Identification of an Erythrocyte Receptor for *Plasmodium falciparum* Glutamic Acid-rich Protein (Pf-GARP)

A thesis submitted by

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Abstract

In the present study, I investigated the underlying basis of *Plasmodium falciparum* glutamic acid-rich protein (Pf-GARP) function in malaria pathogenesis by examining its direct interaction with human red blood cells (RBCs)/erythrocytes, and its impact on RBC aggregation. We hypothesized that specific region(s) of *P. falciparum* proteins bind to human RBCs and regulate malaria pathogenic effects mediated by cell adhesion. We identified Pf-GARP as a novel parasite ligand that binds to the surface of human RBCs. Two overlapping peptide segments of Pf-GARP, identified by two independent phage display cDNA screens, specifically interact with human RBCs. RBC-binding assays as well as CHO-K1 (Chinese Hamster Ovary) cells expressing Pf-GARP confirmed its direct binding to human RBCs. The overlapping segments are likely to cover the core binding-site of Pf-GARP required for its interaction with human RBCs. Using recombinant protein expression methods, we identified and mapped the minimum and stable region of Pf-GARP that binds to human RBCs. Pretreatment of RBCs by chymotrypsin reduced but did not eliminate Pf-GARP binding to human RBCs. Glycophorin B, a known chymotrypsin-sensitive receptor, was ruled out as a host receptor for Pf-GARP using glycophorin B (GYPB)-null (S-s-U-) human RBCs. Importantly, we identified band 3, also called the anion exchanger-1 (AE1) or solute carrier family 4 member 1 (SLC4A1), as a host receptor for Pf-GARP. Fusion proteins from Pf-GARP did not inhibit merozoite invasion in human RBCs under our *in vitro* conditions. However,

synthetic peptides derived from the RBC-binding domain of Pf-GARP induced formation of RBC aggregates. Collectively, our results provide evidence that Pf-GARP plays a role in enhancing the adhesive properties of RBCs. This novel feature of Pf-GARP is mediated either directly by Pf-GARP binding to RBCs or indirectly by modifying the conformational state of band 3. Our findings may also provide a molecular rationale for the well-known phenomenon that *P. falciparum* infection induces RBC adhesion by exposing an epitope within the ectoplasmic domain of band 3. We propose that inhibition of Pf-GARP suppresses the RBC-mediated adhesion events thus unveiling new potential therapeutic strategies to mitigate lesions in cerebral and pregnancy-associated malaria.

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List of Abbreviations

AA – amino acids

ACD – acid citrate dextrose

AE1 – the anion exchanger-1

ANOVA – analysis of variance

AP – alkaline phosphatase

BSA – bovine serum albumin

C – carboxyl

$C_{12}E_8$ – dodecyl octaethylene glycol ether

CAII – carbonic anhydrase II

CBB – Coomassie Brilliant Blue

CD36 – cluster of differentiation 36

CHO-K1– Chinese Hamster Ovary

CIDR – the cysteine-rich interdomain region

Cl^- – chloride ions

CMM – complete malaria media

CO – codon optimized

CO_2 – carbon dioxide

CR1 – complement receptor 1

CSA – chondroitin sulfate A

DBL – Duffy-Binding-Like

DBL1α1 – the first DBL domain

DBR – DIDS-binding region

DIDS – 4,4'-diisothiocyanostilbene-2,2'- disulfonic acid

DPBS – Dulbecco's phosphate buffered saline

DTT – dithiothreitol

E. coli – Escherichia coli

eAE1 – erythrocytes anion exchanger-1

ECL – enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

FBS – fetal bovine serum

FPLC – fast protein liquid chromatography

GAM – goat anti-mouse

GARP-L – Pf-GARP$_{356-552}$

GARP-M – Pf-GARP$_{370-444}$

GARP-M1– Pf-GARP$_{370-416}$

GARP-M2 – Pf-GARP$_{417-444}$

GARP-S – Pf-GARP$_{392-437}$

GC – S-s-U- chymotrypsin treated

gC1qR – globular C1q receptor

GFP – green fluorescence protein

GM-7 – Pf-GARP-M anti-mouse mAb

GPC – glycophorin C

GU – S-s-U- untreated

GYPB – glycophorin B

H^+ – proton

HBA1 – hemoglobin alpha 1

HBA2 – hemoglobin alpha 2

HBB – hemoglobin beta

HCO_3^- – bicarbonate ions

HIV – human immunodeficiency virus

HRP – horseradish peroxidase

HSV gD1 – the herpes simplex virus glycoprotein D protein

HT – host-targeting

ICAM1, CD54 – the intracellular adhesion molecule 1

IL-1 – interleukin-1

IPTG – Isopropyl-beta-D-thiogalactoside

iRBCs – infected RBCs

kAE1 – kidney the anion exchanger-1

KAHRP – knob-associated histidine-rich protein

LB – Luria broth

LCRs – low-complexity regions

Leu – Leucine

LILRB1 – leucocyte immunoglobulin-like receptor B1

mAb – monoclonal antibody

mAbs – monoclonal antibodies

MC – Maurer's clefts

metHb – methemoglobin

MW – molecular weight

N – amino

N_2 – nitrogen

NaCl – sodium chloride

NC – normal chymotrypsin-treated

NCBI – the National Center for Biotechnology Information

Nickel – Ni

NTS – N-terminal

NU – normal untreated

O_2 – oxygen

P-selectin – P stands for platelet

P. – Plasmodium

PBS – phosphate buffered saline

PBST – PBS plus 0.5% Tween-20

PCR – polymerase chain reaction

pen/strep – penicillin-streptomycin

PEXEL – *P. falciparum* export element

Pf-GARP – *Plasmodium falciparum* glutamic acid-rich protein

PfEMP1 – *P. falciparum* erythrocyte membrane protein 1

pfu – plaque-forming unit

pLIC – ligation independent cloning

PMSF – Phenylmethylsulfonyl Fluoride

PMV – plasmepsin V

PSAC – the plasmodial surface anion channel

PV – parasitophorous vacuole

PVM – parasitophorous vacuole membrane

RBCs – red blood cells

rcf – relative centrifugal force

RESA – *P. falciparum* ring-infected erythrocyte surface antigen

RIFINs – the repetitive interspersed families of polypeptides

RPA1 – Palo Alto strain

rpm – revolutions per minute

RPMI 1640 – Roswell Park Memorial Institute Medium

S – serine

S-s-U- – (GYPB)-null

SAO – the Southeast Asian Ovalocytosis

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEMP1 – the small exported *P. falciparum* membrane protein

SLC4A1 – solute carrier family 4 member 1

STEVOR – subtelomeric variant open reading frame

TB – tuberculosis

TBST – tris-buffered saline with 1% Tween-20

TNF-α – tumor necrosis factor

Trx – thioredoxin

Trx-GARP – Pf-GARP cloned into pET32 vector

TSP – thrombospondin

Tyr – Tyrosine

uRBC – uninfected RBCs

WBCs – white blood cells

WD – tryptophan-aspartic acid

WHO – the World Health Organization

ZC3H6 – zinc finger CCCH-type containing 6

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Chapter 1: Introduction

1.1. Malaria: General Introduction

1.1.1. Malaria: Infection

Malaria is a serious disease that affects a massive number of people. Severe malaria is a life-threatening disease that kills about 15-20% of infected people regardless of the mode of treatment with currently existing antimalarial drugs and the efforts made to eliminate the disease (Miller et al., 2013; Rowe et al., 2009). In 2016, the Centers for Disease Control and Prevention (CDC) estimated 216 million cases of malaria infection worldwide resulting in 445,000 deaths mainly children in sub-Saharan Africa (Kumar et al., 2017). According to the World Health Organization (WHO), this estimate has been increased by 5 million cases as compared to 2015 even though the number of malaria deaths remained nearly the same (WHO, 2017a).

