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**OPTIMIZATION OF THE CONDITIONS NECESSARY TO SHOW
BINDING OF THE *PLASMODIUM YOELII* RHOP-3 RHOPTRY
PROTEIN TO MOUSE ERYTHROCYTES**

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**OPTIMIZATION OF THE CONDITIONS NECESSARY TO SHOW BINDING
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ABSTRACT

The *Plasmodium* Rhop-3 rhoptry protein is an erythrocyte binding protein that is secreted into the RBC membrane during merozoite invasion. Anti-Rhop-3 antibodies inhibit merozoite RBC invasion. The C-terminus of the Rhop-3 protein is highly conserved among *Plasmodium* species and antisera from endemic areas reacts with recombinant C-terminus of Rhop-3. The binding domain of the Rhop-3 protein is hypothesized to be within the C-terminal region of the protein.

In the present study I investigated the conditions necessary for binding of the Rhop-3 protein to RBC by expressing recombinant proteins made from partial fragments of the Rhop-3 gene using the vector pDisplayTM. Recombinants were constructed, purified and used to transfect mammalian COS-7 cells. Surface expression of the proteins was detected by immunofluorescence assay (IFA) using Rhop-3 specific antibodies. A rosetting assay was used to determine whether uninfected mouse red blood cells would bind to COS-7 cells expressing Rhop-3 recombinant proteins on the surface.

The pDisplayTM vector was used to express three *Plasmodium falciparum* Rhop-3 recombinants pDIS-PF17, pDIS-PF13, pDIS-PF7 and one *P. yoelii* Rhop-3 recombinant pDIS-PY1412 in COS-7 cells. Surface expression of recombinant Rhop-3 on COS-7 cells was identified using three mouse antibodies (MAb) F1, MAb FL1+FL2, MAb T1

and rabbit antibody # 686. Expression of the recombinant Rhop-3 recombinant pDIS-PY1412 remained consistent.

Binding the recombinant Rhop-3 recombinant pDIS-PY1412 to mouse RBC was obtained once but this binding was not consistent.

The conditions used for the transfection and binding assays were modified to see if consistent binding with pDIS-PY1412 could be maintained. This is the first time the pDisplayTM vector has been used to study *Plasmodium yoelii* erythrocyte binding proteins. Expression of the PvDBP_{II} control remained consistent and binding to human duffy positive RBC was also consistent. Optimizing the conditions for binding of pDIS-PY1412 to mouse RBC would be an essential tool to screen other *Plasmodium yoelii* RBC binding proteins.

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LIST OF ABBREVIATIONS

ABBREVIATIONS

| | |
|---------|---|
| AMA-1 | Apical Membrane Antigen |
| ATCC | American Type Culture Collection |
| CLAG | Cytoadherence-linked Asexual Gene |
| CPD | Citrate Phosphate Dextrose Buffer |
| COS-7 | African green monkey kidney fibroblasts |
| DEPC | Diethylpyrocarbonate |
| DHFR | Dihydrofolate Reductase |
| DHPS | Dihydropteroate synthase |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DNA | Deoxyribonucleic Acid |
| DOC | Sodium Deoxycholate |
| DBP | Duffy Binding Protein |
| EBA-175 | Erythrocyte Binding Antigen |
| EDTA | Ethylenediaminetetraacetic Acid |
| HCL | Hydrochloride |
| HRP | Histidine-rich Protein |
| ICT | Immunochromatographic Test |
| IEM | Immunoelectron Microscopy |
| FBS | Fetal Bovine Serum |
| MSP1-10 | Merozoite Surface Protein |

| | |
|------------------|---|
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| NP40 | Nonidet P40 |
| PABA | p-aminobenzoic acid |
| PCR | Polymerase Chain Reaction |
| PV | Parasitophorous Vacuole |
| PVM | Parasitophorous Vacuole Membrane |
| RhopH-1 | High-molecular-weight Rhoptry Complex |
| RhopH-2 | High-molecular-weight Rhoptry Complex |
| RhopH-3 / Rhop-3 | High-molecular-weight Rhoptry Complex |
| RIA | Radioimmunoassay |
| pLDH | Parasite-specific Lactate Dehydrogenase |
| RESA | Ring Infected Surface Antigen |
| RIMA | Ring Membrane Antigen |
| RNA | Ribonucleic Acid |
| RNAzol B | RNA Isolation Reagent |
| RPMI 1640 | Roswell Park Memorial Institute |
| SDS | Sodium Dodecyl Sulfate |
| SW | Swiss Webster |
| TES | Tris-HCL /EDTA /SDS |
| Tris | Trishydroxymethylaminomethane |

CHAPTER I

INTRODUCTION

1.1 Epidemiology

Malaria is a disease affecting 300-500 million people annually resulting in 2-3 million deaths per year [Ashley et al. 2006]. Pregnant women and children have an increased susceptibility to malaria [Ashley et al. 2006] with mortality predominant in children. According to the United States Centers for Disease Control and Prevention an estimated 75,000-200,000 infants die from malaria associated infections during pregnancy.

Malaria is prevalent in areas of sub-Saharan Africa with nearly 30% of the mortality rate being attributed to malaria [Snow et al. 2001]. Malaria is also transmitted in large areas of Central and South America, the island of Hispaniola, Asia (including areas of India, Indonesia, Iran, Iraq, Sri Lanka, Philippines, Pakistan), Eastern Europe and the South Pacific [Leder et al. 2004] [Fig 1]. Malaria is caused by protozoans of *Plasmodium* spp. There are four *Plasmodium* spp. which infect humans, *P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae*. *Plasmodium falciparum* is the most lethal of the *Plasmodium* sp. in humans. Malaria continues to be a major

public health problem in Africa, Asia and Latin America due to increased resistance of the malaria parasites to antimalarial drugs such as chloroquine and primaquine.

The distribution of *Plasmodium* species varies among the different countries.

Plasmodium falciparum is the predominant species in malaria endemic regions giving rise to the drug resistant strains [Gaye et al.1998]. Climatic factors influence the distribution of malaria. Areas with increased rainfall provide an environmental condition, which promotes mosquito breeding [Grover-Kopec et al. 2006]. Regions with increased temperatures are also important in regulation of the development of mosquito larvae and adult mosquito survival. The parasite multiplies faster at higher temperatures and the mosquitoes also develop faster at these high temperatures [Grover-Kopec et al. 2006].

Plasmodium falciparum accounts for about 80% of malaria cases in Sub-Saharan Africa [Lim et al. 2005]. *Plasmodium vivax* is the most common of the malaria species responsible for 70-80 million cases of malaria annually worldwide [Mendis et al. 2001]. *Plasmodium vivax* is predominantly found in the Middle East, Asia and the Western Pacific but is not as common in the sub-Saharan Africa. *Plasmodium vivax* represents approximately 10 % of the malaria cases in Africa and over half of the malaria cases worldwide [Mendis et al. 2001]. *Plasmodium malariae* is common in many regions of tropical Africa, Sri Lanka, New Guinea, Malaya, Europe and also regions of South America [Schmidt et al. 2000]. *Plasmodium ovale* is the rarest of the human malarial parasites. *Plasmodium ovale* is found mainly in the tropics most commonly on the West Coast of Africa, India, New Guinea, Vietnam and the Philippines [Schmidt et al. 2000].

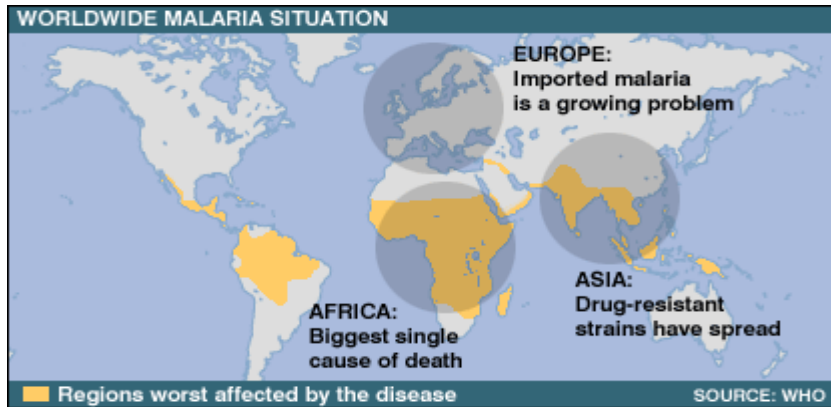


Fig1. Malaria endemic regions of the world

[http://newsimg.bbc.co.uk/media/images/39715000/gif/_39715741_world_malaria_map416.gif]

Other *Plasmodium* species also infect other animals such as birds, reptiles and rodents (Table1). The genus *Plasmodium* is divided into thirteen subgenera, three of which occurs in mammals, four in birds and two in lizards [Garnham 1966]. Malaria parasites belonging to the subgenera *Vinckeia* and *Laverania* occur exclusively in mammals. *Plasmodium falciparum* belongs to the subgenera *Laverania* which occurs in primates. Malaria parasites affecting rodents belong to the subgenera *Vinckeia*. The avian malarial parasites belong to the subgenera *Haemoamoeba*, *Giovannolaia*, *Novyella* and *Huffia* [Garnham 1966]. The pathology in birds is associated with exoerythrocytic schizogony in the capillary endothelium of different organs especially in the brain [Garnham 1966]. Parasite growth in the endothelium results in blockage of vessels and results in birds dying of ‘cerebral malaria’. The parasite can also attack the bone marrow causing the bird to die of aplastic anemia [Garnham 1966]. Whereas, in primates the tissue stages of infection are not harmful. In birds schizonts can also be released in the internal organs such as the heart muscle leading to death. The level of infectivity in birds is influenced by the different subgenera to

which the parasite belongs. Avian malaria has a wider host range compared to mammalian malaria and is also found in more countries of the world [Garnham 1966]. The fact that birds have the ability to migrate from one region to another has made it possible for avian malaria to be found in every continent of the world [Garnham 1966]. The level of infectivity in birds depends on the species of the bird, age and the environmental conditions (*season*).

Malaria parasites infecting reptiles belong to two subgenera *Sauramoeba* and *Carinia*. Of the reptilian malaria parasites known about nine species of *Plasmodium* are found exclusively in America, nine species in Africa, two species in the East Indies and Pacific Islands and two species restricted to Australia. Malaria parasites have been identified in the blood of lizards and snakes [Garnham 1966].

Table 1. Other *Plasmodium* species and their definitive hosts [Garnham 1966]

| Host | <i>Plasmodium</i> species |
|----------------------------|---------------------------|
| <i>Bird Host:</i> | |
| house sparrow | <i>P. biguet</i> |
| blackbird | <i>P. giovannolai</i> |
| wild turkey | <i>P. griffithsi</i> |
| pigeon and doves | <i>P. matutinum</i> |
| Reptile host: | |
| fence lizard | <i>P. chiricahuae</i> |
| chameleon | <i>P. gologoense</i> |
| snakes | <i>P. tomodoni</i> |
| snakes | <i>P. wenyoni</i> |
| <u>Rodent Host:</u> | |
| grass rat | <i>P. aegyptensis</i> |
| mice | <i>P. berghei</i> |
| mice | <i>P. chabaudi</i> |
| mice | <i>P. yoelii</i> |

1.2 Life Cycle

The life cycle of *Plasmodium* spp. [Fig 2] requires two hosts: an invertebrate (mosquito) and a vertebrate (mammal, reptiles and also birds). Malaria parasites are transmitted by the female Anopheles mosquito. There are about 380 species of Anopheline mosquitoes but only 60 are able to transmit the parasite [Schmidt et al. 2000].

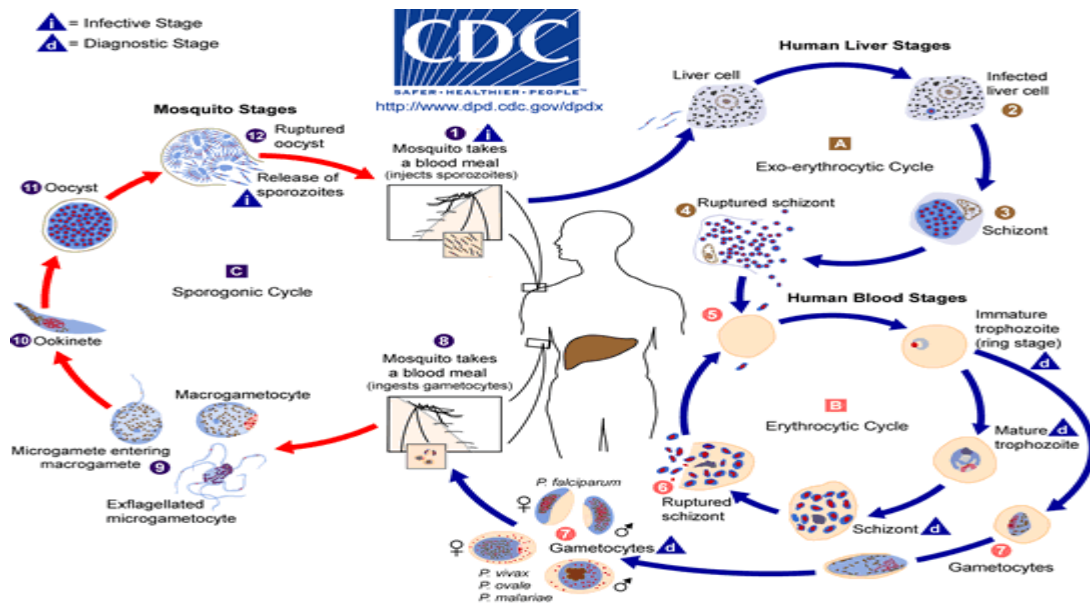


Fig 2. Malaria life cycle in mosquito and a human host
[http://whyfiles.org/shorties/201malaria/images/life_cycle.gif]

1.2.1 Mosquito stage development

Sexual reproduction takes place within a definitive host, the mosquito. When a mosquito takes a blood meal both microgametocytes (male) and macrogametocytes (female) are ingested along with saliva. The macrogametocyte matures into a macrogamete while the microgametocyte undergoes exflagellation. An increase in pH due to the presence of dissolved carbon dioxide in blood stimulates this

transformation [Schmidt et al. 2000]. The microgametocyte matures into a microgamete, which further penetrates a macrogamete fertilizing it to form zygotes. These zygotes become elongate and motile and are known as ookinetes. The ookinetes penetrate the mosquito's stomach wall and migrate to the hemocoel side of the gut where they develop into oocysts. Mature oocysts rupture to release sporozoites that migrate throughout the mosquito's body. Sporozoites migrate to the mosquito's salivary glands and become infective. Depending on the *Plasmodium* spp. sporozoite development takes place from 10 days to two weeks [Schmidt et al. 2000].

