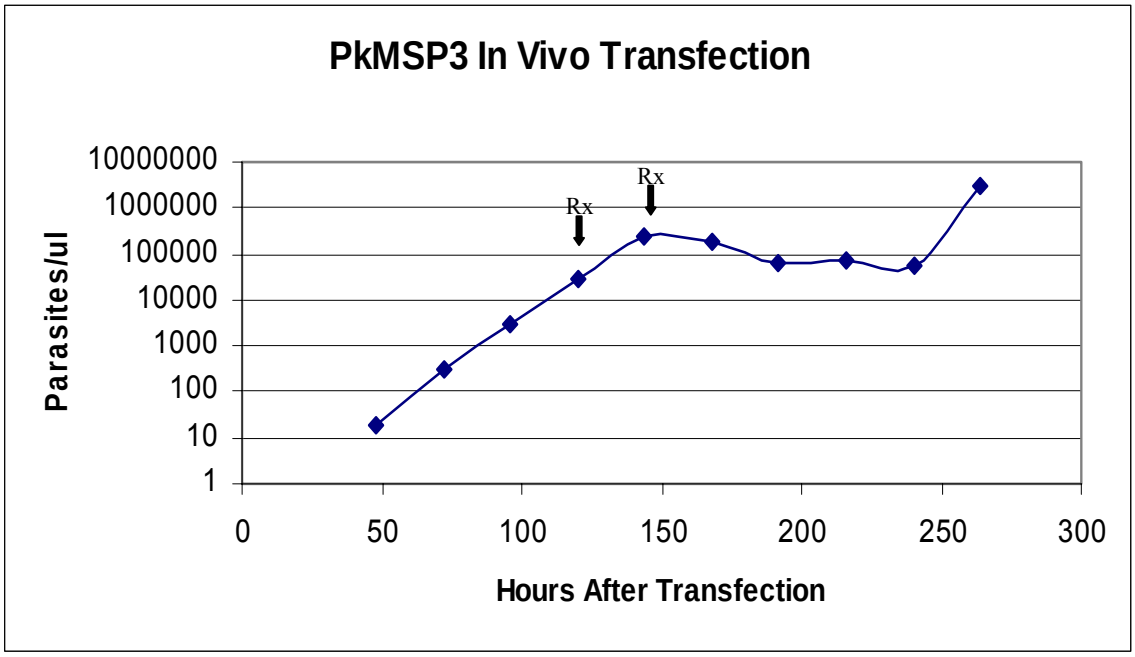

Figure A.4. PkMSP3 control *in vivo* transfection/knock-out experimentation. Merozoite surface protein 3 is a blood-stage antigen whose expression has previously been determined to be unnecessary for propagation of the blood stage. Control transfection experiments were performed against this antigen. Pyrimethamine was administered at the time points indicated (Rx) to select for parasites that had incorporated the transgene. As expected, parasitemia continued to increase after pyrimethamine was administered.

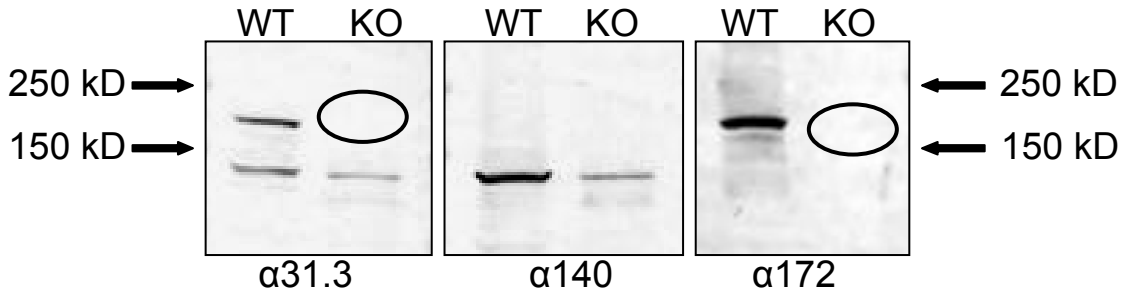

Figure A.5. PkMSP3 protein is absent in transfected parasite samples.