Malaria disease has been documented long time ago but the discovery of the parasites in blood was made in 1880 by Alphonse Laveran (Cox, 2010). Malaria parasites cannot reproduce on their own. They need to live inside a host to be able to survive and replicate. They alter host environment for their own benefits. The parasites use plasma free amino acids as additional source for synthesizing their critical proteins since they have partial ability for amino acid biosynthesis. They also modify host RBC proteins for survival and pathogenesis. The parasites catabolize hemoglobin in their digestive vacuoles and convert it into hemozoin, which forms malaria pigment. Consequently, they use up nearly all hemoglobin derived amino acids for the synthesis of their own essential proteins (Goldberg et al., 1991; Moore et al., 2006; Sherman, 1977). Among ~250 different malaria species, only four species display the capability to infect human; *P.*

falciparum, *P. vivax*, *P. malariae*, and *P. ovale*. *P. knowlesi* is the fifth species that has been detected to infect humans lately although it is known to mainly infect macaque monkeys (Ramasamy, 2014). The various malaria species vary in their specificity, areas where they are endemic, the severity of the disease they cause, their treatment strategies and susceptibility to drug resistance (Bloland, 2001).

1.1.2. Malaria: Geographical Distribution

According to the 2017 Malaria Report published by the WHO, parasite resistance to drugs and mosquito resistance to insecticides play an important role in the failure to eliminate the disease globally. In 2016, approximately 194,000 malaria cases had been reported in Africa, mostly in Nigeria, whereas the South-East Asia Region had the most *P. vivax* cases with India reporting the highest percentage of infections (51%). The Eastern Mediterranean Region is also considered a place for endemic malaria. Globally, ~445,000 deaths occurred annually, mostly in Africa followed by the South-East Asia, the Eastern Mediterranean, the Western Pacific, and the Americas. No death was reported in the European region (WHO, 2017c). *P. knowlesi* is widely distributed in Malaysia and the Southeast Asia Region (Barber et al., 2017).

1.1.3. Malaria: Parasite Life Cycle

The complex life cycle of *P. falciparum* parasite takes place in multiple hosts infecting several different but specific cell types based on specific interactions between parasite proteins and host proteins (Acharya et al., 2017; Lobo et al., 2003; Oh and Chishti, 2005; Paul et al., 2015; Rowe et al., 2009). Malaria parasite is transmitted via the bloodmeal of an infected female *Anopheles* mosquito. The mosquito transmits sporozoites from its saliva into the bloodstream where they migrate to human

hepatocytes. The malaria parasites proliferate in liver producing thousands of merozoites, which are released into the blood stream where they infect red blood cells. Each merozoite can invade a RBC, and subsequently begins the intraerythrocytic stage of maturation characterized by the ring, trophozoite and schizont stages of development. The rupture of infected RBCs (iRBCs) results in the release of anywhere between 10-30 daughter cells (merozoites) initiating the next round of invasion (Hisaeda et al., 2005; Miller et al., 1994; Oh and Chishti, 2005). Several critical steps are required for successful invasion of merozoites to RBCs. These steps include the initial adhesion, re-orientation, propulsion, and finally invagination into host RBCs (Cowman et al., 2012; Mitchell et al., 2004; Oh and Chishti, 2005; Srinivasan et al., 2011). By some poorly understood trigger mechanism, the intraerythrocytic parasites differentiate into female and male gametocytes during the blood stage of parasite development. The parasite-infected RBCs are taken up by the mosquito in the next blood meal, and undergo sexual reproduction inside the mosquito midgut to form sporozoites that have the ability to infect the salivary glands. Thus, the liver cycle of human infection starts again by a subsequent blood meal by the infected mosquito (Aly et al., 2009).

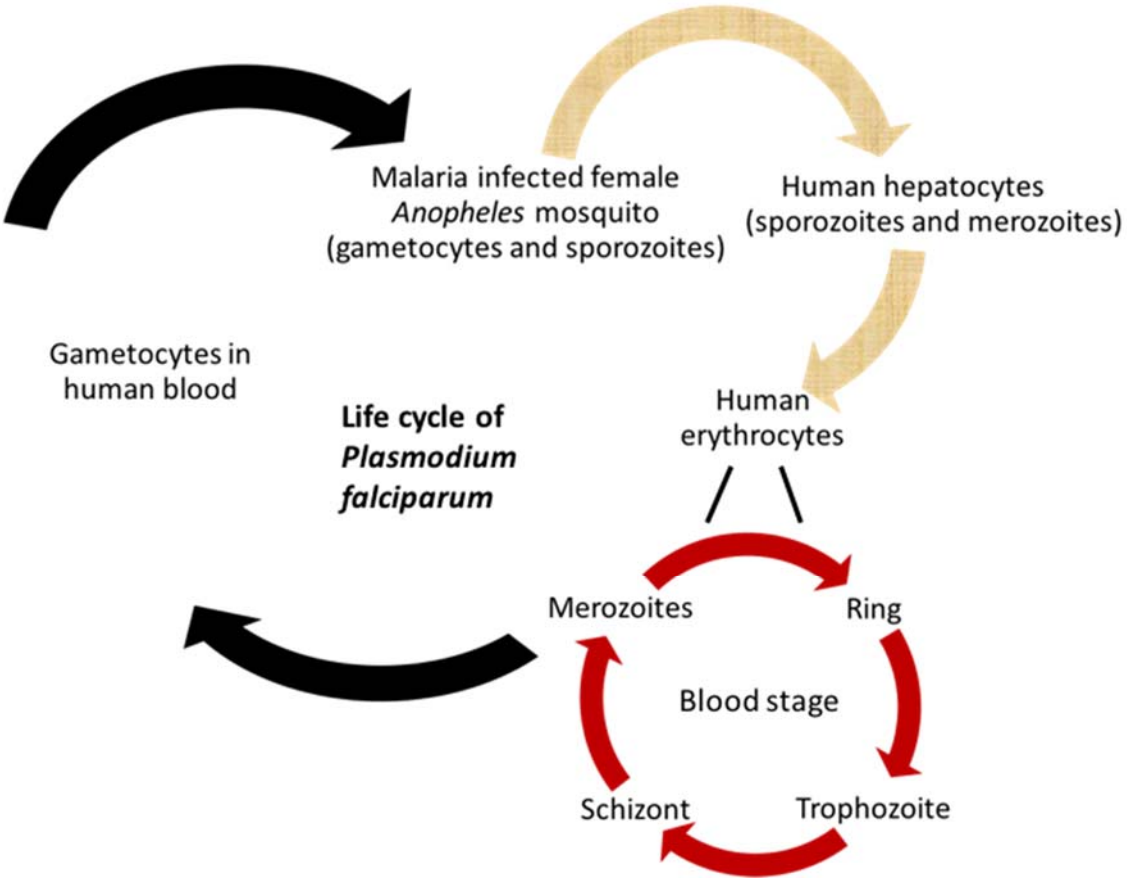


Figure 1.1. The Life Cycle of *Plasmodium falciparum* Involves Mosquito, Human Liver and Blood Stages

Schematic representation of the life cycle of Plasmodium falciparum and other human malaria parasites. The infection starts with the transfer of gametocytes through a blood meal from an infected individual by female Anopheles mosquito. Sporozoites formed in mosquito are then transferred to humans during the next mosquito bite. The parasites initiate liver infection followed by the invasion of RBCs. During the blood stage infection, parasites multiply asexually, and a relatively small percentage also differentiates sexually into gametocytes. The parasite life cycle begins in the mosquito vector during the next blood meal of human host. This figure has been adapted from (CDC, 2016), which is in the public domain and does not require copyright permission. Changes include creation a simplified version of the published figure.

1.1.4. Malaria: Clinical Symptoms and Complications

Although human malaria can be caused by five different species of the genus *Plasmodium*, *P. falciparum* is the most lethal species among the human malaria parasites (Mal, 2011; Rowe et al., 2009). *P. falciparum* infection causes high mortality with ~90% of deaths occurring in sub-Saharan Africa, and children are most vulnerable to infection (Langhorne, 2005). Since the liver stage of malaria infection is asymptomatic, early detection of the disease transmission is not feasible. This limitation is especially critical in malaria endemic areas. The disease symptoms begin to appear mainly at the blood stage of infection. These symptoms include anemia, chills, and high fever due to the rupture of iRBCs and accumulation of toxins in the blood stream which ultimately lead to the activation of macrophages and secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-1 (IL-1) (Chen et al., 2000b; Craig et al., 2012; WHO, 2014). Severe symptoms of malaria include high parasitemia, life threatening anemia due to the hemolysis of iRBCs and uRBCs (uninfected RBCs), hypoglycemia, metabolic acidosis, convulsions, pulmonary edema, and renal failure. Organ dysfunction and coma can occur in very severe cases of malaria infection (Cavaillon, 2001; Chen et al., 2000b; Miller et al., 1994; WHO, 1990; WHO, 2014; WHO, 2015). In fact, the development of cerebral malaria and placental malaria during pregnancy, which may lead to coma and fetal impairment, respectively, are well-known examples of organ failure (Kirchgatter and Del Portillo, 2005; Sherman et al., 2003). Symmetrical peripheral gangrene has also been reported as a rare complication of severe malaria infection (Gupta et al., 2013; Kumar et al., 2017; Sharma et al., 2005).