1.2.2 Exo-erythrocytic stage development

The parasite enters a host when an infected mosquito takes a blood meal. During this process sporozoites are injected in the blood stream of the host. Inside the blood, circulating sporozoites disappear within a few minutes invade hepatocytes [Shin et. al. 1982]. Sporozoites are targeted to the liver through specific ligand-receptor interactions. Within the hepatocytes the parasites undergoes asexual reproduction to produce mature schizonts. Sporozoites transform into trophozoites that feed on the host cell cytoplasm. Depending on the species the time varies for trophozoites to mature and begin schizogony. The parasite transforms into a schizont following the formation of many daughter nuclei (merozoites). Merozoites are released from the liver into the blood to initiate the erythrocytic cycle. Time taken for completion of the exo-erythrocytic stage depends on the *Plasmodium* spp.: 5 ½ to 6 days for *P. falciparum*, 8 days for *P. vivax*, 9 days for *P. ovale* and 13 days for *P. malariae* [Schmidt et al. 2000]. In some species such as *Plasmodium ovale* and *P. vivax*, sporozoites may become dormant cells and remain dormant in the liver stage

becoming reactivated (relapse) causing malaria several months or years after the mosquito bite. These dormant cells are called hypnozoites [Schmidt et al. 2000].

1.2.3 Erythrocytic stage development

Merozoites released from the liver penetrate the red blood cells where they undergo further asexual development. Inside the erythrocytes, merozoites transform into trophozoites that feed on the cytoplasm of the host cell to produce a large food vacuole [Fig 3]. At this stage the parasite appears as a ring within the erythrocyte with a visible nucleus to one end. As the parasite grows and feeds on the host cytoplasm the food vacuoles disappear and pigment granules called hemozoin become visible in the vacuoles. The parasite further develops into early schizont then mature schizonts. Mature schizonts contain numerous erythrocytic merozoites that are released into the blood when the schizont ruptures. Released merozoites continue the cycle and invade uninfected red blood cells. Rupture of the host cell releases parasite metabolic wastes and hemozoin into the blood, a stage that is associated with symptoms of the disease. Red cells infected with the malaria parasite undergo hemolysis. Active Kupffer cells of the liver ingest infected red blood cells killing the malaria parasites [Murthi et al. 2006]. Some merozoites invade red blood cell also develop into schizonts others invade and develop into gametocytes. These gametocytes are then ingested by a susceptible mosquito when it takes a blood meal.

1.2.4 Red blood cell invasion

Organelles of the apical complex (microneme, rhoptries and dense granule) are involved in host cell invasion, formation and maintenance of the parasitophorous

vacuole. Host cell invasion involves initial recognition and attachment to the host cell by surface components of the merozoites [Sam-Yellowe 1996]. The merozoite first interacts with the surface of the red blood cells and re-orientates the apical end of the complex towards the membrane. The contents of the apical organelles are released and a moving junction is formed between the merozoite and the red blood cell membrane. The parasite enters the vacuole by invagination of the red blood cell membrane around the parasite forming the parasitophorous vacuole (PV). The invasion and invagination of the red blood cells is facilitated by discharge of contents from the rhoptry and microneme. Following parasite entry into the host cell the red blood cell membrane is resealed [Aikawa et al. 1978].

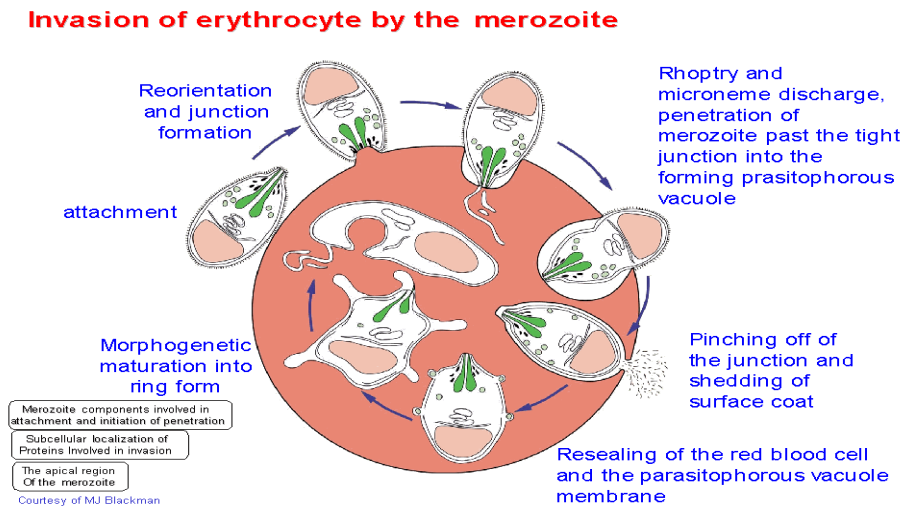


Fig 3. Erythrocyte invasion

[http://www.nimr.mrc.ac.uk/parasitol/blackman/images/invsion_full.gif]

1.3 Types and Prevalence of Malaria

Plasmodium sp. infects humans, birds, rodents, primates and reptiles. The different *Plasmodium* sp. cause different types of malaria. *Plasmodium falciparum* causes malignant tertian malaria. Malignant tertian is a very severe form of fever. A very high fever (hyperpyrexia) develops and the body temperature may continue to increase until eventually death may occur.

Plasmodium falciparum accounts for approximately 50% of all deaths from malaria in tropical zones of the world [Schmidt et al. 2000]. Signs associated with malignant tertian malaria are: darkening of the gray matter of the brain and abundant pigment in other tissues of the body as seen in patients who succumb to the disease [Schmidt et al.2000]. *Plasmodium falciparum* requires high temperatures for optimal development and is almost exclusively predominant in areas of sub-Saharan Africa and South East Asia. *Plasmodium vivax* causes benign tertian malaria and accounts for more than 50% of all malaria cases in the world [Mendis et al. 2001]. Tertian malaria recurs every 48 hours in infected patients [Schmidt et al. 2000]. Symptoms of tertian malaria are myalgia, headache, diarrhea, nausea, vomiting and a cough [Oh et al. 2001]. *Plasmodium vivax* is prevalent in temperate regions of the world.

Plasmodium vivax merozoites are unable to penetrate mature red blood cells and invade only young erythrocytes known as reticulocytes. *Plasmodium vivax* merozoites penetrate only erythrocytes containing the Duffy antigen which serves as the receptor *P. vivax* merozoites [Schmidt et al. 2000]. Quartan malaria caused by *Plasmodium malariae* and *P. ovale* and tertian malaria caused by *P. ovale* are the two less common types of malaria. Quartan malaria causes a fever with paroxysms that

recur every 72 hours or every fourth day due to the schizogony and release of merozoites from infected cells, with invasion of new erythrocytes by *Plasmodium malariae* [Schmidt et al 2000].

1.4 Symptoms and Pathogenesis

Early symptoms of malaria resemble the onset of a flu followed by periods of chills. These symptoms are mainly due to the host inflammatory response to the parasite. Destruction of the red blood cells gives rise to anemia and jaundice. Rupture of the infected red blood cells to release merozoites is correlated with the increase in body temperature giving rise to a fever. Body temperature rises rapidly fluctuating between this high temperature and normal body temperature within two to three hours. The time period for the stages of benign tertian is between 8 to 12 hours. The time period for stages in quartan malaria is a recurrence every 72 hours. As the disease progresses patients may feel symptoms such as headaches, body aches, sweating, malaise, nausea and vomiting. Specific symptoms are also associated with the different species. Complications associated with malaria vary according to the infecting species.

A *Plasmodium falciparum* infection can lead to severe anemia due to the destruction of both parasitized and nonparasitized erythrocytes, circulation of iron bound hemozoin and bone marrow suppression. Cerebral malaria is a one of the common complications of a *Plasmodium falciparum* infection. The progression of cerebral malaria involves the sequestration of *Plasmodium falciparum* infected red blood cells to the blood brain barrier endothelium [Tripathi et al 2007]. Adherence of RBCs involves the knob proteins and direct interaction with the endothelial cell

surface [Robert et al. 1996]. Cerebral malaria accounts for 10% of *Plasmodium falciparum* cases and if left untreated results in death. Cerebral malaria is a disease of the brain and is commonly accompanied by an extremely high temperature above 108°F and psychotic symptoms. The disease is accompanied by generalized convulsions and a persistent coma. Mortality due to cerebral malaria is between 15-20% [Adhikari 2002].

Fluid accumulation in the lungs (pulmonary edema) is also associated with *Plasmodium falciparum* infection. Symptoms include difficulty breathing, coughing up blood, excessive sweating, anxiety and pale skin. This is often due to an over administration of intravenous fluids that can lead to a coma or death within a few hours [Schmidt et al. 2000]. Algid malaria occurs in 0.37% of malaria cases [Popov 2005] and is characterized by a rapid development of shock. Blackwater fever is another condition associated with a *Plasmodium falciparum* infection. This is characterized by kidney failure, hemolysis and hemoglobinuria [Schmidt et al. 2000]. Kidney failure can result in a coma or eventually death. Lysis of erythrocytes releases hemoglobin and its breakdown products into the blood. Free hemoglobin destroys the glomerulus in the kidneys and leaks into the urine [Schmidt et al. 2000]. The result is a dark discoloration in urine due to the presence of hemoglobin.

1.5 Diagnosis

The diagnosis of malaria is confirmed by identifying the parasite in the blood of a patient. Accurate detection and treatment of malaria requires methods of rapid diagnosis. Direct visualization of a well-prepared thick or thin stained blood smear

using a microscope is one method of diagnosing the disease. Two types of blood smears can be prepared, thick or thin smears. Thick smears are used to identify the parasite in the blood when parasitemia is low. The thin smear is used to identify the specific *Plasmodium* species. *Plasmodium falciparum* can be detected by two fast and simple immunochromatographic tests.

Where microscopes are not available malaria antigens can be detected using various kits to do a rapid diagnostic test (RDTs). These tests are quick and results are obtainable in minutes. These tests are based on the dipstick principle [Iqbal et al. 2004]. These tests use specific antibodies bound to a membrane to detect circulating parasite antigens (Gaye et al. 1998). The immunochromatographic test (ICT Malaria P.f/P. v) detects a *Plasmodium falciparum* histidine-rich protein (HRP-2) antigen. OptiMAL detects a parasite- specific lactate dehydrogenase (pLDH) [Gaye et al. 1998]. Presence of the parasite in human host can also be detected by molecular diagnosis by means of polymerase chain reaction (PCR) using genus and species specific primers. Serological diagnosis can also be used to detect malaria antibodies in individuals who have previously had *Plasmodium* infections. *Plasmodium* sp. schizonts are used as the antigens to detect for malaria-specific antibodies in a patients serum [CDC] by indirect immunofluoresence assay (IFA).

None of these newer methods has had as much success as the standard peripheral blood smear study. Various stains are used in identifying the parasite and the species. Common stains used are Romanowsky [Lillie 1978], Giemsa [Shute et al.1963] Lieshman's [Chatterjee 1980]. Smears are often prepared by finger prick, ear lobe stab or venipuncture. Malaria pigments can also be identified in circulating phagocytic

leucocytes in cases of partially treated infections and in bone marrow aspirates [Sheikh et al. 2003].

1.6 Treatment

The type of treatment and drug used depends on the parasite species causing the infection, clinical status of the host (age, sex, body type, immune status, symptoms, and associated health conditions), geographical area of the malaria diagnosis [CDC]. Chloroquine is used to treat *P. falciparum* sensitive strains of malaria and primaquine is used effectively against all strains of all species. Current suppressive treatment involves the use of chloroquine to suppress the erythrocytic stage of the parasite during development. Chloroquine is effective in destroying *P. vivax* gametocytes. In areas endemic with malaria all cases of fever are treated as malaria cases with doses of chloroquine being administered to a patient. Chloroquine is however ineffective in destroying *P. falciparum* gametocytes and as a result does not prevent its spread.

In areas endemic with *P. falciparum* a single dose of both primaquine and chloroquine is usually administered. The constant recurrence of the disease is one of the main problems of controlling the disease. Recurrence is often due to incomplete treatments, re-infection, absence of effective immune responses and relapse of the disease as in the case of *P. ovale* and *P. malariae* where dormant hypnozoites can be re-activated. *Plasmodium vivax* can remain in the blood for months to years and cause recurrence of the disease. *Plasmodium vivax* or *P. falciparum* infection can recur within 8-10 days after the initial infection. *P. vivax* and *P. ovale* are known for causing recurrence of malaria [Omonuwa et al.2002].

Malaria recurrence of more than 12 months is less likely to be *Plasmodium*

falciparum [Omonuwa et al. 2002]. *Plasmodium falciparum* recurrence is caused by reinfection and recrudescence. Cases of recrudescence can be due to incomplete or inadequate treatment, antigenic variation or multiple infections by different strains [Omonuwa et al.2002]. Another major factor affecting treatment is the increase in the resistance of parasites to the antimalarial drugs. Drug resistance has been increasing over the years and also continues to spread across endemic regions [Tilley et al. 2006].

1.7 Drug resistance

Plasmodium falciparum has developed resistance to nearly all the antimalarial drugs currently available. *Plasmodium vivax* infection is normally treated with a dose of 25 mg salt/kg of chloroquine over 36-48 hours + primaquine for 14 days. *Plasmodium falciparum* infection is treated by administering chloroquine or pyrimethamine with sulphadoxine /quinine/mefloquine/artemisinin and/or combinations as suppressive therapy. Primaquine is administered as a gametocytocidal in a single dose. Specific treatment depends on the sensitivity of the patient and the severity of the infection.