There are two PkMSP3 proteins expressed in *P. knowlesi* that are either 172 kD or 140 kD in size. The transfections conducted were against the 172 kD protein. Western blot analysis was performed using mature blood-stage wild-type *P. knowlesi* parasites (WT) and mature blood-stage transfected *P. knowlesi* parasites (KO). Samples were probed with three different antibodies, the anti-31.3 antibody recognizes the common C-terminus of both the 172 kD and 140 kD proteins, the anti-140 antibody specifically recognizes the 140-kD protein, and the anti-172 antibody specifically recognizes the 172-kD protein. PkMSP3 172 kD protein is absent in transfected parasites.

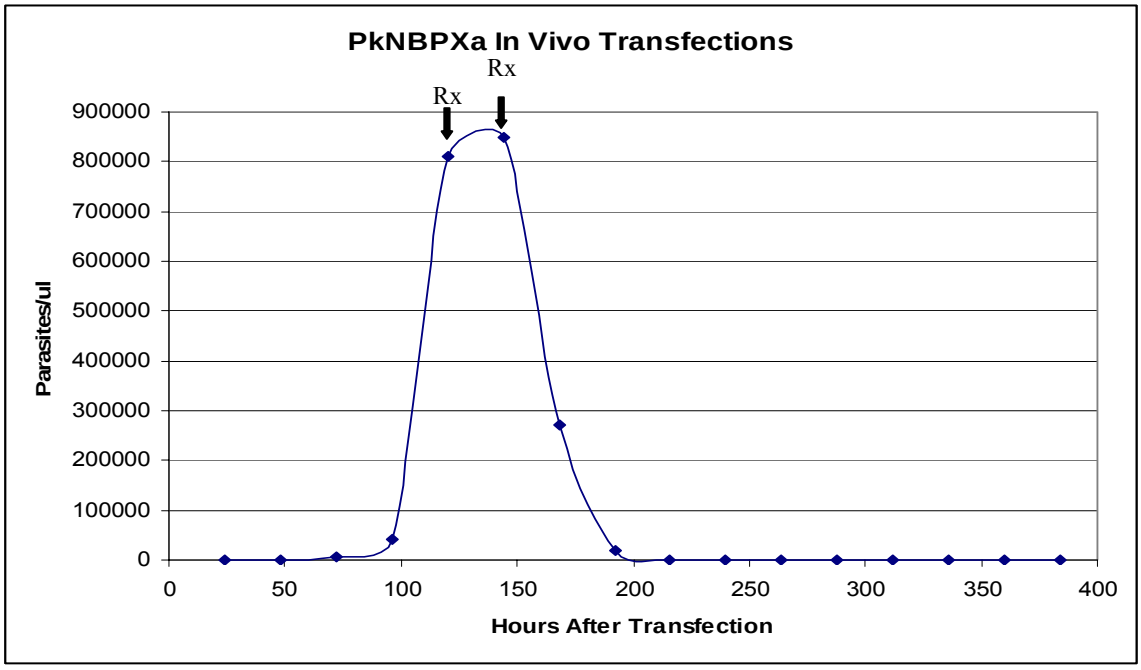

Figure A.6. PkNBPXa *in vivo* transfection/knock-out experimentation.

Pyrimethamine was administered at the time points indicated (Rx) to select for parasites that had incorporated the PkNBPXa transgene. The parasitemia was monitored for approximately one week after pyrimethamine adminstration and unlike for the control transfections, the parasitemia never increased after administration of pyrimethamine. The PkNBPXa protein appears to be necessary for merozoite invasion of erythrocytes and subsequent propagation of the blood-stage of the life cycle.