1.1.5. Malaria: Treatment Options

Current malaria treatment guidelines slightly differ between the CDC and the WHO. The detailed treatment and management protocols are available via published sources (Bloland, 2001; CDC, 2017a; CDC, 2017b; WHO, 2015; WHO, 2017b). Briefly, under both guidelines, the treatment options depend on malaria parasite species, severity of the disease, susceptibility to drug resistance and patient's condition. For example, the patient might be young, pregnant, obese, non-immune or has co-infections such as human immunodeficiency virus (HIV) or tuberculosis (TB). A general recommendation is to use combinational therapy of drugs acting via different mechanisms of action to inhibit the occurrence of severe malaria due to drug resistance. For instance, co-formulated effective drugs in a single tablet are available to enhance the patient compliance. Intramuscular, intravenous and rectal mode of drug combinations are also available in severe cases of infection. Current widely-used medications are artemisinin compounds, quinine and its derivatives, antifolate combination drugs and antibiotics. Their precise mechanisms of action are not fully understood. These drugs are believed to kill the parasites at specific stages of development either by inhibiting heme detoxification step by interfering with the polymerization of hemoglobin toxic by-product to the non-toxic hemozoin, inhibiting biosynthesis of nucleic acids and protein synthesis, and other known and unknown mechanisms (WHO, 2015). The drugs targeting iRBCs can reach their targets by either facilitated or active diffusion through the RBC lipid bilayer when they are uncharged or by passing though the parasite-induced channels on the surface of iRBCs to facilitate the uptake of nutrients and other solutes from plasma, e.g. the plasmodial surface anion channel (PSAC) (Basore et al., 2015). It is also recommended to treat severe malaria

complications by using antipyretics, anti-emetics and anticonvulsants depending on patient specific symptoms (WHO, 2015).

1.1.6. Remodeling of Malaria Infected Erythrocytes

1.1.6.1. Export of Virulence Proteins by Malaria Infected RBCs to Specific Destinations

Following invasion, severe modifications of iRBCs occur mainly by the export of hundreds of proteins synthesized inside the parasite and translocated to the RBC cytoplasm and membrane via complex trafficking mechanisms. These proteins are usually expressed at the trophozoite and schizont stages of parasite development (Florens et al., 2004), and believed to play a unique role in *P. falciparum* surface virulence and survival due to enhanced iRBC adhesiveness (Beck et al., 2014; Elsworth et al., 2014; Maier et al., 2008). These features are distinct from the malaria invasion steps that are mainly mediated by the merozoite surface proteins (Beeson et al., 2016).

Host-targeting (HT) motif or *P. falciparum* export element (PEXEL), is a conserved motif across all *P. falciparum* species that is responsible for protein translocation through the parasitophorous vacuole membrane (PVM), the membrane surrounding the parasite in iRBC (Hiller et al., 2004; Marti et al., 2004). PEXEL motif is recognized, cleaved, and processed by the endoplasmic reticulum protease enzyme termed plasmepsin V (PMV). Acetylation of the new amino terminus of the cleaved protein occurs, which then it moves to parasitophorous vacuole (PV), passes through PVM, and translocates to other destinations (Beck et al., 2014; Elsworth et al., 2014; Goldberg and Cowman, 2010; Haase and de Koning-Ward, 2010; Hiller et al., 2004;

Marti et al., 2004; Soni et al., 2016). Notwithstanding this established export mechanism, there are alternate trafficking pathways for exporting PEXEL-containing proteins that are not cleaved by PMV as well as PEXEL-negative proteins (Boddey et al., 2013).

RBC modifications, which have been extensively investigated and reviewed, are believed to affect RBC sequestration (Acharya et al., 2017; Chen et al., 2000b; Kirchgatter and Del Portillo, 2005). Sequestration is a strategy used by the parasite to avoid immune cell detection and spleen clearance of iRBCs by their adhesion to vascular endothelium; thus, avoiding removal from the peripheral circulation (Chen et al., 2000b; Rowe et al., 2009). Several factors are believed to influence sequestration phenomenon. For example, reduced RBC deformability is one of the main alterations that negatively affect flow of iRBCs through small blood vessels. This feature occurs in proportion to the severity of disease, and is not limited to iRBCs. In fact, the reduced flexibility has been detected even in uRBCs (Chen et al., 2000b; Dondorp et al., 1997). Increased stickiness of iRBCs, and to a lesser extent uRBCs, is one of the main alterations that contributes to the virulence of *P. falciparum* (Balaji and Trivedi, 2013; Treutiger et al., 1992). It is presumed that the adhesion effect of blood cells can be classified into binding of iRBCs to endothelial receptors (cytoadherence), binding of uRBCs to iRBCs (rosetting), binding of iRBCs to other iRBCs and platelets (auto-agglutination or platelet-mediated clumping) (Rowe et al., 2009) and binding of uRBCs to each other (RBCs aggregation) (Balaji and Trivedi, 2013; Dondorp et al., 2000).

1.1.6.2. Reduced Deformability of RBCs

RBCs are normally deformable. They become rigid upon *P. falciparum* infection (Dondorp et al., 2000). Exported proteins and their interactions with the RBC cytoskeleton have been shown to be the main contributory factors for iRBC rigidity (Cooke et al., 2001; Hosseini and Feng, 2012; Maier et al., 2008). The change in rigidity of iRBCs is believed to affect iRBCs sequestration in *P. falciparum* infection mainly, since the *P. vivax*-infected RBCs oppositely become more deformable upon infection and they are not known to sequester in small blood vessels (Handayani et al., 2009; Suwanarusk et al., 2004). Targeted gene disruption methodologies and generation of mutant *P. falciparum* cell lines have identified several important genes whose genetic disruption caused a significant reduction in the stiffness of iRBCs. One example is *P. falciparum* ring-infected erythrocyte surface antigen (RESA) (Maier et al., 2008; Silva et al., 2005). In addition, the secreted *P. falciparum* proteins are also believed to play a role in decreasing the deformability of RBCs since it was observed that the plasma membrane of uRBCs became more rigid in *P. falciparum* cultures *in vitro* (Chen et al., 2000b; Dondorp et al., 1997; Naumann et al., 1991).

1.1.6.3. Increased Cytoadherence in *P. falciparum* Malaria

Adherence of iRBCs to host vasculature, particularly to the endothelial cells of small blood vessels in several organs, specific epithelial placental cells and immune cells has been observed in severe malaria patients (Craig et al., 2012; Cserti-Gazdewich et al., 2012; David et al., 1983; Rowe et al., 2009). The precise molecular mechanisms of cytoadherence and sequestration that lead to microvascular occlusion remain poorly understood. However, several ligand-receptor interactions involved in iRBC

cytoadhesion process have been identified. For instance, the antigenically variant *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, which is the main virulence and adhesive ligand, had been identified on the surface of iRBCs in association with surface knobs (Cooke et al., 1998; Crabb et al., 1997). Knobs are electron-dense protrusions on the surface of iRBCs and are believed to contribute to increased RBC adhesive properties (Crabb et al., 1997; Oh et al., 2000; Raventos-Suarez et al., 1985). Parasite-encoded knob-associated histidine-rich protein (KAHRP) is also a key component of knobs, expressed at the cytoplasmic side of the RBC membrane (Maier et al., 2009; Oh et al., 2000). However, there is some evidence that PfEMP1 is also expressed in knobless parasites that they can maintain their iRBCs cytoadherence capability, however, some reduction in cytoadherence of iRBCs that lack knobs has been reported several times due to decreased PfEMP1 surface expression (Stanisic et al., 2016).