A mixed *Plasmodium vivax*, *P. falciparum* infection is treated by administering chloroquine or related drugs. Administering chloroquine+Primaquine in a single dose treats chloroquine sensitive strains of *Plasmodium falciparum*. Within 48 hours after drug administration the number of parasites should be reduced by 75%. Treatment for chloroquine resistant strains requires different combinations of various drugs. The following drugs are usually administered, in tablet form: Quinine + Pyrimethamine/ Sulfa, Quinine + Tetracycline /doxycycline, Artesunate + Mefloquine; Mefloquine +

Pyrimethamine/Suipha. Drugs used to treat malaria target different stages of the parasite life cycle. Combinations of some of the named drugs can also be administered in the form of injections. Table 2 shows the classes of antimalarial drugs and the mode of action of the different types of drugs [Olliaro 2001].

Table 2. Modes of action and resistance of the main classes of antimalarial drugs [Olliaro 2001].

| Pathway | Drug Class | Target | Mechanisms of Action | Mechanisms of Resistance |
|-------------------------|--|--|--|---|
| Nucleic acid Metabolism | Type-1 antifolates; Sulfonamides, Sulfones | Formation of dihydropteroste from PABA+ pteridine catalysed by DHPS | Mimic PABA; Compete for active site of DHPS | Mutations at the binding site |
| | Type-2 antifolates: Pyrimethamine, biguanides (proguanil, cycloguanil), trimethoprim | Reduction of di- to tetra- hydrofolate (cofactor for the biosynthesis of thymidylate, purine nucleotides, aminoacids) by DHFR using NAPDH as cofactor. | Mimic dihydrofolate: compete for active site of DHFR | Mutations at binding site |
| | Naphthoquinones (atovaquone) | Mitochondrial functions (electron transport chain), blockade of pyrimidine synthesis | Inhibits DHODase; mimics ubiquinone: competes for complex III | Mutations at Coenzyme Q binding site |
| Heam detoxification | Type-1 (4-amino-quinolines) | Heam crystallization | Inhibition / termination of β -haematin formation | Multi-gene; altered accumulation at FV (reduced influx or increased efflux) |
| | Type-2 quinolines (aryl-amino alcohols) | Same as Type-1 | Same as Type-1 | Same as Type-1 |
| Oxidative Stress? | Artemisinin-type compounds | Alkylation of unidentified target? Hydroxylation | Free radical formation through activation of the peroxide by binding with Fe(II)PPIX | Unknown |

1.8 Phylum Apicomplexa

Plasmodium belongs to the phylum Apicomplexa. All members of this phylum are obligate intracellular parasites [Sam-Yellowe 1996]. Other members of the phylum include: *Toxoplasma*, which is transmitted by eating contaminated food, and can result in gastrointestinal diseases, *Babesia* another protozoan causes babesiosis and is transmitted to people by the bite from an infected tick. Within the phylum are numerous parasites known to be involved in macrophage, lymphocyte, endothelial cell and erythrocyte invasion [Sam-Yellowe 1996]. Members of Apicomplexa possess specialized secretory organelles termed apical organelles [Sam-Yellowe 1996]. Apical organelles are found at the anterior end of the invasive stage of the parasite [Fig 4]. Three of these organelles; the rhoptries, micronemes and dense granules play a role in host cell invasion and modification of the host cells during infection [Sam-Yellowe 1996]. Within the apical complex are two to eight rhoptries, micronemes and several dense granules.

1.9 The Apical Complex organelle

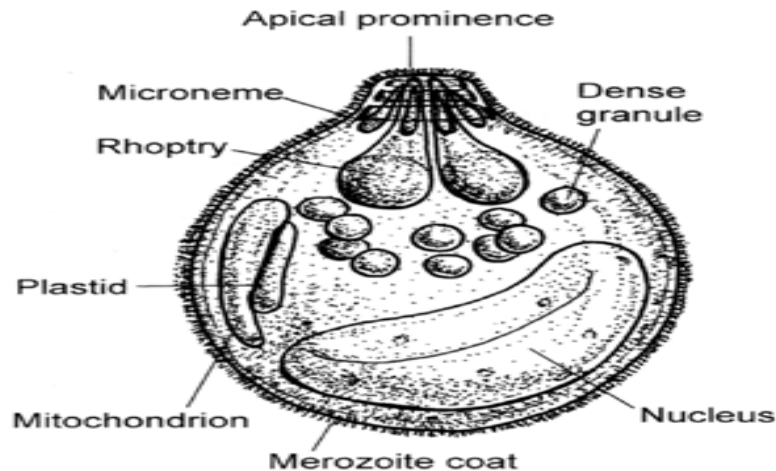


Fig 4. Diagram of the Apical Complex

[http://www.impact-malaria.com/FR/EPS/Formations_et_cours_internationaux/Formation_de_la_Liverpool_School_LSTMH/cours_liverpool/malaria_template_fr/Section_1/images/merozoite.gif]

The rhoptries are membrane-bounded organelles and are thought to be involved in erythrocyte attachment, invasion and formation of the parasitophorous vacuole membrane (PVM). The PV is described as a membrane-bound sack or vacuole, which is seen upon invasion of the erythrocytes. Erythrocytic stages of the parasite reside and develop within the parasitophorous vacuole [Aikawa et al. 1978]. The parasite's surface is separated from the host cell cytoplasm by the PVM which acts as an interface at which host parasite interactions take place [Lingelbach et al 1997].

1.10 Microneme proteins

The micronemes like the rhoptries are membrane bound and consist of many types of proteins [Blackman et al. 2001]. Erythrocyte membrane glycoproteins of the Duffy binding protein family act as receptors for *P. knowlesi* and *P. vivax* merozoites [Miller et al. 1975, Miller et al. 1976]. *Plasmodium knowlesi* and *P. vivax* merozoites do not invade erythrocytes lacking the Duffy glycoprotein [Wertheimer et al. 1989, Haynes et al. 1988]. A 175-kDa protein ligand has been identified in *P. falciparum* known as erythrocyte binding antigen EBA-175. EBA-175 binds to erythrocytes [Orlandi et al. 1990] and may be involved in junction formation [Jakobsen et al. 1998]. At least ten micronemal proteins have also been identified in *Eimeria* and *Toxoplasma* [Blackman et al. 2001]

1.11 Dense granule proteins

The dense granules are also membrane-bounded vesicles that release their granular contents by exocytosis into the parasitophorous vacuole (PV). A number of dense granule proteins have been isolated from different *Plasmodium* species. Four dense granule proteins have been identified in *P. falciparum*: ring infected surface antigen (RESA), ring membrane antigen (RIMA), two serine proteases, PfSUB- 1 and PfSUB-2 [Blackman et al. 2001]. A number of other proteins have been localized in the dense granules of *Toxoplasma* tachyzoites (GRA1-GRA8). The dense granule proteins localized all have a form of association with the PV [Blackman et al. 2001].

1.12 Erythrocyte binding proteins and their identified binding domains

Several erythrocyte binding proteins from the merozoite surface and the apical complex have been characterized. Erythrocyte-binding antigen (EBA-175) [Sim et al. 1998], shown to mediate invasion of red cells [Preiser et al. 1999], apical membrane antigen AMA-1 [Fraser et al. 2001], merozoite surface protein MSP-1 [Perkins and Rocco 1998], Duffy binding protein (DBP) [Xainli et al. 2003], RhopH [Sam-Yellowe et al. 1991] and Py235 [Ogun and Holder 1996] binds to the erythrocyte surface membrane. In *Plasmodium falciparum*, merozoite invasion is initiated by binding of the merozoite surface protein MSP-1 to the red blood cells [Perkins and Rocco 1998].

To date approximately 10-11 such proteins have been identified, MSP-1 to MSP-11 [Sherman 1998]. MSP-1 binding is followed by interaction of the apical complex proteins including proteins from the micronemes and the rhoptries. Merozoite surface protein MSP-1 interacts with sialic acids on erythrocyte glycoprotein [Perkins and Rocco 1988]. Following this interaction other apical complex proteins are then able to bind to and interact with erythrocytes mainly rhoptry proteins. MSP-1 is part of a family of merozoite surface proteins and is found to be expressed in all species of *Plasmodium* [Barnwell et al. 1998].

A 21 amino acid sequence from EBA-175 of *Plasmodium falciparum* binds to glycophorin A on erythrocytes [Jakobsen et al. 1998]. This amino acid sequence (aa1076-96) binds via an initial sialic acid dependent step to erythrocytes. Delineation of this sequence gives a 12 amino acid sequence (aa1085-96) capable of binding to erythrocytes independent of sialic acid [Jakobsen et al. 1998]. The EBA-175 peptide

(aa1076-96) induces antibody formation in mice after conjugation with a purified protein derivative [Jakobsen et al. 1998]. AMA-1 is present in the apical complex organelle often times on the surface of *Toxoplasma* and *Plasmodium* zoites. AMA-1 is thought to have erythrocyte binding function and has a role in invasion [Barnwell et al. 1998]. The MAEBL gene family proteins have also been localized by immunoelectron microscopy (IEM) in *Plasmodium yoelii* and localized to the surface of merozoites within schizonts. These MAEBL proteins have been identified as erythrocyte binding proteins.

AMA-1 (apical membrane antigen 1) a micronemal protein was found to be highly conserved among *Plasmodium spp.* and is thought to have an adhesive or receptor-binding function during red blood cell invasion [Barnwell et al. 2005]. In *Plasmodium yoelii* YM AMA-1 domains 1 and 2 facilitate erythrocyte binding to mouse and rat erythrocytes [Fraser et al. 2001]. Antibodies against *Plasmodium falciparum* AMA-1 are shown to inhibit cell invasion by the parasite [Barnwell et al. 2005]. The gene encoding AMA-1 has been cloned in several *Plasmodium* species including *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. chaubaudi* and *P. fragile*. AMA-1 is an 83-kDa protein in *P.falciparum* [Barnwell et al. 2005] and is conserved among apicomplexans.

1.13 Rhoptry proteins

Several rhoptry proteins have been characterized to date (Table 3). The Rhop-H/Clag proteins or high molecular mass rhoptry protein complex contain three distinct proteins, the 155/140 RhopH-1, 140/130 RhopH-2 and the 110/105 kDa RhopH-3 proteins [Barnwell et al. 2005]. RhopH-1 has been identified as a member of the

cytoadherence-linked asexual gene (clag) family [Barnwell et al. 2005]. Clag 9-specific antibodies immunoprecipitate the RhopH complex and Clag 9 was detected in the complex purified by antibodies to RhopH2 [Barnwell et al. 2005]. These data suggests that the RhopH complex could be a mix of clag components [Kaneko 2001]. Five members of the RhopH-1/clag family have been evaluated in *Plasmodium falciparum* HB3 parasites, clag2, clag3.1, clag3.2, clag 8 and clag9 [Kaneko et al. 2005].

The rhoptry-associated protein (RAP) consists of three lower-molecular-mass proteins, the 86 kDa RAP-1 and 42/39 kDa RAP-2, RAP-3 proteins [Baldi et al. 2002]. The rhoptry-associated membrane antigen (RAMA) is a 60 kDa GPI-linked protein formed from a 170 kDa precursor, this protein is discharged from the rhoptries during invasion and is found in the PV during early ring stages of the parasite life cycle [Barnwell et al. 2005]. Other molecules associated with the rhoptries include a 52 kDa protein, and a novel *Plasmodium falciparum* rhoptry protein, PfRhop 148 [Lobo et al. 2003]. The 229 kDa repetitive organellar protein (ROPE) is also thought to be involved in the invasion process and may be involved in the mimicry of spectrin [Werner et al. 1998].

Due to their high conservation among *Plasmodium* spp. these asexual stage proteins have been targeted as malaria vaccine candidates [Sam-Yellowe et al. 2000]. A majority of the rhoptry proteins are secreted during merozoite invasion, many of which disappear during early development of the parasite suggesting that their role is in invasion. The Rhop-3 protein has been found to be conserved among *Plasmodium falciparum*, and the rodent species *P. berghei*, *P. chabaudi* and *P. yoelli* [Anthony et

al. 2000, Sam-Yellowe et al. 1998].

Table 3. Characteristics of the major rhoptry proteins [Modified from Sam-Yellowe et al. 1996]

| Protein | Size (kDa) | Features | References |
|------------------------------|------------|--|----------------|
| Plasmodium falciparum | | | |
| Rhoptry proteins: | 240/225 | Localized in the neck of the each rhoptry | 30,34 |
| RhopH complex: | | | 30,34 |
| RhopH-1 | 155/140 | RBC-binding protein | 30,34 |
| RhopH-2 | 140/130 | RBC-binding protein | 30,34 |
| RhopH-3 | 110/105 | RBC-binding protein | |
| RAP-1 | 86 | Associated with the lamellar membrane-like structures released from rhoptries of free merozoites. | 15, 35,34 |
| RAP-2 | 42/39 | Associated with the lamellar membrane-like structures released from rhoptries of free merozoites. | 30,34 30,34 |
| RAP-3 | 37 | - | 12 |
| Serine proteases | 80/76 | Serine protease/ GPI anchored | 30,34 |
| Pf60 | 60 | - | 8 |
| MCP-1 | 60 | Oxidoreductase domain | 16 |
| CLAG 2. | - | Merozoite-erythrocyte interactions | 20 |
| CLAG 3.1 | - | Merozoite-erythrocyte interactions | 20 |
| CLAG 3.2 | - | Merozoite-erythrocyte interactions | 20 |
| CLAG 9 | | Implicated in cytoadherence and the binding of infected erythrocytes to host endothelial cells | 20 20 |
| Rhop148 | - | Transmembrane protein, possible role in rhoptry biogenesis | |
| RAMA | 60 | GPI anchored protein Interacts with LMW and HMW complexes. Secreted during invasion and binds to RBC surface. | 44 |
| Stomatin | | Interacts with RhopH2 in detergent resistant micodomain (DRM). Secreted during invasion and transferred to the outer leaflet of the PVM in young rings. Possible role in raft-based invagination of the host cell during invasion. | 20 |
| RSP-2 | 42 | Discharged onto the erythrocyte membrane during contact with erythrocytes. | 10 |
| p52 | 52 | - | 43 |
| ROPE | 229 | Invasion by mimicry of spectrin | 47 |

1.13.1 RhopH-3

RhopH-3 is distinct gene product of the high molecular weight complex, RhopH.