Table A.2. Percent Inhibition of Invasion compared to pre-immune rabbit IgG

Target Region	Untreated	Chymotrypsin	Trypsin	Neuraminidase
PkNBPXb-II	11%	40%	10%	38%
PkNBPXb-C	28%	21%	19%	36%

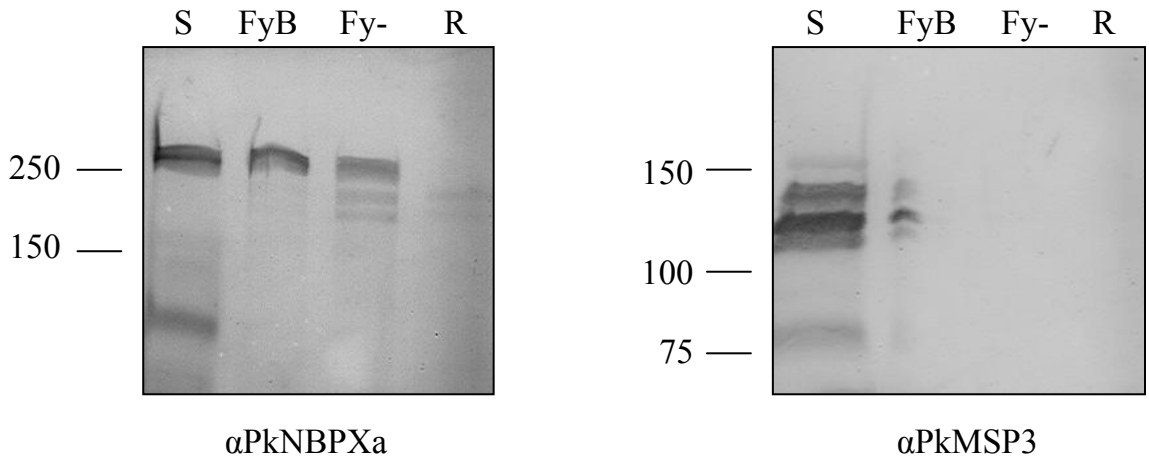

Figure A.7. PkNBPXa binds to Duffy negative and Duffy positive human erythrocytes.

Culture supernatants (S) containing soluble merozoite proteins were electrophoresed and transferred to nitrocellulose membranes. Protein samples eluted from human Duffy positive (FyB) and Duffy negative (Fy-) erythrocytes after incubation with supernatants were also electrophoresed and transferred to nitrocellulose membranes. A PkNBPXa specific antibody was used to probe the membranes and PkNBPXa was detected in the supernatants and eluates from Duffy positive and Duffy negative erythrocytes. As a control, erythrocytes were incubated in the absence of supernatants (R) and no PkNBPXa specific bands were detected in those samples. A PkMSP3 specific antibody was also used to probe the same samples (S, FyB, Fy-, R) as this protein is known to be present in supernatants but does not bind to erythrocytes. PkMSP3 was only found in the supernatants (the bands in the FyB sample are from spillover from the supernatant samples). The binding of PkNBPXa is specific to the Duffy positive and Duffy negative erythrocytes.

Table A.3. Primer Sequence for regions cloned into pDisplay vector

Gene	Region	Primer Sequence
pknbpxa	I (pDisplay)	Sense: 5'-ccccagatctGCAAGCTGGGAACAAGTT-3 Antisense: 5'-ccccgtcgacCTCAATAATACTAACAATGCT-3'
	II (pDisplay)	Sense: 5'-ccccagatctTACACGTGCGAAGAAATTG-3' Antisense: 5'-ccccgtcgacCCCTTCGTAACTTTGTATAG-3'
	III (pDisplay)	Sense: 5'-ccccagatctAGCATTGTTAGTATTATTGAG-3' Antisense: 5'-ccccgtcgacATCTGTATAGTTGCCCATATT-3'

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