Numerous studies have shown that PfEMP1 plays a key role in the cytoadherence and rosetting of iRBCs (Langhorne, 2005; Maier et al., 2009). PfEMP1 consists of highly variable extracellular domain and semiconserved intracellular segment (Maier et al., 2009; Smith and Craig, 2005). Duffy-Binding-Like (DBL) domain and the cysteine-rich interdomain region (CIDR) are the main adhesive modules that have been identified to bind various receptors such as the intracellular adhesion molecule 1 (ICAM1, CD54), which is expressed on endothelial cells and leukocytes, and cluster of differentiation 36 (CD36), which is expressed on endothelial cells, epithelial cells, platelets, macrophages, monocytes, and adipocyte (Baruch et al., 1997; Chen et al., 2000a; Cooke et al., 1998; Gullingsrud et al., 2013; Mayer et al., 2009; Rowe et al., 2009; Smith et al., 1998; Smith

et al., 2000). Little evidenced showed CD36 expression on the surface of RBCs (Handunnetti et al., 1992; Rowe et al., 2009). Additionally, the DBL domains of VAR2CSA, a protein that belongs to the PfEMP1 family, have been demonstrated to bind chondroitin sulfate A (CSA) in the placenta, and may contribute to pregnancy-associated malaria (Adams et al., 2006; Andersen et al., 2008; Andrews et al., 2005; Buffet et al., 1999; Obiakor et al., 2013; Resende et al., 2009; Resende et al., 2008).

PfEMP1-mediated cytoadhesion has become an important area of study in the malaria field to discover new strategies for reducing malaria pathogenesis associated with the adhesion of iRBCs. Several *P. falciparum* proteins have been identified that regulate PfEMP-1 trafficking, its localization on the surface of iRBCs, and/or its correct conformation (Maier et al., 2008; Nacer et al., 2015; Oberli et al., 2016). In addition, several other protein families have been identified that play functional roles in the PfEMP1-independent cytoadhesion process. One such example is as the repetitive interspersed families of polypeptides (RIFINs), which were have been recently shown to bind leucocyte immunoglobulin-like receptor B1 (LILRB1) expressed on immune cells and hence inhibit their activation in malaria infection (Saito et al., 2017).

1.1.6.4. Rosetting

Rosetting pertains to binding of multiple uRBCs to one or two iRBCs. This phenomenon has been linked to severe malaria thorough the enhancement of RBC sequestration, mostly during the trophozoite and schizont stages of iRBCs (Carlson, 1993; Ch'ng et al., 2016; Doumbo et al., 2009; Fernandez et al., 1998; Ho et al., 1991; Treutiger et al., 1992; Udomsangpetch et al., 1989). Partial inhibition of blood flow *ex*

vivo occurred with rosetting-negative and cytoadherent-positive parasites while complete vascular occlusion was detected with rosetting and cytoadherent positive parasites (Kaul et al., 1991; Treutiger et al., 1992). Upon rosetting, the parasites were sequestered in cerebral blood vessels of patients died from cerebral malaria (Treutiger et al., 1992).

A functional role of several malaria ligands and RBC receptors has been identified in rosetting formation. The extracellular regions of PfEMP1 from the VarO variant of *P. falciparum*; specifically, the N-terminal segment (NTS) and the first DBL domain (DBL1α1) expressed on the surface of iRBCs were found to be involved in rosetting via binding to heparan sulfate (Vogt et al., 2003). Although heparin was able to inhibit rosetting formation under these conditions, this strategy was not an appropriate treatment option for complicated malaria since heparin infusion caused severe bleeding (Adams and Rowe, 2013; Angeletti et al., 2015; Chen et al., 1998; Juillerat et al., 2010; Juillerat et al., 2011; Treutiger et al., 1992). However, sevuparin, which has similar anti-adhesive properties to heparin but does not have any antithrombin activity, is currently under investigation for anti-rosetting and anti-cytoadherence activities (Saiwaew et al., 2017). Besides, monoclonal antibodies (mAbs) against DBL-1 domain of PfEMP1 and antibodies against the NTS-DBLα were able to inhibit and disrupt rosette formation induced by 89F5 Palo Alto VarO and TM284varl parasites, respectively (Adams and Rowe, 2013; Guillotte et al., 2016). Nevertheless, functional involvement of rosetting ligands other than PfEMP1 in *P. falciparum* infection cannot be excluded at this stage.

Human RBCs from blood groups A and B that were infected with the rosette-forming *P. falciparum* clone, Palo Alto strain (RPA1), were able to form rosettes more

efficiently than iRBCs of blood group O (Moll et al., 2016; Pipitaporn et al., 2000; Udomsangpetch et al., 1993). In fact, *P. falciparum*–encoded RIFINs have been demonstrated to be expressed on the surface of iRBCs and are involved in cytoadherence and rosetting formation especially when using RBCs from blood group A, whereas PfEMP1-mediated rosetting of RBCs occurs mainly in RBCs from blood group O (Fernandez et al., 1999; Goel et al., 2015; Kyes et al., 1999). Additionally, a recent study showed that a multi-gene family encoding subtelomeric variant open reading frame (STEVOR), which is expressed on the surface of iRBCs, is responsible for rosetting formation via binding to RBC glycophorin C (GPC) in a way that was independent of PfEMP1 interaction (Niang et al., 2014). Moreover, erythrocytes lacking complement receptor 1 (CR1) and infected with *P. falciparum*-rosetting laboratory strains were unable to form RBC rosettes (Rowe et al., 1997; Rowe et al., 2000). Together, these studies suggest that multiple mechanisms may account for the rosetting phenomenon observed in severe malaria infection caused by *P. falciparum*.

The hypothesis that the soluble parasite proteins shed from iRBCs and their binding to uRBCs enhances invasion and/or rosetting by acting as receptor(s) for merozoites or other iRBCs has been tested before by measuring the ability of normal uRBC or cultured uRBCs harvested from *P. falciparum* culture to disrupt preformed rossettes. It was concluded that this hypothesis might not be true under these conditions since both groups of uRBCs had the ability to form new rosettes similarly (Treutiger et al., 1992; Wahlgren et al., 1989; WHO, 1986). However, effect of secreted proteins from iRBCs in culture media on rosettes formation was not eliminated. Thus, there remains a possibility that secreted proteins might boost parasites' detrimental effects since

formation of huge rosettes consisting of ~40 uRBCs bound to ~15 iRBCs or even ~10 uRBCs bound to one iRBC has been documented (Treutiger et al., 1992). Clearly, the surface area of one RBC is not sufficient to bind ten or more cells and there is no direct binding of all uRBCs to the iRBC (Treutiger et al., 1992), suggesting that iRBCs secrete proteins that recruit uRBCs for binding, possibly by their ability to self-associate while retaining the RBC-binding functionality.

1.1.6.5. Platelet-mediated Clumping of Infected RBCs

Platelet-mediated clumping is another phenomenon that has been believed to be associated with the development of severe malaria and sequestration via infected erythrocytes-induced aggregates formation with platelets (Chotivanich et al., 2004; Mayor et al., 2011; Pain et al., 2001; Wassmer et al., 2008). Several receptors have identified to be involved in this mechanism, such as CD36, P-selectin (P stands for platelet) and globular C1q receptor (gC1qR) (Arman et al., 2013; Biswas et al., 2007; Mayor et al., 2011; Pain et al., 2001; Wassmer and Grau, 2017; Wassmer et al., 2008). However, the precise mechanism of platelet-mediated clumping remains unclear at this stage.

1.1.6.6. Aggregation of Uninfected RBCs

Autopsy of patients died from severe cerebral malaria showed aggregates of both iRBCs and uRBCs detected in the obstructed cerebral vessels. Serum proteins and *P. falciparum* secreted knob antigens were also detected in the blocked areas (Aikawa, 1988; Treutiger et al., 1999; Wahlgren et al., 1989). In fact, both serum proteins and *P. falciparum* secreted or released proteins are believed to enhance rouleaux formation

(stacking of RBCs) and RBCs aggregation (Balaji and Trivedi, 2013; Dondorp et al., 2000; Treutiger et al., 1999; Yamamoto, 1986). Methemoglobin (metHb), an oxidized form of hemoglobin released upon RBC lysis, has also been demonstrated to form larger RBC aggregates in *P. falciparum* culture (Balaji and Trivedi, 2013). Nevertheless, there are many studies on malaria rosetting process rather than uRBCs aggregation alone indicating the functional importance of this phenomenon in severe pathology.