The Rhop-3 protein is secreted into the erythrocyte membrane during invasion and is an erythrocyte binding protein [Sam-Yellowe et al. 1988, Sam-Yellowe and Perkins 1991]. The gene encoding the Rhop-3 protein consists of 7 exons (Fig 5.) [Brown and Coppel 1991].

The most conserved regions of the protein lies within the C-terminus (the second half of exon 6 amino acid #548 to #665) and the beginning of exon 3 (amino acid #59 to #210) [Wang et al. 2006].

There is a strong human immune response to the C-terminus of the Rhop-3 protein among sera from individuals in different malaria endemic regions [Yang et al. 1996]. This region of the protein is highly conserved among *Plasmodium* species. Immunization of mice using proteins encoded by the C-terminus of *P. yoelii* Rhop-3 and the full *P. berghei* Rhop-3 protein have also been shown to induce protection against the rodent parasites, *P. yoelii* and *P. berghei* [Wang et al. 2006]. The Rhop-3 protein is therefore considered a strong candidate for an asexual stage malaria vaccine. The Rhop3 protein is an erythrocyte binding protein involved in red blood cell invasion however, the binding domain of the Rhop-3 protein has not yet been characterized.

The 140/130/110 kDa Rhop-H/Clag complex binds directly to intact mouse erythrocytes, this complex also binds to membranes and inside-out vesicles of mouse, human, saimiri, rhesus rat and rabbit erythrocytes [Sam-Yellowe et al.1991]. This complex was also found to bind specifically to liposomes made using different types of phospholipids [Sam-Yellowe et al. 1991]. Phosphatidylethanolamine (PE) containing phospholipids block binding of the rhoptry protein complex to mouse cells [Sam-Yellowe et al. 1991]. Results of these studies suggest that the complex may interact directly with sites in the lipid bilayer of the red blood cell membrane [Sam-Yellowe et al. 1991]. The binding domains for a number of erythrocyte-binding proteins have already been characterized, however the binding domain of the Rhop-3

proteins is not yet known. The aim of this study is to identify the binding domain the Rhop-3.

1.14 Hypothesis

The binding domain of the Rhop-3 protein lies within the C-terminus of the protein.

1.15 Aim

To identify the erythrocyte binding domain of the *Plasmodium* merozoite rhoptry protein Rhop-3.

1.16 Significance of Project

Knowledge of the domain will be important to determine if the binding epitope can induce protective immune responses.

If the binding domain induces an immune response it may be a suitable protein target for vaccine studies.

1.17 Objectives

1. Clone Rhop-3 exons using the vectors, pDisplay (Invitrogen), pRE4 and pEGFPNI and express clones in COS-7 cells.
2. Use erythrocyte rosetting assays to determine Rhop-3 binding to erythrocytes and use Rhop-3 specific antibodies in inhibition studies to evaluate specificity of binding.

CHAPTER II

MATERIALS AND METHODS

2.1 Parasite maintenance

Plasmodium berghei obtained from Dr. Mark Wiser, Tulane University and *Plasmodium yoelii* obtained from Dr. Carole Long, National Institute of Health, infected Swiss Webster mice were maintained as described [Sam-Yellowe et al.1998]. Infected erythrocytes (1×10^6 cells/ml) were collected by cardiac bleeding of SW mice. Infected blood was collected in citrate phosphate dextrose buffer (CPD) and washed 3X in non-sterile RPMI 1640 and centrifuged for 5 minutes each. Infected erythrocytes were used for weekly passaging of parasites into female SW mice maintained at the animal facilities of Cleveland State University. *Plasmodium falciparum* strain FCR-3 were maintained and grown in human erythrocytes according to the method of [Trager and Jensen 1976].

2.2 Culture of mammalian cells

COS-7 green monkey kidney cells obtained from the American Type Culture Collection (ATCC) were cultured and used to express recombinant proteins. Cells

were grown in open screw capped 25cm² flasks and maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) plus 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, CA) in a 37°C incubator, gassed with 5% CO₂, 95% air. Cell monolayers were grown to confluence and routinely passaged 3-4 days by removing spent culture medium and replacing with fresh DMEM [Fraser et al. 2001].

2.3 Isolation of genomic DNA

Schizont-infected erythrocytes of *P. berghei*, *P. yoelii* and *P. falciparum* were lysed with tris pH 8.8 followed by centrifugation at 15,000 rpm (JA-20 rotor Beckman) for 10 minutes to obtain schizont pellets. The pellets were then homogenized in 600ul TES buffer (10mM Tris-HCL p117.6, 50 mM EDTA pH8.0, 0.1 % SDS) containing 1mg/ml proteinase K. Lysed erythrocytes were then incubated in a 37°C water bath for 30 minutes. 500ul phenol chloroform (1:1) mix was used to extract schizont DNA and the DNA precipitated in 70% ethanol and resuspended in distilled water. Isolated DNA was separated by agarose gel electrophoresis using a 0.7% agarose gel, visualized using ethidium bromide. Isolated DNA was used as templates for PCR. DNA concentration was obtained using Beckman DU Series 500 Spectrophotometer.

2.4 RNA Isolation

RNA was isolated from schizont pellets using RNAzol B Isolation method (Tel-Test Inc). Pellets were homogenized with RNAzol B. RNA was extracted in chloroform and precipitated in isopropanol. The white-yellow RNA pellet was washed with 75% ethanol, air dried and dissolved in 25ul DEPC water. Isolates were analyzed by formaldehyde RNA gel electrophoresis on a 1% agarose gel.

2.5 Protein extraction.

Plasmodium berghei, *P. yoelii* and *P. falciparum* protein samples were extracted by adding 100-150µl 1% Triton X 100 to schizont pellets. The pellets were vortexed and placed on ice for 5 minutes and vortexed again. This vortexing, ice incubation cycle was repeated 5 times for a combined 30 minutes extraction. The suspension was then centrifuged for 5 minutes at 14,000 rpm and the supernatant removed and collected into a fresh tube and the supernatant and pellets frozen at -20°C. The protein concentration was then determined by using the method of Bradford. Protein samples were used for SDS-PAGE analysis, transferred to nitrocellulose paper (NCP) and western blotting techniques performed using specific anti-Rhop-3 antibodies Ab”686” [Yang et al, 1996], Mab 1B9 [Sam-Yellowe et al., 1988], Ab “F1”, Ab “T1” and pooled Ab “F1”+ “F12” [Wang et al., 2006]. Normal rabbit antisera NR701 and normal mouse antisera were used as negative controls.

2.6 PCR Amplification

PCR amplification was performed in a 50 µl reaction mixture using 10X Mg⁺⁺ free PCR Buffer (500mM KCL, 100mM Tris HCL (pH 9.0), 15mM MgCl₂, 200 µM of each dNTP, 10pM of each primer, 5 units of DNA polymerase using the following cycling parameters: one cycle of 2 min at 94°C, 40 cycles of 15s at 94°C, 30s at 45°C, and 2 min at 68°C followed by 1 cycle of 5 min at 68°C and cooled to 4°C. 2µl of template DNA was used in the reaction mixture. A negative control tube

containing no DNA was also included. A 1% ethidium bromide stained agarose gel was used to analyze and visualize the PCR products.

2.7 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The total RNA from the rodent *Plasmodium* parasite was reverse transcribed into complementary DNA (cDNA) and the strands amplified using designed primer pairs based on the sequences of the Rhop-3 exons. The primer pairs for exons 1-3, mini-exons 4/5, exons 6-7 and full-length were designed for amplifying the exons by RT-PCR.

2.7.1 Plasmid Amplification

A plasmid vector was used to transfect mammalian cells for expression of the recombinant proteins. The plasmid is a mammalian expression vector designed to target recombinant proteins to the surface of mammalian cells.

2.7.2. Feature of the Plasmid

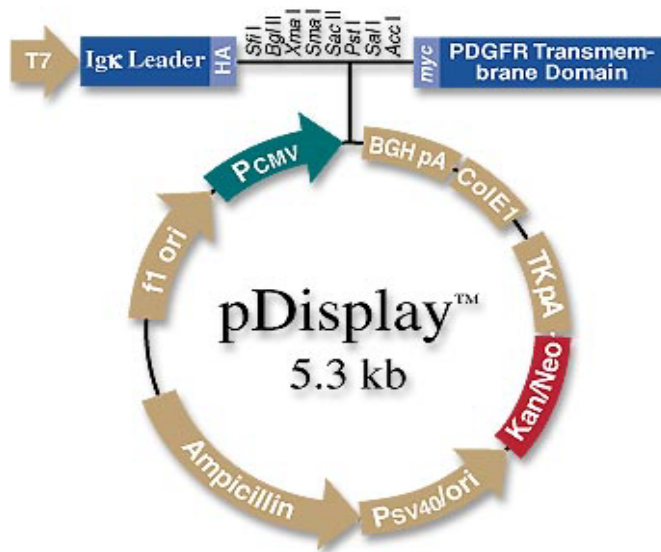


Figure 7. Diagram of Plasmid pDisplay™ (Invitrogen)

Plasmid pDisplay (Fig. 7) has an N-terminal cell surface targeting signal and a C-terminal transmembrane anchoring domain and has the advantage of having the protein of interest expressed on the cell surface. The plasmid facilitates directional cloning of the desired insert within its multiple cloning site. Recombinant proteins expressed have a hemagglutinin A and *myc* tag fused to the C-terminus of the protein allowing for detection of the protein by immunofluorescence or western blot.

Proteins expressed are fused at the N-terminus to the murine Ig k-chain allowing the protein to enter the secretory pathway and fused at the C-terminus to the platelet derived growth factor receptor (PDGFR) transmembrane domain for anchoring the expressed protein to the cell surface (www.invitrogen.com).

2.8 Construction of Recombinant Plasmids

Plasmid pDisplay was used to construct recombinants containing the full length Rhop-3 gene and fragments of the Rhop-3 gene. Primers containing the appropriate restriction sites were used to amplify Rhop-3 by PCR and RT-PCR and the products were gel purified. The purified products were cut at their appropriate restriction sites and the digested products separated by gel electrophoresis on a 1% agarose gel. DNA was visualized using ethidium bromide. Separated bands were further excised from the gel and gel purified using (S.N.A.P Gel Purification kit). Purified products were ligated to the plasmid vector digested with the same restriction enzymes. Ligation was carried out overnight at 12°C (Thermolyne ThermoKool, CA, U.S.A). The recombinant plasmid was used to transform *E. coli* strain DH5 α cells (Invitrogen). Transformants were selected after growth on antibiotic selective media and inoculated into LB broth. Overnight cultures were used to isolate purified recombinant using an endotoxin-free purification kit (Qiagen). The isolated recombinant plasmid was used to transfect COS-7 cells to optimize protein expression.

2.9 Seeding of COS-7 cells

Media was removed from a confluent flask of cells. 2mls of trypsin EDTA was added to dislodge adherent cells. The cells were washed in 10-12ml incomplete media (DMEM without serum) and spun for 5 minutes at 1250rpm. The supernatant was discarded and the cells resuspended in 2ml incomplete media. 10 μ l of the cell suspension was used to determine the concentration using a hemacytometer. A cell suspension of 160,000 cells/ml was made up in incomplete media. 250 μ l of the cell

suspension was added to each well of a Poly-D-Lysine coated 24-well plate or 8-well slide. The cells were then let to settle for 3hrs at 37°C in the incubator.

2.10 Transfection with Lipofectamine

A 3% Lipofectamine solution was made up in incomplete media and incubated at RT for 15 minutes. DNA solutions were made in incomplete media (100ng/well), combined with an equal volume of Lipofectamine solution and incubated at rtp for 20 minutes. Incomplete media was added to each DNA solution (200µl/well). 250µl of each DNA solution was added to each well and the plates incubated for 4hrs at 37°C. Media on the transfected cells was replaced with complete media (DMEM with 10% serum) and the cells incubated for 42-44hrs.

2.11 Erythrocyte binding assay using spent culture supernatant

Mouse and human blood were used to carry out erythrocyte binding assays. Mouse blood was collected by cardiac bleeding of BALB/c mice into citrate-phosphate-dextrose (CPD) and washed 3X in RPMI-1640. Erythrocyte binding assay was performed as described previously (Perkins and Rocco 1988). Spent culture supernatant (SCS) was collected from *P. falciparum* cultures and from short-term cultures of rodent *Plasmodium* schizont-infected erythrocytes. Unlabeled SCS (300-600µl) was added to mouse erythrocytes (100µl) resuspended gently and incubated for 30 minutes at room temperature. The cell suspension was then layered over 0.5ml silicone fluid (Dow Coming 550 fluid) in a 1.5ml eppendorf tube and centrifuged for 2 minutes at 14,000 rpm. The supernatant was then collected and the cell pellet resuspended in 0.5ml RPMI-1640. The cell suspension was layered over 0.5ml

silicone fluid and centrifuged for 2 minutes. Supernatant was removed and discarded and the cells gently resuspended in 0.5M NaCl and incubated for 10 minutes at RT. The suspension was then centrifuged for 2 minutes at 14,000 rpm. The eluate was removed and mixed with protein electrophoresis sample buffer and analyzed on a 5 to 15% SDS-gradient gel [Perkins and Rocco 1988].

2.12 Protein Expression and detection

Recombinant plasmids containing the genes of interest isolated from bacterial cells were used to transfect COS-7 cells (African Green Monkey Kidney Fibroblasts) using Lipofectamine 2000TM (Invitrogen), Lipofectin, Lipofectamine 2000TM and Plus reagent (Invitrogen), Lipofectin and Plus reagent. The plasmid used allowed for the encoded proteins to be expressed on the surface of the mammalian cells. Expression of the protein of interest was analyzed by Immunofluorescence Assay (IFA) with Rhop-3 specific antibodies.

2.13 Rosetting Assay

Erythrocyte rosetting technique was used to study the binding of Rhop-3 to erythrocytes. The assay was carried out to evaluate the binding of uninfected erythrocytes to COS-7 cells expressing Rhop-3 on the surface membrane. The rosetting assays were performed using a standard procedure [Adams et al. 2000]. COS-7 cells were plated into Poly-D-Lysine coated 24-well culture plates and Poly-D-Lysine coated 8-well culture slides. COS-7 cells were transfected with recombinant pDisplay DNA using Lipofectamine 2000TM (Invitrogen), Lipofectin, Lipofectamine 2000TM and Plus reagent (Invitrogen), Lipofectin and Plus reagent in

Dulbecco modified Eagle medium (DMEM; Sigma) without serum. After a 4hr incubation the transfection medium was replaced by DMEM with 10% fetal bovine serum and incubated for 24hr, 42hr, 44hr or 60hrs. The transfected cells were incubated for 2hr at room temperature with human or mouse erythrocytes (10% final suspension washed in DMEM without serum). The cells were then washed three times in PBS to remove non-adherent erythrocytes. Rosettes were counted in 30 fields at a magnification of X200 to evaluate the binding. Rosettes were counted as positive when adherent erythrocytes cover more than 50% of the cell surface. These experiments were carried out in three replicates and repeated three times [Adams et al. 2000].

CHAPTER III

RESULTS

3.1 Western Analysis

Western blot assays using anti-Rhop3 mouse antibodies T2, FL1+FL2 and F2 were used to identify Rhop-3 in *Plasmodium falciparum* (lane 3), *P. berghei* (lane 2) extracts separated by SDS-PAGE gel electrophoresis [Fig 8]. The mouse antibodies T2, FL1+FL2, F2 recognized the rodent *Plasmodium* Rhop-3 [Fig. 8 (a), (b), (d) lane 2] with no Rhop-3 identified in *Plasmodium falciparum* [Fig 8. (a), (b), (d) lane 3]. No proteins were detected using the normal mouse antibody [Fig 8. (e)]. Rabbit antibody #686 identified Rhop-3 in the human malaria species and also in the rodent species [Fig 8. (c) lane 3 and 2 respectively] .

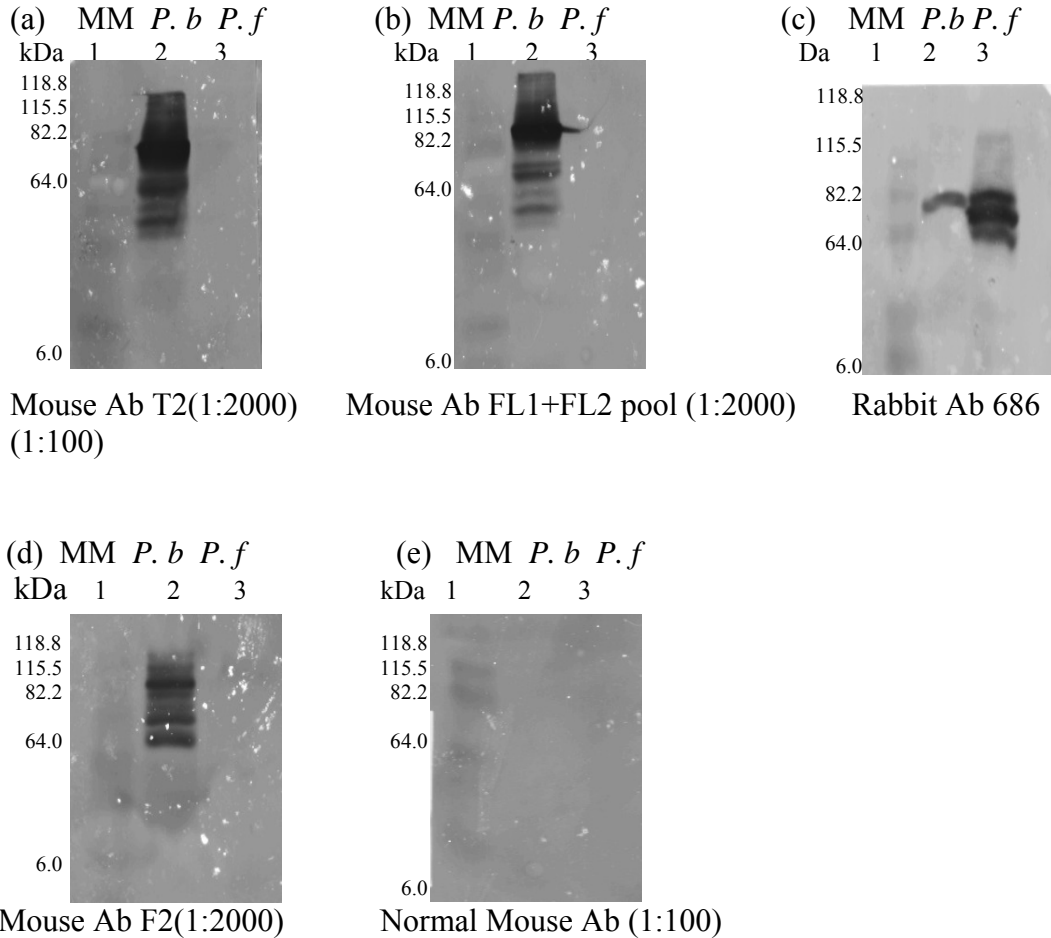


Figure 8. Western Blots using Rhop-3 specific antibodies. The antibodies react with the 110-kDa protein from *P. berghei* and *P. falciparum*.

3.2 Recombinant Constructs

PY1412 sequence was amplified from *Plasmodium yoelii* and *P. berghei* genomic DNA by PCR to obtain a partial fragment of the Rhop-3 gene to be inserted into the vector pDisplay. The amplified fragment was ~960bp [Fig 9. lanes 2-5] and was conserved in *Plasmodium yoelii* and *P. berghei*

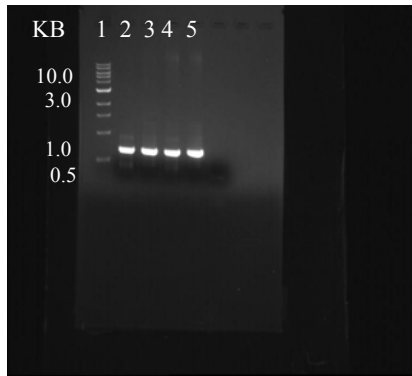


Figure 9. PY1412 PCR products amplified from genomic *P. yoelii* and *P. berghei*. *P. berghei* PY1412pDisplay (lane 2), *P. berghei* PY1412/pEGFPN1 (lane 3), *P. yoelii* PY1412/pDisplay (lane 4), *P. yoelii* PY1412/pEGFPN1 (lane 5), water control (lane 6)

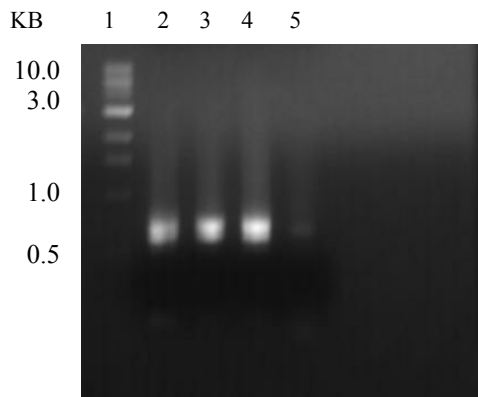


Figure10. PY1412 PCR products amplified from recombinant pDisplay/PY1412. PY1412/pDIS (lane 2-4),water control (lane 5) Purified *Plasmodium yoelii* PY1412 was digested using the restriction enzymes *Bgl*III and *Sal*I and ligated to *Bgl*III +*Sal*I digested pDisplay. The ligation mixture was used to transform *Escherichia coli* DH5 α cells and the recombinant DNA isolated. The *Plasmodium yoelii* PY1412 sequence was amplified from the recombinant DNA and ~960bp fragment recovered [Fig 10. lanes 2-4].

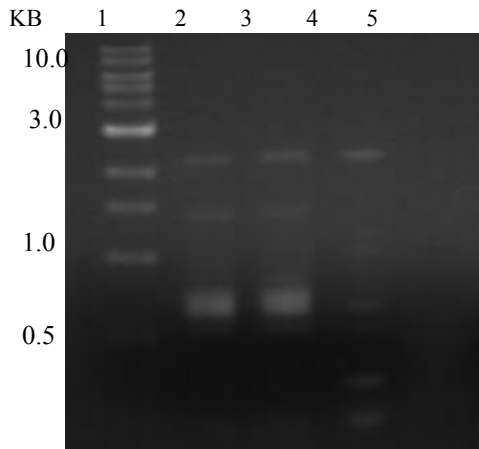


Figure 11. PY1412 PCR products amplified from recombinant pEGFPN1 PY1412/pEGFPN1(lanes 2-3), control exon 7 primers (lane 4), (5) water control water control (lane 5). Purified *Plasmodium yoelii* PY1412 was digested using the restriction enzymes BamHI and HindIII and ligated to BamHI +HindIII digested pEGFPN1. The ligation mixture was used to transform *Escherichia coli* DH5 α cells and the recombinant DNA isolated. The *Plasmodium yoelii* PY1412 sequence was amplified from the recombinant DNA and recovered a fragment around 960bp, however there were also two other bands recovered at ~ 1.5kb and 2.0kb [Fig 11 lanes 2-3].

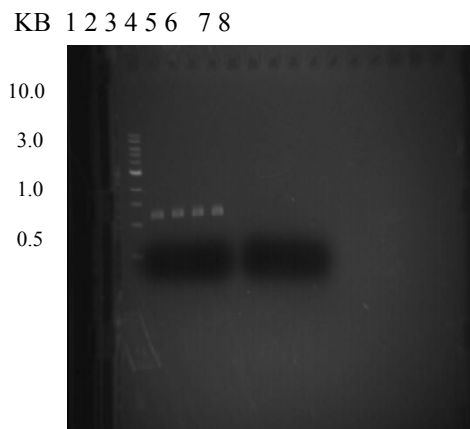


Figure 12. Exon 1 to 3 RT-PCR products amplified from *P. falciparum* RNA (lanes 2-5) exon 1-3 (lane 6) water control (lane 7) enzyme control. Using designed primers exon 1 to exon 3 of

Plasmodium falciparum was amplified from isolated *P. falciparum* RNA by RT-PCR for insert into the vector pDisplay. The amplified fragment was ~1.1kb [Fig 12 lanes 2-5]

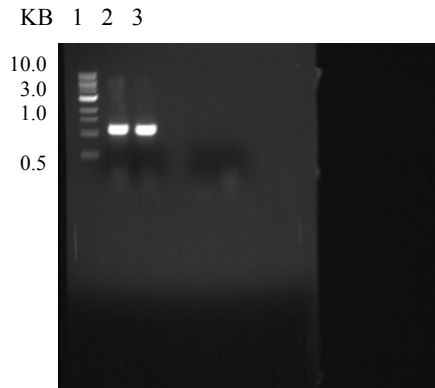


Figure 13. Exon 1 to 3 RTPCR products amplified from *P. falciparum* Dd2 (lane 2-3) exon 1-3 (lane 5) water control (lane 6) enzyme control Using designed primers exon 1 to exon 3 of *Plasmodium falciparum* was amplified from isolated *P. falciparum* RNA by RT-PCR for insert into the vector pDisplay. The amplified fragment was ~1.1kb [Fig 12 lanes 2-3].

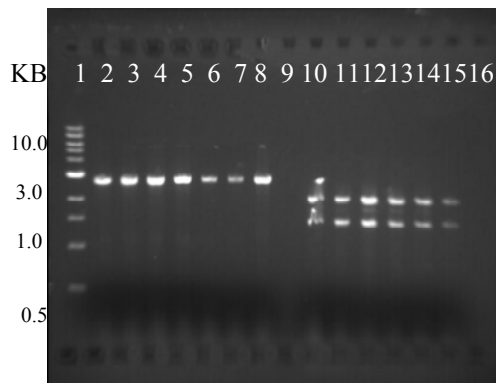


Figure 14. Exon 1 to 7 and 6-7 RTPCR products amplified from *P. falciparum* Dd2 exon 1-7 (lanes 2-8), exon 6-7 (lane10-15), water control (lane 16). Using designed primers exon 1 to exon 7 and exon 6 to exon 7 of *Plasmodium falciparum* were amplified from isolated *P. falciparum* RNA by RT-PCR for insert into the vector pDisplay. The amplified fragment exon 1-7 was ~2.7kb [Fig 14 lanes 2-8], the sequence from exon 6 -7 was not amplified [Fig 14 lanes 10-15].

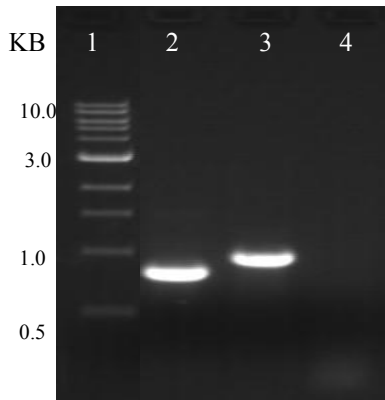


Figure 15. Exon 6 and 7 PCR products amplified from *P. falciparum* FCR-3 DNA exon 7 (lane 2), exon 6 (lane 3), water control (lane 4). Using designed primers exon 6 and exon 3 of *Plasmodium falciparum* was amplified from isolated *P. falciparum* genomic DNA by PCR for insert into the vector pDisplay.

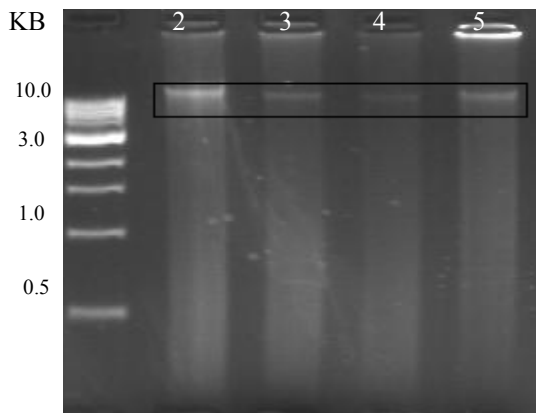


Figure 16. Full-length recombinant *P. falciparum* Rhop-3 DNA (2-5) exon 1-7 recombinant pDisplay/1-7. *Plasmodium falciparum* recombinant DNA encoding exon 1-7 was isolated and purified from transformed *Escherichia coli* DH5 α [Fig 16 lanes 2-4].