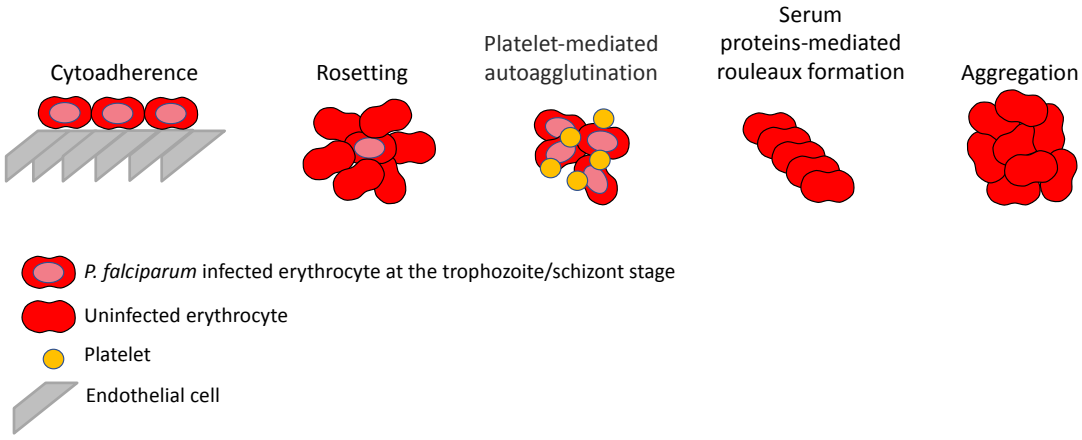


Figure 1.2. Factors Contributing to Sequestration in Severe *Plasmodium falciparum* Infection
Models of different *P. falciparum* virulence phenomena at the blood stage infection are presented. Multiple aggregation models are believed to trigger blood vessel obstruction and organ damage by forming sticky clumps in severe malaria. This figure has been adapted and reproduced with permission from "Rowe, J. A., Claessens, A., Corrigan, R. A., & Arman, M. (2009). Adhesion of Plasmodium falciparum-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. *Expert Reviews in Molecular Medicine, 11*, E16. Doi:10.1017/S1462399409001082. Figure 2. Page 4. © Cambridge University Press". Changes include addition of other iRBC-adhesion phenomena: Serum proteins-mediated rouleaux formation (Treutiger et al., 1999) and RBC aggregation (Balaji & Trivedi, 2013).

1.2. Strategies to Eradicate Malaria

1.2.1. Current Status of Protein-based Malaria Vaccine

Developing an effective vaccine against malaria, mainly *P. falciparum* infection, and its specific pathogenic effects offers the most promising avenue to decrease mortality, reduce severe complications and prevent transmission (Mal, 2011). However, this milestone has represented an enormous challenge for investigators mainly due to the fact that pathogenesis of human malaria triggered by *P. falciparum* cannot be tested on accessible animal models (Vaughan et al., 2012). Several *P. falciparum* malaria vaccine candidates to block several stages of malaria infection are at preclinical or clinical stages of development. RTS,S/AS01 is the only vaccine thus far developed that is based on recombinant protein components derived from pre-erythrocytic stage antigens, mainly the sporozoite surface *P. falciparum* circumsporozoite protein. Although this vaccine has completed the Phase 3 clinical trial with positive regulatory evaluation, complete protection of children enrolled in the study was not achieved. Accordingly, the vaccine needs to be assessed in depth before reaching the market (Barry and Arnott, 2014; WHO, 2016). A general perception is that the RTS,S/AS01 vaccine may not be very effective because it does not include blood stage antigens responsible for the clinical symptoms of malaria infection initiated by the RBC reinvasion of merozoites or the cytoadherence properties of iRBCs, as well as lack of any gametocyte and oocyst antigens responsible for the transmission of the disease (Barry and Arnott, 2014). The vaccine only targets the sporozoite antigens, and if only a single sporozoite successfully reaches the hepatocyte, it will produce ~10,000 merozoites thus re-initiating the infection (Barry and Arnott, 2014). Thus, an ideal malaria vaccine would include a combination of multiple protein antigens

against multiple stages of malaria infection to reduce morbidity and mortality especially since a small fraction of infections can be transmitted congenitally or via blood transfusion from affected patients (Draper et al., 2015; Josling and Llinas, 2015; WHO, 2016).

1.2.2. Phage Display Technology to Identify New Malaria Ligand-receptor Interactions

Notwithstanding recent progress, relatively little is known about hundreds of undefined malaria ligands and their corresponding host receptors. There is an urgent need for the identification of putative novel malarial mediators that play a functional role during the pathogenesis of the malaria disease.

Phage display cDNA screening technology is one of the most effective methods to identify potential vaccine candidates via screening millions of independent proteins and peptides displayed on the surface of bacteriophage virions as fused proteins, without affecting the infective capability of the bacteriophages towards *Escherichia coli* (*E. coli*) bacteria. The displayed peptides are screened by virtue of their binding to particular receptors or molecules, and specific bound phage particles can be eluted and amplified in bacterial cultures through several rounds of biopanning (Bazan et al., 2012a; Bazan et al., 2012b; Hamzeh-Mivehroud et al., 2013; Kay and Hoess, 1996; Lanzillotti and Coetzer, 2008; Smith, 1985).

The Chishti laboratory has used phage display technology to identify ligand-receptor interactions at the blood stage of malaria infection (Baldwin et al., 2015; Li et al., 2008; Li et al., 2012). The *P. falciparum* merozoite surface protein (MSP-1), which is a promising component of the second generation of malaria vaccine, was identified as

one of the putative antigens (Baldwin et al., 2015; Barry and Arnott, 2014). In this thesis work, we have identified a novel antigen termed Pf-GARP from two independent phage display cDNA libraries. Pf-GARP has a potentially promising role at the blood stage of malaria infection as discussed later. We identified several overlapping segments of Pf-GARP that bind to intact RBCs from phage clones displaying millions of independent *P. falciparum* peptides. We have confirmed and validated the RBC binding ability of Pf-GARP through different means. Moreover, we have mapped the core binding site of Pf-GARP that is sufficient for its interaction with human RBCs. Finally, we have identified the RBC receptor of Pf-GARP. The implications of these findings will be discussed in the context of malaria pathogenesis caused by *P. falciparum*.

1.2.2.1. Pf-GARP is a Novel Ligand Identified by Multiple Phage Display cDNA Library Screens

Pf-GARP (PF3D7_1133400, PFA0620c), comprised of 26% of glutamic acid residues (Triglia et al., 1988), is one of the exported proteins with a PEXEL motif (Maier et al., 2008). We identified Pf-GARP as a novel ligand using phage display *P. falciparum* 3D7 cDNA library screen based on its interaction with human RBCs. The overall objective was to establish the biochemical basis of Pf-GARP mediated recognition of RBCs, and determine the functional role of Pf-GARP in contributing to the multiple *P. falciparum* virulence phenomena.

Pf-GARP, whose function has not yet been identified, is detected in rings, trophozoite and schizont stages of parasite development with the last two stages being more related to cytoadherence phenomenon (Aurrecoechea et al., 2009; David et al., 1983; Florens et al., 2004; Maier et al., 2008). One study showed that Pf-GARP

antibodies have been detected in the plasma of children resistant to malaria and a polyclonal Pf-GARP antibody inhibited trophozoite development of *P. falciparum* 3D7 parasites by 99% (Raj et al., 2016). Homologues of Pf-GARP have been detected in different isolates of *P. falciparum* but this gene has not been detected in other species of human malaria parasites including *P. vivax* (Maier et al., 2008; Triglia et al., 1988). More recent studies revealed the existence of Pf-GARP in chimpanzee parasite species termed *P. reichenowi* CDC and *Plasmodium gaboni*, which are closely related to the human malaria parasite, *P. falciparum* (Liu et al., 2010; Otto et al., 2014; Sundararaman et al., 2016). These findings suggest that Pf-GARP plays a functional role that is unique to the lesions caused by *P. falciparum*.