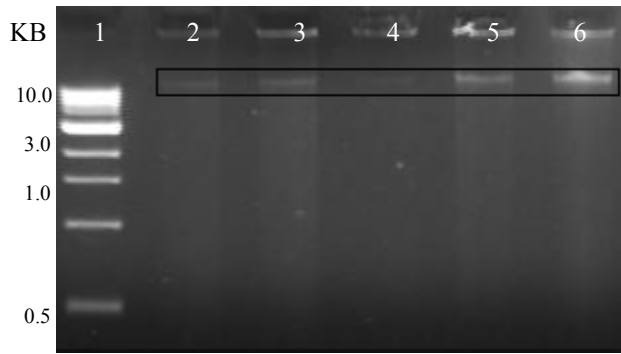


Figure 17. Recombinant *P. falciparum* Rhop-3 DNA exon 1-3 (2-6) exon 1-3 recombinant pDisplay/1-3 *Plasmodium falciparum* recombinant DNA encoding exon 1-3 was isolated and purified from transformed *Escherichia coli* DH5 α [Fig 17].

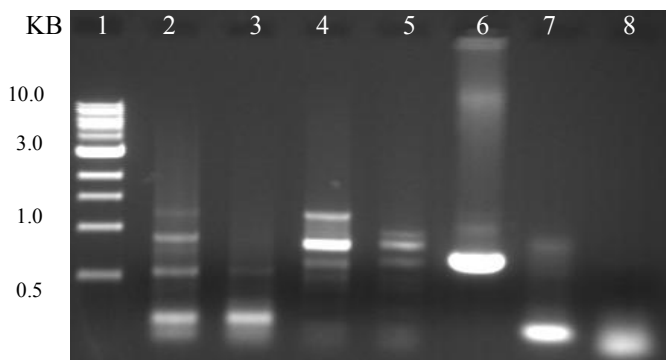


Figure 18. Recombinant *P. falciparum* Rhop-3 PCR products (2-3) exon 1-7 (4-5) exon 1-3 (6,7,) controls (8) water control primers for exon 1 and exon7 and exon 1 and exon 3 were used to amplify these fragments from the recombinant DNA. From the gel the water control appears to be contaminated and the products do not correspond with the fragments amplified from RNA.

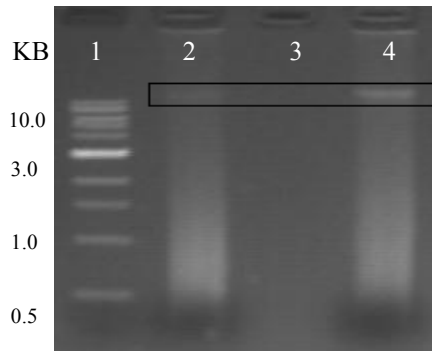


Figure 19. Recombinant *P. falciparum* Rhop-3 exon 1-3 and exon 7 (2) recombinant exon 1-3 (4) recombinant exon 7. *Plasmodium falciparum* recombinant DNA encoding exon 7 was isolated and purified from transformed *Escherichia coli* DH5 α [Fig 19].

3.3 Erythrocyte binding Assay

COS-7 cells were transfected with 100ng of recombinant DNA using a LipofectamineTM reagent and incubated for 44hr to allow for protein expression. After 44hrs COS-7 cells expressing the protein of interest were used to carry out erythrocyte binding assays. 50 μ l of a 10% RBC suspension was added to each well as demonstrated below [Table 4]. *SalI* the Duffy binding protein was used as a positive control. The recombinant *SalI* expresses region II of the Plasmodium vivax Duffy binding protein (PvDBP_{II}) which binds to Duffy positive human RBC.

Table 4. Seeding and Transfection layout of 24 well plates.

| | | | | | | |
|--------------|---------|---------|---------|-------------------|-------------------|-------------------|
| Mouse RBC | Sal1 | Sal 1 | Sal 1 | pDisplay/ 1412 | PDisplay/ 1412 | PDisplay/ 1412 |
| | pEGFPNI | pEGFPNI | pEGFPNI | -VE | -VE | -VE |
| Human RBC | Sal1 | Sal 1 | Sal 1 | pDisplay/ 1412 | PDisplay/ 1412 | PDisplay/ 1412 |
| | pEGFPNI | pEGFPNI | pEGFPNI | -VE | -VE | -VE |

COS-7 cells were transfected with 100ng of recombinant PDIS-PY1412 DNA using a Lipofectamine™ reagent and incubated for 44hr to allow for protein expression. After 44hrs COS-7 cells expressing the protein of interest were used to carry out erythrocyte binding assays. 50µl of a 10% mouse RBC suspension was added to each well transfected with PDIS-PY1412 and 50µl of a 10% human RBC suspension added to the *SALI* positive control as demonstrated below [Table 5]. There was a high percentage of binding of uninfected mouse RBC to the PY1412 recombinant protein with 20-30 RBC attached to each COS-7 showing resetting [Table 5]. There was some binding seen in the positive control also. Human RBC did not bind to the PY1412 protein and mouse RBC did not bind to the *Plasmodium vivax* DBP.

Table 5. Results from Binding Assay showing binding of Recombinant *Plasmodium yoelii* pDisplay/PY1412 to COS-7 cells

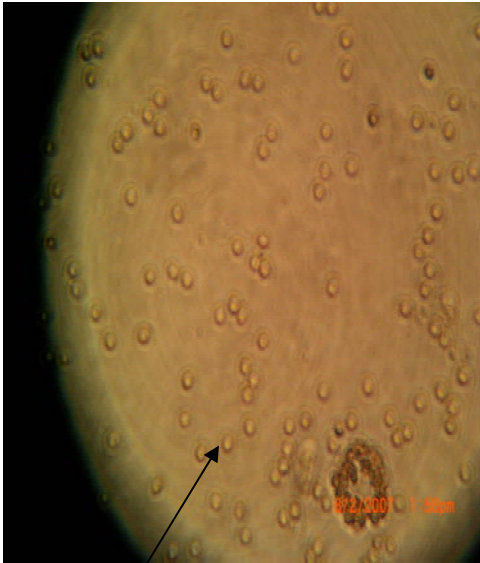
| | <i>Pv</i> -DBP _{II} Sal1 | | | Recombinant PY1412/pDisplay | | |
|-----------|-----------------------------------|---|---|--------------------------------|----|----|
| | Mouse RBC | 0 | 0 | 0 | 49 | 45 |
| | 0 | 0 | 0 | 0 | 0 | 0 |
| Human RBC | 14 | 9 | 7 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 | 0 |

Using the PvDBP_{II}Sal1 as control, the number of rosettes formed with the *Plasmodium vivax* protein was considered total binding and taken to be 100% binding. The number of rosettes of the *Plasmodium yoelii* protein were then compared to the total number of rosettes from *P. vivax* [Table 6].

Table 6. Percent Binding of *Plasmodium yoelii* recombinant Rhop-3 protein to COS-7 cells compared to binding in the Sal as control.

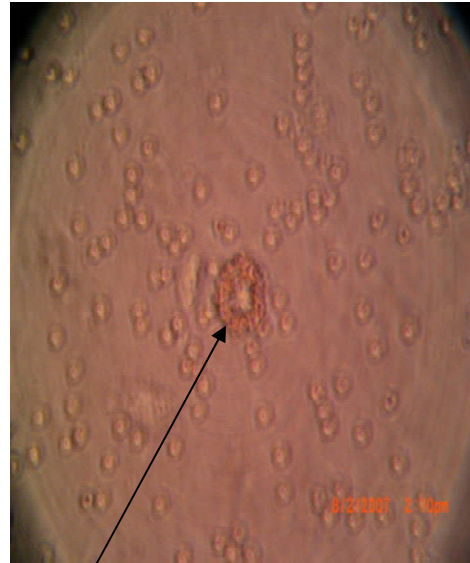
| | <u>Sample</u> | |
|-----------------------------|-----------------------------------|---|
| | <i>Pv</i> -DBP _{II} Sal1 | <i>P. yoelii</i> Recombinant pDisplay PY1412 |
| <i>Average</i> (3 wells) | 10 | 52.3 |
| % Binding | 100 | 523 |

Rosettes were counted as positive when more the half the surface of the COS-7 cell had attached RBC's as seen in the photographed fields below [Figure 21] taken of the PvDBP_{II}SalI positive control. The total number of rosettes seen in 30 fields were counted.



free RBC

Figure20. Rosetting of Human RBC to expressing Pv-DBP_{II}Sal1



COS-7 cell with more than 50% of the surface covered with RBCs

Figure 21. Rosetting of Human to COS-7 cell RBC COS-7 cell expressing Pv DBP_{II}Sal1

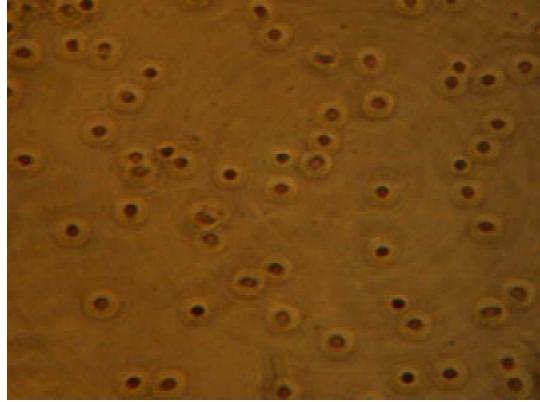


Figure 22. Control well showing unbound RBCs

Using the vector pDisplay three recombinant *Plasmodium falciparum* Rhop-3 proteins including the full-length Rhop-3 protein were expressed using the COS system, the presence of the protein was detected by IFA's using 4 different anti-Rhop-3 specific antibodies. The pDisplay vector was also used to express a protein encoded within the C-terminus of the *Plasmodium yoelli* Rhop-3 gene which was also detected by IFA using anti-Rhop-3 antibodies [Table 7].

Table 7. Recombinants used in Transfection and Binding Assays

| Recombinants Attempted | Clones Made | Transfection & Binding | Rosettes | Results | IFA | Results |
|---|--------------------|-----------------------------------|-----------------|--------------------------|-------------------|--|
| <i>P. falciparum</i> Vector- pDisplay Exon 1 Exon 2 Exon 3 Exon 6 Exon 7 Exon 1-3 Exon 6-7 Exon 1-7 | YES YES YES | YES YES YES | NO NO NO | No binding No binding | YES YES YES | Fluorescence Fluorescence Fluorescence |
| Vector pEGFPN1* Exon 1 Exon 2 Exon 3 Exon 6 Exon 7 | | | | | | |
| Vector- pEGFPN1a Exon 1 Exon 2 Exon 3 Exon 6 Exon 7 | | | | | | |
| <i>P. yoelii</i> Vector -pDisplay PY1412 | YES | YES | YES | *Some binding | YES | Fluorescence |
| Vector pEGFPN1 PY1412 | YES | YES | | | | |
| Vector- pEGFPN1a PY1412a | | | | | | |

Expression and binding of the *Plasmodium vivax* DBP_{II} to human RBC was observed in eight independent experiments and expression and binding of the *Plasmodium yoelii* PY1412 mouse RBC observed in one independent experiment [Table 8].

Table 8: Results obtained from attempted Binding assays

| Date | Assay | DNA | Results | | | | |
|------------|--|-----|--|--------------------|----------------------------|------------------|---|
| | | | Human Blood | Rosettes | Mouse Blood | Rosettes | |
| 7/7/2007 | Transfection & Binding Assay * regular plates | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | (untransfected cells with blood added) | | | | |
| | | | positive control Sal 1 | 0 | positive control Sal 1 | 0 | |
| 7/30/2007 | Transfection & Binding Assay *poly-D-Lysine plates | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 49,45,43 | |
| | | | pEGFPN1 | 0 | pEGFPN1 | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 14, 9, 7 | positive control Sal 1 | 0 | |
| 7/31/2007 | Transfection & Binding Assay *poly-D-Lysine plates | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | pEGFPN1 | 0 | pEGFPN1 | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 36,30,60 | positive control Sal 1 | 0 | |
| 8/11/2007 | Transfection & Binding Assay *poly-D-Lysine plates | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | pEGFPN1 | 0 | pEGFPN1 | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 36,30,60 | positive control Sal 1 | 0 | |
| 8/7/2007 | Transfection & Binding Assay *poly-D-Lysine plates & slides IFA | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 0 | positive control Sal 1 | 0 | |
| 8/8/2007 | Transfection & Binding Assay *poly-D-Lysine slides (IFA) | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 0 | positive control Sal 1 | 0 | |
| 8/14/2007 | Transfection & Binding Assay *poly-D-Lysine | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 0 | positive control Sal 1 | 0 | |
| 10/8/2007 | Transfection & Binding Assay *poly-D-Lysine | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | Full length (1-7) | 0 | Full leghFull-length (1-7) | 0 | |
| | | | Exon 1-3 | 0 | Exon 1-3 | 0 | |
| | | | Exon 7 | 0 | Exon 7 | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 14,20,16 | positive control Sal 1 | 0 | |
| 10/10/2007 | Transfection & Binding Assay *poly-D-Lysine | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | Full length (1-7) | 0 | Full leghFull-length (1-7) | 0 | |
| | | | Exon 1-3 | 0 | Exon 1-3 | 0 | |
| | | | Exon 7 | 0 | Exon 7 | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 0,0,0 | positive control Sal 1 | 0 | |
| 10/11/2007 | Transfection & Binding Assay *poly-D-Lysine plates | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | Full length (1-7) | 0 | Full leghFull-length (1-7) | 0 | |
| | | | Exon 1-3 | 0 | Exon 1-3 | 0 | |
| | | | Exon 7 | 0 | Exon 7 | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 5,8,5 | positive control Sal 1 | 0 | |
| 10/31/2007 | Transfection & Binding Assay *poly-D-Lysine slides for (IFA) | 1ug | pDisplay 1412 recombinant | | | | |
| | | | Negative Control | | | | |
| 11/1/2007 | Transfection & Binding Assay *poly-D-Lysine plates Balb /cJ & Balb / cBy J mice -6 wk old | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | Negative Control - | 0 | Negative control | 0 | |
| | | | positive control Sal 1 | 0 | positive control Sal 1 | 0 | |
| 11/28/2007 | Transfection & Binding Assay *poly-D-Lysine plates Balb /cJ & Balb / cBy J mice newly made and transformed PY1412 recombinant | 1ug | pDisplay1412 recombinat - | 0 | pDisplay 1412 recombinant | 0 | |
| | | | 5ug | Negative Control - | 0 | Negative control | 0 |
| | | | positive control Sal 1 | 0 | positive control Sal 1 | 0 | |

Poly-D-lysine coated 8-well slides were seeded with COS-7 cells. COS-7 cells were transfected with recombinant PDIS-PY1412 and incubated 44hr for protein expression. After incubating slides were fixed in 2% formaldehyde and incubated for 1hr in primary antibody F1/T1/FL. After incubation slides were washed 3X for 5 minutes each in PBS. Slides were then incubated for 1hr in secondary antibody normal mouse antibody conjugated to FITC in the dark. After incubation slides were then washed 3X PBS and once in water and mounted in Vectashield. Slides were examined using fluorescence microscope for surface expression of PY1412 [Figure 24].