Using transcriptome analysis, Pf-GARP gene expression was found to be up-regulated in parasites that lack the small exported *P. falciparum* membrane protein (SEMP1), a newly characterized Maurer's clefts (MC) membrane protein that is partially translocated to the membrane of iRBCs (Dietz et al., 2014). This finding indicates that Pf-GARP can interact with proteins that have the ability to interact with host the RBC membrane. Pf-GARP has been shown to be differentially regulated in adhesive *P. falciparum* parasites, FCR-3-CSA and FCR-3-CD36 (Davies et al., 2016; Ralph et al., 2005). Both Pf-GARP gene and peptide expression were increased in parasites isolated from Tanzanian children with malaria (Vignali et al., 2011), indicating its direct or indirect role in promoting severe malaria in children. The effect of multiple specific *P. falciparum* gene deletions on the surface expression of PfEMP1, the foremost cytoadherence protein, was tested by Maier et al (Maier et al., 2008). They generated malaria parasite specific gene knockout in CSA *P. falciparum*, a strain known to express

a specific variant of PfEMP1 encoded by VAR2CSA gene that binds to CSA. Therefore, effect of genes deletion on the adherence of iRBC was studied. They used antibodies specific for the PfEMP1 VAR2CSA segment to detect its change in expression on the surface of parasitized RBCs. Pf-GARP gene knockout in the CSA *P. falciparum* clone resulted in a significant decrease in the reactivity of antibodies to PfEMP1 indicating that lack of Pf-GARP expression has a critical impact on PfEMP1 surface expression on the surface of iRBCs. To further validate that decrease in reactivity, they treated RBCs infected by Pf-GARP-knockout-CSA parasites with trypsin to measure the effect of Pf-GARP gene deletion on the surface expression of the trypsin-resistant segment of PfEMP1. Although no direct strong effect on the expression of PfEMP1-trypsin-resistant segment was detected, subsequent experiments showed that the adherence of iRBCs to CSA was decreased by 70% or more indicating the critical importance of Pf-GARP in promoting the adhesive properties of iRBCs, probably in a PfEMP1-independent manner or possibly affecting PfEMP1 proper conformation for enhanced adhesion. No clear effect on the rigidity of iRBCs was observed upon Pf-GARP gene deletion (Maier et al., 2008).

Pf-GARP consists of multiple low-complexity regions (LCRs) between amino acids (AA) 70-78, 118-166, 262-334, 372-444 and 545-660 as indicated in the database (http://plasmodb.org) (Aurrecoechea et al., 2009; Davies et al., 2016). LCRs are sequences within proteins that include tandem repeats of one or few amino acids. Those repeats in Pf-GARP contribute to 44% of the mature protein sequence (Davies et al., 2016). LCRs are mostly hydrophilic, flexible, and do not have stable three-dimensional structures. Further analysis of these LCRs revealed that they are highly abundant in *P.*

falciparum proteins. Thus, it was hypothesized that multiple repeats in *P. falciparum* proteins may play an important role in their antigenic diversity (Brocchieri, 2001; Coletta et al., 2010; Zilversmit et al., 2010). Additionally, another study showed that proteins containing LCRs are likely to have more binding capabilities and participate in a variety of interactions with other proteins (Coletta et al., 2010). Pf-GARP contains three distinct lysine-rich repeats AA 119-163, 253-340, and 372-446, which are responsible for the localization of the protein to the RBC plasma membrane as detected by the translocation of GFP-tagged proteins in transfected *P. falciparum* 3D7 parasites (Davies et al., 2016). In fact, we identified the AA 417-444 region, which overlaps with the third lysine-rich repeat identified by Davies et al., (Davies et al., 2016) as a blood-stage pathogenic antigen of *P. falciparum* that binds to human erythrocyte receptor as discussed later.

1.2.2.2. Role of Erythrocyte Band 3 in Malaria Infection. Band 3 as a Host Receptor for Several Malaria Proteins

Band 3, the anion transport protein, is the most abundant integral membrane protein in human erythrocytes. It was recognized almost 50 years ago based on the Coomassie Brilliant Blue (CBB)-staining of its polypeptide as a prominent band in the human erythrocyte membranes by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Fairbanks et al., 1971; Lenard, 1970; Yu and Steck, 1975). It is encoded by the SLC4A1 gene, and expressed in erythrocytes (eAE1) and acid-secreting intercalated cells of the kidney collecting duct (kAE1), with respective molecular weights of 101.8 kDa and 94.2 kDa due to the truncated N-terminus segment in the smaller kidney isoform (Kollert-Jons et al., 1993; Tanner et al., 1988; The UniProt, 2017). Band

3 migrates on SDS-PAGE as large diffuse band at ~90-100 kDa due to its glycosylation content (Ideguchi et al., 1982; Low, 1986).

Band 3 consists of 14 transmembrane segments. Although the exact topology of these segments has not been completely resolved, several amino acid positions have been identified to play a specific role in band 3 structure (Arakawa et al., 2015; Hirai et al., 2011; Jarolim et al., 1997; Lux et al., 1989; Reithmeier et al., 2016). For example, Asparagine (Asn)-642 has been identified as a site for N-glycosylation (Groves and Tanner, 1994; Hirai et al., 2011; Reithmeier et al., 2016; Tam et al., 1994). Two distinct domains, the N-terminus ~55-60 kDa and C-terminus ~35 kDa, are released after external cleavage of band 3 with chymotrypsin at Tyrosine (Tyr) 553, Tyr 555 and Leu (Leucine) 558 on the third extracellular loop (Ideguchi et al., 1982; Jarolim et al., 1997; McPherson et al., 1993; Okubo et al., 1994; Rao, 1979; Reithmeier et al., 2016).

The main physiological function of erythrocyte band 3 in circulation is the exchange of chloride (Cl^-) for bicarbonate (HCO_3^-) ions to facilitate the release of carbon dioxide (CO_2) from RBCs (Jay, 1996; Jennings, 1984; Low, 1986). The carboxyl (C)-terminal transmembrane region of band 3 is detected to be the main segment involved in gas transport function since it contains the binding site for carbonic anhydrase II (CAII), which converts CO_2 and water to proton (H^+) and $HCO3^-$ (Jennings, 1984; Reithmeier et al., 2016; Sterling et al., 2001). The amino (N)-terminal cytoplasmic portion of band 3 maintains the structure and stability of intact RBCs since it is known to associate with major RBC membrane proteins such as ankyrin, protein 4.1, and protein 4.2 and to cytoplasmic proteins including hemoglobin and glycolytic enzymes (Casey et al., 1989; Cordat et al., 2003; Czerwinski et al., 1988; Jay, 1996; Low, 1986; Peters et al., 1996;

Puchulu-Campanella et al., 2013; Satchwell et al., 2011). Mutations in the SLC4A1 gene generate several blood group antigens, and define the genetic basis of several human diseases (Denomme, 2004; Jarolim et al., 1995; Jarolim et al., 1998; Reithmeier et al., 2016). For instance, deletion of band 3 within AA 400-408 region resulted in the Southeast Asian Ovalocytosis (SAO), characterized by rigid erythrocytes and resistance to malaria invasion (Allen et al., 1999; Cheung et al., 2005; Jarolim et al., 1991; Wrong et al., 2002).

Band 3 is believed to exist as dimers, tetramers, and forms complexes with multiple RBC proteins including glycophorin A (Aoki, 2017; Baldwin, 2015; Baldwin et al., 2015; Denomme, 2004; Lux, 2016; Satchwell et al., 2011). The abundant surface expression of band 3 and associated proteins in erythrocytes could be one possible reason for the malaria parasite to utilize this protein complex as a major host receptor site for invasion. In fact, liposomes containing human band 3 and some associated membrane proteins were able to inhibit RBC invasion by *P. falciparum in vitro* (Okoye and Bennett, 1985). Subsequently, band 3 was identified as an invasion receptor for MSP-1(Goel et al., 2003; Oh and Chishti, 2005). More recently, our lab identified band 3 and glycophorin A complex as a major host receptor for multiple malaria parasite proteins involved in RBC invasion (Baldwin, 2015; Baldwin et al., 2015). Similarly, a modified form of band 3 has been identified in *P. falciparum* iRBCs that plays a robust role in facilitating increased cytoadherence properties of iRBCs (Winograd and Sherman, 2004). The molecular mechanism of band 3-mediated cytoadherence phenomenon remains poorly understood.