3.4. Immunofluorescence Assay

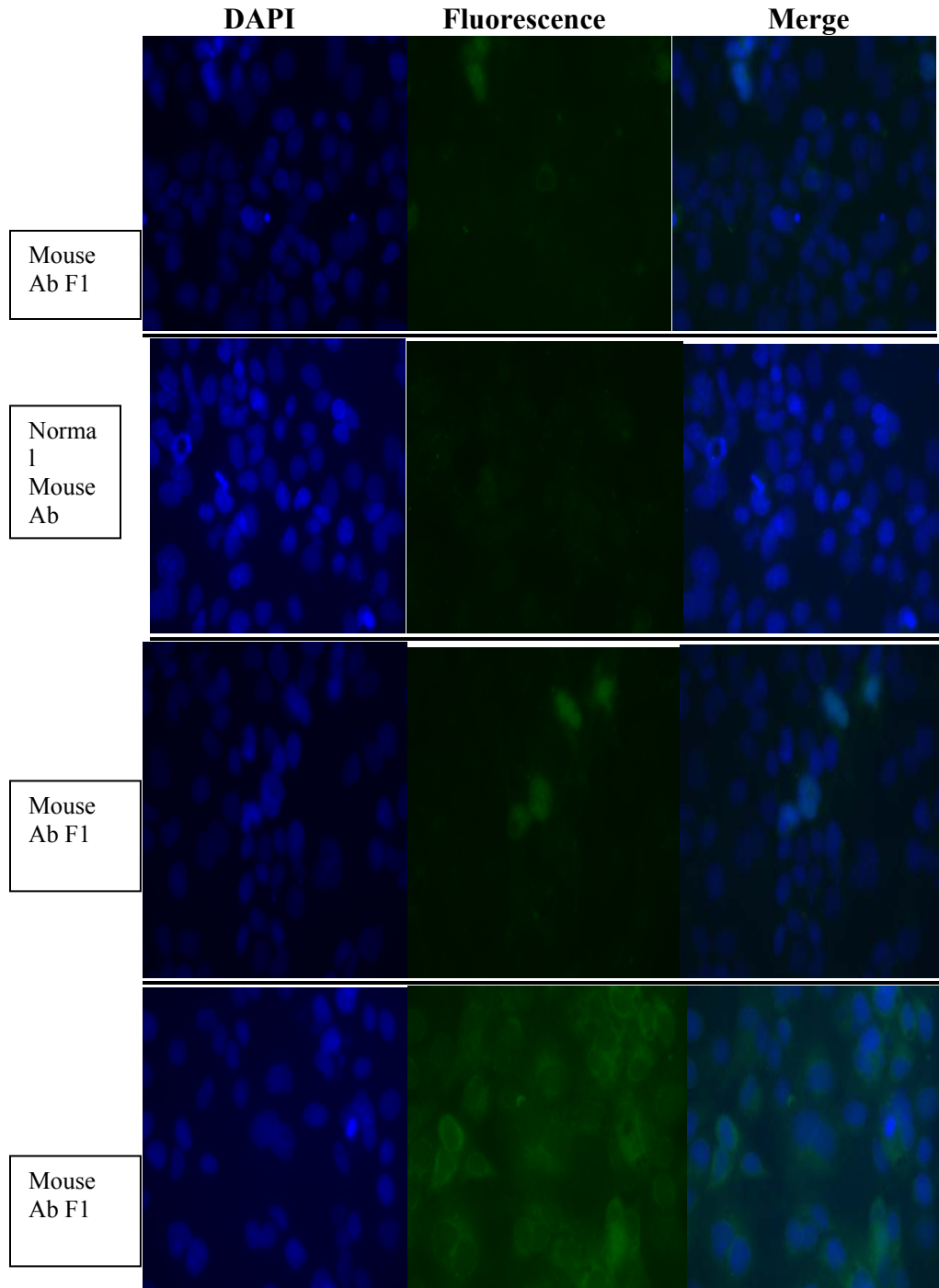


Fig 23. Immunofluorescence localization of the PY1412 C-terminal Rhop-3 pDisplay recombinant.

Formalin fixed slides were incubated in mAb or normal mouse antibody and FITC-goat-anti-mouse antibody. C-terminalRhop-3 recombinant showing surface expression by immunofluorescence

using mAb F1 COS-7 cells Expressing C-terminal recombinant incubated in normal mouse antibody show no fluorescence.

COS-7 cells expressing full-length *Plasmodium falciparum* Rhop-3 recombinant protein were examined for surface expression by IFA using a rabbit anti-Rhop-3 antibody. Expression was detected using the rabbit antibody and the normal mouse antibody showed some cross reactivity [Fig 24].

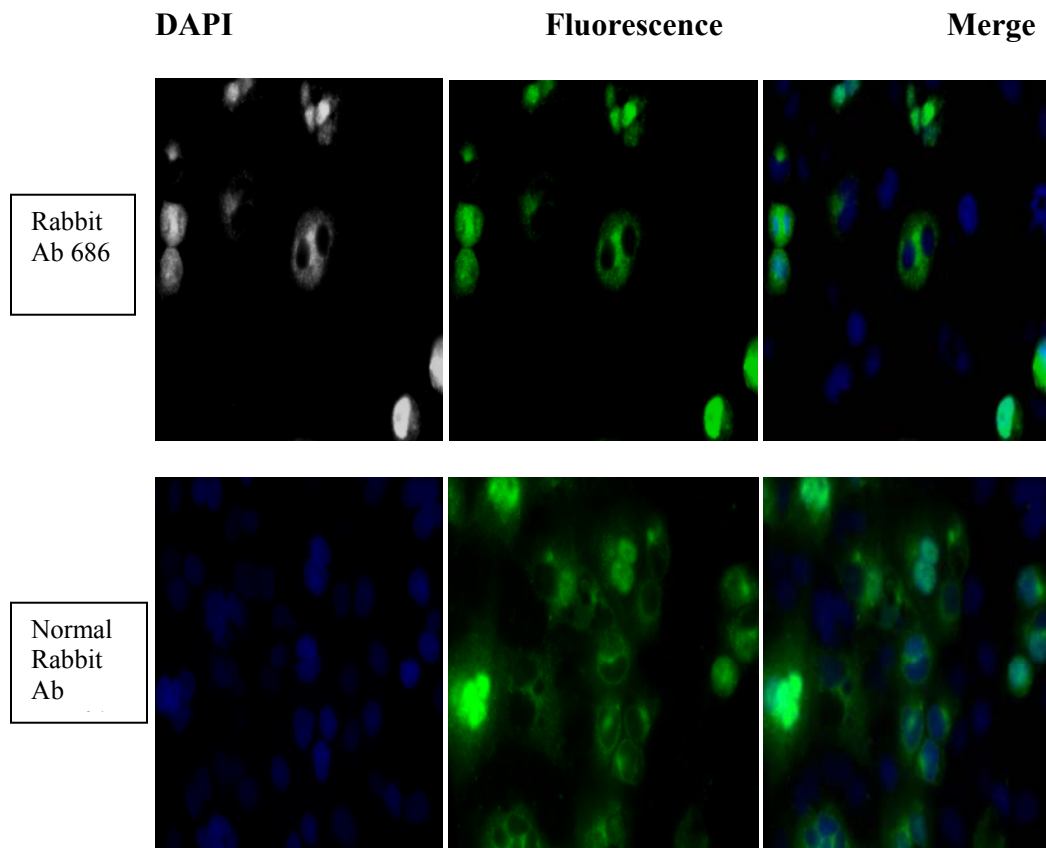


Fig 24. Immunofluorescence images of expressed *P. falciparum* full-length Rhop-3 recombinant pDisplay/1-7, 44hr post transfection. Immunofluorescence localization of the full-length Rhop-3 pDisplay recombinant. Formalin fixed slides were incubated in rAb or normal rabbit antibody and FITC-goat-anti-rabbit antibody. Full-length Rhop-3 recombinant showing expression by immunofluorescence using rAb #. COS-7 cells expressing full-length recombinant incubated in normal mouse antibody show some fluorescence.

COS-7 cells expressing the *Plasmodium vivax* PvDBP_{II} protein were also detected by IFA. The protein is expressed as GFP fusion protein for easy detection in mammalian cells without the use of antibodies [Fig 25].

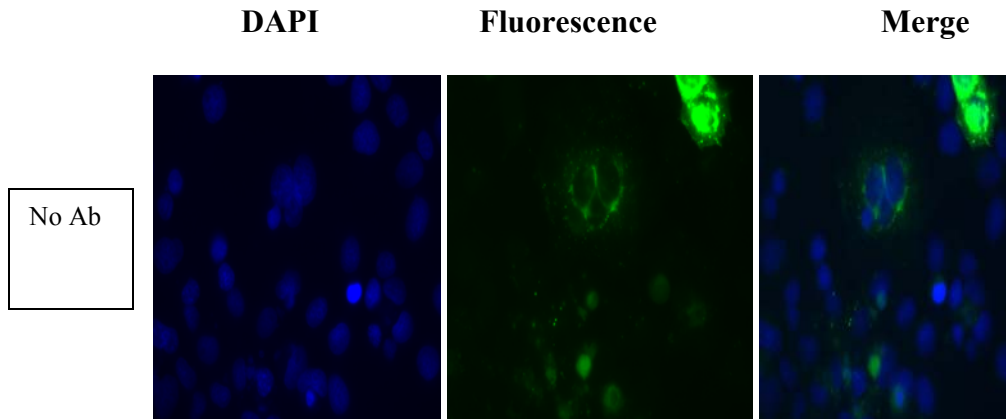


Fig 25. Immunofluorescence images of expressed Pv-DBP_{II} SalI 44hr post transfection. .

Immunfluoresence localization of the PvDBP_{II}SalI GFP control. Formalin fixed slides were mounted in Vecta shield and examined under the microscope, expression of region II of *Plasmodium vivax* DBP.

COS-7 cells expressing *Plasmodium falciparum* Rhop-3 recombinant protein encoded by exons 1 to 3 were examined for surface expression by IFA using a rabbit anti-Rhop-3 antibody. Expression was detected using the rabbit antibody and the normal mouse antibody showed some cross reactivity [Fig 26].

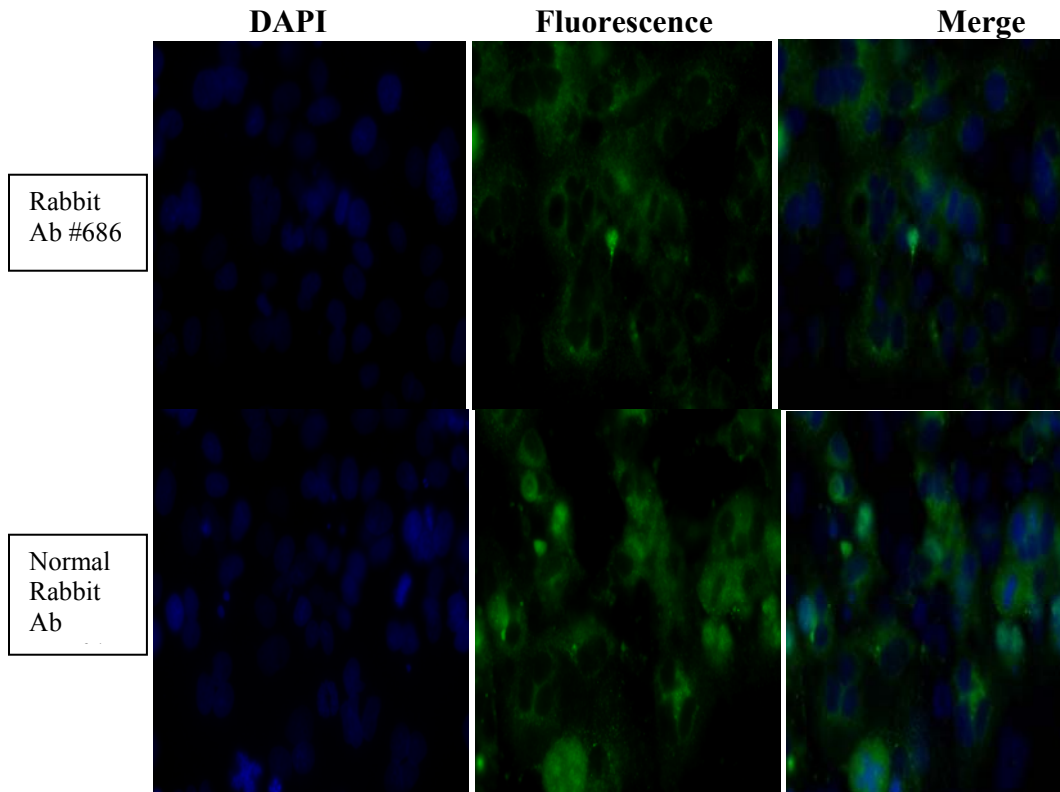


Figure 26. Immunofluorescence images of expressed *P. falciparum* pDisplay exon 1-3,44hr post transfection. Immunofluorescence localization of the exon 1-3 Rhop-3 pDisplay recombinant. Formalin fixed slides were incubated in rAb or normal mouse antibody and FITC-goat-anti-rabbit antibody. Partial Rhop-3 recombinant exon 1-3 showing expression by immunofluorescence using rAb #686. COS-7 cells exon 1-3 Rhop-3 recombinant incubated in normal rabbit antibody show some fluorescence.