1.3. Significance of this Project

Although several treatment modalities have been developed to target the blood-stage malaria infection, resistance has been demonstrated to most current antimalarial drugs (Sinha et al., 2014). Therefore, a better understanding of the molecular mechanism of parasite life cycle at the blood stage is essential for the future development of novel therapies targeting severe complications. All previously mentioned phenomena are associated with severe malaria resulting in capillary obstruction, organ failure, and death. There is much interest in understanding the mechanisms of adhesion to develop effective adjuvant anti-adhesion therapies that inhibit or reverse the lesions or ultimately develop the anti-adhesion vaccines (Gullingsrud et al., 2015; Rowe et al., 2009). Since many malaria symptoms, particularly by *P. falciparum*, are caused by the adhesion mediated phenomena, a detailed molecular understanding of these processes is essential to design therapies against the lethal effects of this devastating disease.

1.4. Hypothesis

We hypothesized that specific region(s) of Pf-GARP mediate its direct binding to human RBCs, and these interactions regulate iRBC rosetting, invasion, and adhesion phenomena in malaria infection.

1.5. Specific Aims

In my thesis project, I used the phage display cDNA technology and established cell-based and protein-based binding assays to evaluate the potential function of a novel ligand-receptor interaction at the blood stage of malaria infection.

1.5.1. Characterization of the Functional Role of Pf-GARP

A1a: Use of recombinant protein expression methods to identify and map the minimum and stable region of Pf-GARP that binds to human RBCs.

A1b: Express stable recombinant segment of Pf-GARP on the surface of CHO-K1 cells, and evaluate the interaction of human RBCs to transfected CHO-K1 cells.

A1c: Investigate whether specific Pf-GARP segment identified by the phage display screen enhances or interferes with the invasion of RBCs by merozoites. These experiments will help to clarify the functional role of GARP in the invasion of host RBCs.

1.5.2. Identification of Human RBC Receptor that Binds to Pf-GARP

A2a: Test the binding activity of Pf-GARP to enzyme-treated human RBCs by using recombinant stable segment of Pf-GARP identified in our phage screens. Binding sensitivity of this stable Pf-GARP peptide to specific enzyme-treated RBCs will enable us to narrow down the identity of potential host receptors.

A2b: Perform blot overlay assays using human RBC ghosts as bait with Pf-GARP fusion protein to determine the size of potential host receptor(s). The precise identify of the host receptor will be determined by Western blotting and/or mass spectrometry of the bands identified in the assay.

A2c: Use of a "reverse cDNA screen" approach to identify the RBC receptors that bind to Pf-GARP. Recombinant segments of Pf-GARP will be used as bait to screen new phage display cDNA libraries made from human erythroid precursors and/or from human reticulocytes.

A2d: Perform pull-down assays to identify the captured RBC receptors that bind to Pf-GARP recombinant protein.

Chapter 2: Materials and Methods

2.1. Phage Display Library Screen

Phage display screening method was employed to identify human RBC binding proteins of *P. falciparum* (Lauterbach et al., 2003). The phage cDNA library was constructed from *P. falciparum* 3D7 mRNA using T7 select system (Novagen). The number of independent clones (phages) in 3D7 cDNA library was 9×10^5 pfu (plaque-forming unit). *P. falciparum* FCR-3 library was kindly provided by Dr. Theresa L. Coetzer (Lauterbach et al., 2003). Phage clones were selected through four rounds of biopanning using human RBCs. The first round was performed by incubating 100 µL of 50% hematocrit of human RBCs with 3×10^9 pfu. of *P. falciparum* phage lysate. Thus, each independent phage was screened more than 3000 times. The binding buffer was autoclaved PBS (Phosphate Buffered Saline, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4-$7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) containing 3% BSA (Bovine Serum Albumin, Gold Biotechnology, A-420-100). The total volume of the reaction was 700 µL and the incubation of the binding assay was performed for two hours while rotating at room temperature on a rotator (Tube Revolver / Rotator, Thermo Fisher Scientific™ 88881001, 11-676-341).

The mixture was centrifuged through 500 µL silicone oil, to separate RBCs pellet from supernatant, at 12,000 revolutions per minute (rpm) or 13,400 rcf (relative centrifugal force) using Eppendorf® Microcentrifuge 5415D. Phage bound RBCs were washed three times with PBS at 1800 rpm. Bound phages were eluted by incubating the RBCs with 3 M NaCl (sodium chloride) for 20 minutes and amplified in BLT5403 *E. coli* (*Escherichia coli*) bacterial culture by shaking for 3 hours at 37^0C. Next rounds were

performed by using 10 μL of the amplified phage stock from previous round. Plaque assays were performed as described (Novagen's T7Select® System Manual). Phage inserts were amplified from independent plaques by polymerase chain reaction (PCR) and further purified using E.Z.N.A.® Cycle-Pure Kit (VWR, 101318-904), and sequenced at Tufts University Core Facility. Positive hits were used for additional experiments.

Reverse phage display screens were attempted to identify possible human RBCs receptors that bind to Pf-GARP. Using T7 select system, libraries were constructed from human reticulocyte mRNA with either random primed or oligo dT primed cDNA synthesis methods. The number of independent clones were 6 x 10^6 pfu for random primed library and 1.8 x 10^7 pfu for oligo dT primed library. ELISA (enzyme-linked immunosorbent assay) plates were washed with deionized water and coated with Pf-GARP fusion proteins as described below at final amount of 10 μg/mL in PBS overnight at 4^0C. Wells were washed three times with PBS, blocked with blocking buffer (1% BSA in PBS), washed five times with deionized water, and then kept at 4^0C until used. 1x 10^8 pfu of random primed library and 1 x 10^9 pfu of dT primed library were diluted in PBST (PBS plus 0.5% Tween-20 (Fisher Scientific, BP337-500)), added to coated wells and incubated for 30 minutes. Plates were washed five times with PBST. Excess liquid was removed by tapping inverted wells. Bound phages were eluted by 3 M NaCl and amplified as described above. Total of four rounds of biopanning per one screen were performed. All cDNA libraries were prepared by Dr. Toshihiko Hanada.

2.2. Construction of the Expression Plasmids of GARP

The GARP coding sequences, Pf-GARP$_{356-552}$ (GARP-L) of 3D7 strain and Pf-GARP$_{392-437}$ (GARP-S) of FCR-3 strain, were amplified by PCR from the phage using primers with EcoRI and HindIII adaptors, and cloned in pET-32b vector (Novagen), which adds His x 6 and Trx (thioredoxin)-Tag as fusion protein. PCR products were purified using E.Z.N.A.® Cycle-Pure Kit (VWR, 101318-904) and plasmid was purified from the bacterial culture using E.Z.N.A. Standard Plasmid Isolation Mini Kit (VWR, 101318-898). The PCR products and plasmid were digested with EcoRI and HindIII (New England Biolabs: NEB), and electrophoresed in agarose gel. The corresponding DNA bands were excised from the gel with a new razor blade (VWR, 300090-990) and purified using E.Z.N.A.® Gel Extraction Kit (VWR, 101318-976). Purified inserts and plasmid were ligated and the ligation products were used to transform NEB 5-alpha Competent *E. coli* (VWR, 200067-176). After confirming the successful cloning by colony PCR and DNA sequencing, NEB BL21 (DE3) competent *E. coli* (Fisher Scientific, 50-429-4) was transformed with the plasmid for recombinant protein expression. The primers used to amplify and sequence the GARP-S and GARP-L proteins were T7 Up 5-GGAGCTGTCGTATTCCAGTC-3 and T7 Down 5-AACCCCTCAAGACCCGTTTA-3. The primers used for colony PCR and the sequencing of the inserts in pET-32b vector were 5-CGAACGCCAGCACATGGACA-3 (Forward, pETF) and 5-TGCTAGTTATTGCTCAGCGG-3 (Reverse, pETR).