COS-7 cells expressing *Plasmodium falciparum* Rhop-3 recombinant protein encoded by exon 7 were examined for surface expression by IFA using a rabbit anti-Rhop-3 antibody. Expression was detected using the rabbit antibody and the normal mouse antibody showed some cross reactivity [Fig 27].

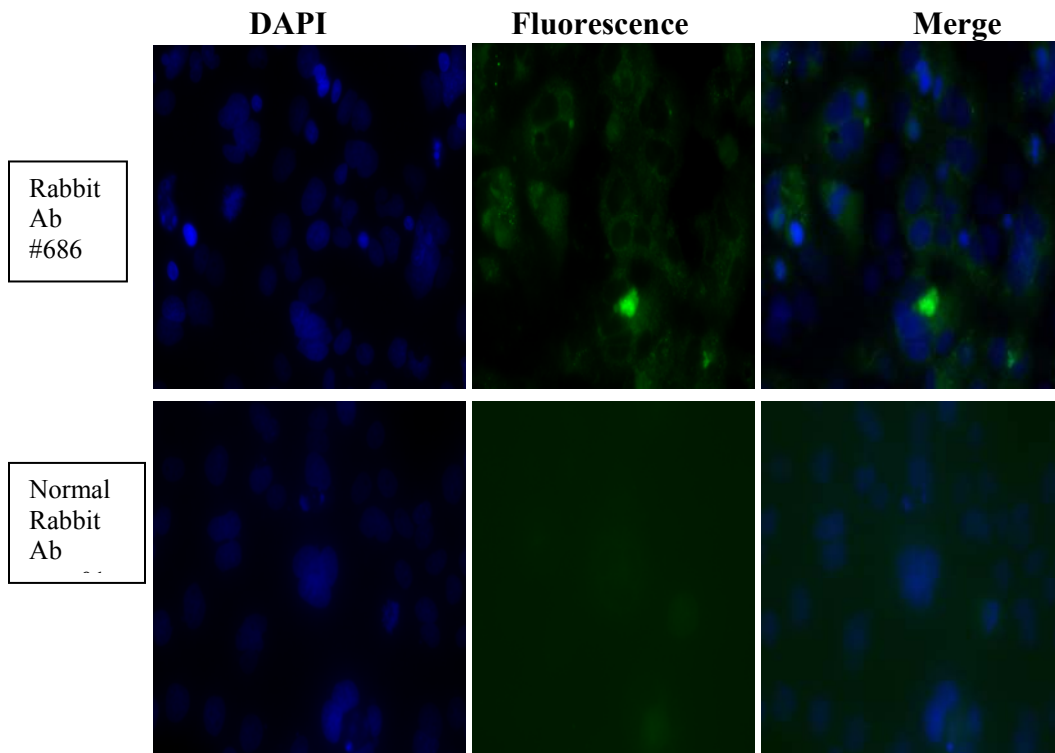


Figure 27. Immunofluorescence images of expressed *P. falciparum* pDisplay exon 7, 44hr post transfection. Immunfluorescence localization of the exon 7 Rhop-3 pDisplay recombinant. Formalin fixed slides were incubated in rAb or normal mouse antibody and FITC-goat-anti-rabbit antibody. Partial Rhop-3 recombinant exon 7 showing expression by immunofluorescence using rAb #686. COS-7 cells exon 7 Rhop-3 recombinant incubated in normal rabbit antibody show no fluorescence.

CHAPTER IV

DISCUSSION

The malaria rhoptry protein Rhop-3 is an erythrocyte binding protein involved in the recognition and invasion of host cells by merozoites. The C-terminus of Rhop-3 generates immune responses in individuals living in malaria endemic regions and antibodies against the protein can inhibit red blood cell invasion *in vitro*. The 110 kDa Rhop-3 protein is encoded by a 7 exon gene, however the binding domain of the protein has not yet been reported. The purpose of this study was to identify the binding domain of the Rhop-3 protein. Different regions of *P. falciparum* Rhop-3 and the C-terminus from of Rhop-3 from the rodent malaria parasite *P. yoelii* were expressed on the surface of COS-7 cells and subsequent erythrocyte binding assays (EBAs) carried out to examine the degree of binding.

Using cDNA from *P. falciparum* the full-length Rhop-3 gene (2.7 kb) pDIS-PF17, regions I-III (exon 1–3, 1.1 kb) pDIS-PF13, and region VI (exon 7, 0.6 kb) pDIS-PF7 were cloned into the vector pDisplay. PDIS-PY1412 encoding a protein in the C-terminus from Rhop-3 (~1 kb) also cloned into the vector pDisplay. To test recombinant protein binding to erythrocytes the purified clones were transfected into

COS-7 cells and recombinant proteins expressed on the surface of the COS cells. Erythrocyte binding assays were carried out using mouse and human cells and the cells checked for rosetting. Region II of the *P. vivax* Duffy Binding Protein (DBP_{II}) which binds to Duffy positive human RBC was used as a control for binding. The transfection efficiency of COS-7 cells was determined by immunofluorescence assays.

In these experiments, it was shown that the recombinant clones expressed the proteins of interest on the surface of the COS cells. Using rabbit antibody #686, which recognizes the *P. falciparum* Rhop-3 protein, I was able to show that pDIS-PF17, pDIS-PF13 and pDIS-PF7 were expressed on the surface of the COS cells. The recombinant *P. falciparum* Rhop-3 proteins did not bind to human or mouse RBCs

Using mouse antibody F1, which recognizes the *P. yoelii* Rhop-3 protein I was able to show that pDIS-PY1412 was expressed on the surface of the COS cells. In the EBAs pDIS-PY1412 bound to mouse erythrocytes but not to human erythrocytes. A very high level of binding was seen in one independent EBA experiment, however attempts to reproduce the binding in other independent assays was not successful. Erythrocyte invasion by merozoite is dependent on binding of parasite proteins to receptors on the RBCs [Adams et al., 1992]. Antibodies directed against erythrocyte binding proteins may block erythrocyte invasion making such ligands attractive candidates for malaria vaccines [Pandey et al., 2002].

A small number of malaria proteins have been extensively studied for their binding properties using the COS-7 system, mainly *P. falciparum* EBA-175 [Sim et al., 1994]

P. vivax Duffy Binding Protein [Chitnis & Miller 1994, Adams et al., 1992, Michon et al., 2001, VanBurskirk et al., 2004] and *P. yoelii* AMA-1 [Fraser et al., 2001].

An important aspect of the COS-7 system binding system is that the vector used enables surface expression of the parasite proteins of interest. The first vector used to express malaria protein to the surface of COS cells was an engineered vector pRE4 [Cohen et al. 1988]. Plasmid pRE4 contains the coding sequence gD-1 and the Rous sarcoma virus promoter was designed to subclone the gD gene a virion envelope glycoprotein of herpes simplex virus (HSV). Mutations were made in the gD-1 gene and the proteins expressed in mammalian cells to study the effects of mutations on expression of gD [Cohen et al., 1988]. From these studies they found that synthesis of gD-1 was sensitive to changes involving cysteine residues. pRE4 HSV gD has a 25 amino acid signal peptide and a 24 amino acid hydrophobic transmembrane region which targets proteins to the surface of mammalian cells. pRE4 (provided by Drs. Gary Cohen and Roselyn Eisenberg, University of Pennsylvania, Philadelphia, PA) has been the main vector of choice used to target different regions of malaria parasite proteins to COS-7 cells to study binding. At the beginning of my project I obtained the pRE4 vector from Dr. Cohen, however I was not able to use it to construct any of clones due to poor amplification of the vector.

Using the pRE4 vector the binding domain of a *P. vivax* and *P. knowlesi* erythrocyte binding ligand was identified to lie within region II of these proteins [Chitnis & Miller 1994]. For each protein different regions of the protein were targeted to the surface of COS cells and binding assays performed to identify which expressed proteins also showed rosetting to human RBC's. The *P. vivax* DBP protein

binds only to human erythrocytes expressing the Duffy group antigen. Using the same vector the binding domain of *P. falciparum* EBA-175 was identified as region II, a region containing a cysteine-rich motif and requirement for glycophorin A and sialic acid for erythrocyte recognition [Sim et al., 1994]. The *P. yoelii* AMA-1 erythrocyte binding domain was also identified using the pRE4 vector, region 1/2 mediated binding to mouse and rat erythrocytes but not human erythrocytes [Fraser et al., 2001].

The pRE4 vector has been effective in targeting different portions of malaria ligands to the surface of COS-7 cells to study binding, however, having a commercially available vector with the ability to target malaria proteins to the surface of mammalian cells would be more beneficial to researchers interested in studying binding.

Another designed vector which has been used to study erythrocyte binding proteins is HSVgD1/DBP_{II} plasmid pEGFP-N1 (Clontech) which has been engineered to express the chimeric protein as a recombinant fusion at the N-terminus of green fluorescent protein (GFP). The HSVgD1-DBP was amplified from a pRE4-DBP_{II} construct and inserted into the appropriate site of the pEGFP-N1 vector. This designed construct pEGFP-DBP_{II} has been used to further study the *P. vivax* DBP [Michon et al., 2000, Michon et al., 2001, VanBuskirk et al., 2004].

Besides *Plasmodium yoelii* AMA-1 [Fraser et al. 2001] the binding properties of rodent *Plasmodium* rhoptry proteins have not been investigated to show that a commercially available vector can also be successfully used to target proteins to the surface of mammalian cells to study erythrocyte binding. pDisplay has been used to

target other proteins such as hepatitis C virus envelope E2 protein [Forns et al.1999] and endoglin [Guerrero-Esteo et al. 2002] to the cell surface but has not been used to express malaria binding proteins.

Along with using the COS system erythrocyte binding activity has also been studied by means of using parasite culture supernatant, recombinant proteins and peptides. The two-stage binding of the *P. falciparum* EBA-175 antigen was identified using parasite labeled culture supernatant [Kain et al.1993]. In these assays uninfected erythrocytes were incubated with culture supernatant and bound proteins eluted with a high salt buffer. Erythrocytes can also be pre-treated with enzymes to study erythrocyte surface receptors required for protein recognition to facilitate binding. The binding properties of *P. falciparum* EBA-175 has also been extensively studied using peptides [Jakobsen et al., 1998, Sim et al., 1990] and recombinant proteins [Pandey et al., 2002]. Erythrocyte binding assays using peptides are useful in identifying specific sequence residues within known binding domains that are sufficient for binding.

The binding properties of *P. falciparum* AMA-1 has been studied using synthetic peptides [Urquiza et al.2001, Valbuena et al.2006], RAP-1 [Patarroyo et al. 2006] an extensively studied *Plasmodium vivax* rodent rhoptry protein and RAP-2 [Patarroyo et al. 2004] have been studied using synthetic peptides. Another extensively studied protein are the MSP proteins, MSP-1 has been the most characterized MSP protein. These proteins are involved in the initial interaction of the merozoite with erythrocyte receptors during invasion [Blackman et al.1991]. The binding properties of MSP

proteins have also been extensively studied using synthetic peptides [Patarroyo et al. 2005, Patarroyo et al.2006, Patarroyo et al.2007].

Erythrocyte binding assays using the COS system take a molecular approach to studying parasite ligand interaction with erythrocytes, this method can also be used to screen erythrocyte binding proteins that may be potential candidates for malaria vaccines.

The *Plasmodium falciparum* is highly (A + T) which makes it extremely difficult to express and maintain clones in *Escherichia coli*. The overall (A+T) composition of *P.falciparum* is 80.6% an up to ~ 90% in introns [Gardner et al. 2002] From my studies it is seen that, despite the great difficulty with working with an AT-rich genome of *Plasmodium falciparum*, it is possible to construct a full-length Rhop-3 recombinant protein. How stable the expressed protein is has not yet been determined but from IFA's there is expression. Mouse RBC bound to COS-7 cells expressing the PY1412 protein following transfection and binding assay using a fresh batch cloned DNA. Due to the (A+T) rich genome is frequent rearrangements of *Plasmodium* genes when expressed in different hosts causing those genes to not always be stable. Rearrangements in *P. yoelii* can cause the genes to be even less stable as its genome is even more AT rich than that of *P. falciparum*. This would give an account for the variability in binding seen with the *P. yoelii* P1412 recombinant protein.

The conditions used for the transfection and binding assays were modified to see if the binding results obtained with pDIS-PY1412 could be reproduced, however these were not done with fresh cloned DNA. These modifications included: (1) different incubation times for protein expression: 24hr, 42hr, 44hr, 48hr and up to 60 72hrs (2)

length of time incubated in transfection media: 4 hr and 24hr (3) transfection reagent: Lipofectamine, Lipofectin, Lipofectamine with Plus reagent and Lipofectin with Plus reagent (4) incubation time with RBCs: 2hr, 4hr, 6hr and overnight. (5) temperature for incubation with RBCs: RT and 37°C for 2hr, 4hr and overnight. One of my limitations during this study was available human red blood cells expressing the Duffy Blood group antigen to use with the PvDBP_{II} positive control. Due to this, binding using the Pv-DBP_{II} was not consistent suggesting that there were times which the blood obtained from Interstate Blood Bank may have contained erythrocytes not expressing the Duffy Blood group antigen. The Duffy antigen is not routinely checked for by the blood bank during the processing procedure.

Findings from the current studies and others using rodent rhoptry proteins will be of benefit to the investigation of other rhoptry proteins that may be involved in erythrocyte recognition and invasion. Due to the inconsistent binding obtained with PY1412 the binding conditions in future binding assays can be performed using other fragments within the C-terminus of the Rhop-3 protein. IFA results showed that the PY1412 protein was expressed on the cell surface however the epitope recognized by the Rhop-3 antibodies may not be the same epitopes recognized by receptors on the RBCs. This suggests that the inconsistency in binding may be a due to the conformation of the expressed protein and the availability of the epitope necessary for recognition by receptors on the erythrocytes. These studies can be to identify the specific sequence residues that are sufficient for binding. The specificity of Rhop-3 binding can be determined by using antibodies to see if this binding can be inhibited.

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