Pf-GARP$_{370-444}$ (GARP-M) construct, which was kindly suggested by Dr. James Baleja using PSIPRED sequence analysis, was ordered from GenScript USA Incorporation as a codon optimized DNA cloned into pUC57 vector with PvuII and ApaI restriction sites linkers to be cloned into pRE4 vector forming pRE4-GARP-M construct. It was digested from pUC57 vector and cloned into pRE4 vector. The primers used to sequence pRE4 vector were 5-CGGCAAATATGCCTTGGCGG-3 (Forward, pRE4F) and 5-CCTCCAAGAGGGCCGAATCC-3 (Reverse, pRE4R). GARP-M was also cloned in pET32b to express Trx-GARPM recombinant protein. The primers used to amplify and clone GARP-M into pET-32b vector were 5-GCCGAATTCGACCCCAGAAG-3 (Forward, COGARP-MpET32bF) and 5-GCCAAGCTTCTTGACGACGTG-3 (Reverse, COGARP-MpET32bR).

Subclones of GARP-M, Pf-GARP$_{370-416}$ (GARP-M1) and Pf-GARP$_{417-444}$ (GARP-M2) were cloned in pGEX2T for GST fusion protein expression (BamHI and EcoRI retriction sites). Primers used for the cloning were 5-GCGGATCCACCCCAGAAGAGCATAAG-3 (Forward, GARPMF1), 5-GCGAATTCGGACTTGTGCTCCTTGCT-3 (Reverse, GARPMR1), 5-GCGGATCCAAGGGCAAGAAGGACAAG-3 (Forward, GARPMF2) and 5-GCGAATTCCTTGACGACGTGCTTCTT-3 (Reverse, GARPMR2). Trx-GARP-M1$_{(370-416)}$ and GARP-M2$_{(417-444)}$ clones were made by moving the inserts from pGEX2T to pET32a. GARP-S, GARP-M1, and GARP-M2 were kindly cloned by Dr. Toshihiko Hanada. His-GARP-M was kindly cloned by Dr. James Schiemer into pLIC-His plasmid (ligation independent cloning

vector with His-tag at the N-terminus) according to the previous publication (Cabrita et al., 2006) using the following primers 5-CCAGGGAGCAGCCTCGACCCCAGAAGAGCATAAGGAAGGCG-3 (Forward, pLIC-GARP-M-Fwd) and 5-GCAAAGCACCGGCCTCGTT ACTTGACGACGTGCTTCTTGACCTTT-3 (Reverse, pLIC-GARP-M-Rev). All primers were synthesized by Integrated DNA Technologies.

2.3. Protein Expression and Purification

Recombinant proteins were expressed in *E. coli* BL21 (DE3). Transformed BL21 (DE3) was cultured in LB (Luria Broth, Affymetrix, VWR, 101170-308) supplemented with 70 μg/ml ampicillin (Fisher Scientific, ICN19014805). Expression was induced with 0.5 mM IPTG (Isopropyl-beta-D-thiogalactoside, Gold Biotechnology, I2481C25). Cells were harvested by centrifugation for 20 minutes at 9000 rpm using Sorvall RC5C Plus Centrifuge with SS-34 rotor (Thermo Electron Corporation) at 4^0C. Cells were lysed by sonication in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole (OmniPur*, VWR, EM-5720)) containing 100 μg/mL lysozyme from chicken egg white (Sigma-Aldrich, L7651) and 0.1 M PMSF (Phenylmethylsulfonyl Fluoride, VWR, 97064-900). The insoluble materials were pelleted by high-speed centrifugation at 9000 rpm using Sorvall RC5C Plus Centrifuge with SS-34 rotor at 4^0C. Recombinant proteins were purified from the cleared supernatant.

His-tag GARP proteins were purified through Nickel (Ni) affinity chromatography using high density Ni agarose beads (Gold Biotechnology,

H-320-25) or Cobalt resin (HisPur™ Cobalt Resin, Thermo Scientific) for small volume purification. For larger volume, fast protein liquid chromatography (FPLC) equipped with Nickel column was used. We followed manufacturer's protocol (Novagen's pET system manual) for protein purification. Briefly, soluble supernatant was incubated with Ni-chelating resin for 2 hours at 4^0C on a rotary shaker using PBS as a binding buffer. Protein bound beads were washed three times by centrifugation at 500g using washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole). All proteins were eluted by the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 400 mM Imidazole) and dialyzed against sterile PBS overnight at 4^0C. Small aliquots of proteins were stored in -80^0C. For long time storage, 0.1% glycerol (The British Drug Houses, VWR, BDH1172-1LP) has been added. In some cases, proteins were dialyzed again against PBS after thawing. Protein concentration was determined using NanoDrop® ND-1000 spectrophotometer/software version 3.8., Protein A280, according to the molecular weight and extinction coefficient of the protein. Protein bands were analyzed by 12% SDS-PAGE (Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad, 4561044) followed by CBB staining.

2.4. Preparation of Human RBCs

Human blood was collected from healthy donors in ACD buffer (Glass Blood Collection Tubes with Acid Citrate Dextrose, BD Vacutainer™), or human RBCs were ordered from Red Cells AS-1 500 mL Mnf AB+ (Red Cross, 3361164). GYPB-null blood was kindly provided by Ms. Jigna Rami,

ITxM Clinical Services, Rosemont IL 60018. All blood samples were washed three times in DPBS (Dulbecco's Phosphate Buffered Saline) without calcium and magnesium (Fisher Scientific, BW17-512Q) or RPMI 1640 (Roswell Park Memorial Institute Medium, Gibco, 11875-093), each time for 10 minutes at 500 rcf with slow acceleration using Beckman Coulter^{TM} Allegra X-30R Refrigerated Centrifuge (acceleration 9, deceleration 3) at 4^0C. The thin top layer of blood containing white blood cells (WBCs) was removed after first wash. Washed blood containing mostly RBCs was saved in RPMI at 4^0C for up to one month maximum.

2.5. Enzyme Treatment of Human RBCs

Human erythrocytes treated with enzymes to remove certain molecules from the surface were used for red blood cell binding assay and for blot overlay assay. We followed the protocol as described previously with slight modifications (Goel et al., 2003). Basically, intact human erythrocytes were incubated with the following enzymes in PBC for one hour at 37^0C on a rotator. Enzymes used were α-chymotrypsin from bovine pancreas (0.5- 1.0 mg/ml, SIGMA), trypsin from bovine pancreas (0.5- 1.0 mg/ml, SIGMA) or neuraminidase from *Clostridium perfringens* in 100 mM sodium acetate, 2 mM $CaCl_2$, pH 5.0 (5 Units/ml, SIGMA). After incubation, treated erythrocytes were washed with PBS three times by centrifugation at 500 rcf. Chymotrypsin and trypsin-treated RBCs were incubated with trypsin-chymotrypsin inhibitor from Glycine max (Soybean, Sigma) for 20 min

rotating at room temperature. RBCs were washed subsequently three times with PBS and used for the assays.

2.6. RBC-binding Assay

The protocol for RBC-binding assay of recombinant proteins was described previously (Baldwin et al., 2015; Li et al., 2012). Protein concentrations were measured by BCA Protein Assay Kit or Nano-drop. Equal amounts (1μM final concentration) of the recombinant proteins and control protein were mixed with 1.0 mg/mL BSA (0.1%) in PBS or DPBS with calcium and magnesium (Gibco, 14040133) and 20 μL of fresh packed RBCs (80 μL of 25% hematocrit) and then rotated gently for 2 hours at room temperature, and finally centrifuged on 500 μL silicone oil at 12,000 rpm. In some experiments, we added 0.5 mM DTT (Dithiothreitol) to the binding buffer. The pellet was washed with PBS three times. Bound proteins were eluted from RBCs by the addition of 40 μL of 1.5 M NaCl in PBS. The bound Trx-tagged proteins were detected by western blotting using anti-Trx monoclonal Ab (GenScript, Fisher Scientific, 50272466). Input (proteins only) and elution (bound proteins) were run on 12% SDS-PAGE and transferred to nitrocellulose membrane (BioRad, 1620115) in transfer buffer solution (20 mM Tris base, 150 mM Glycine, 20% Methanol (Fisher Scientific, A412P-4) in distilled water, 200 mA). Non-specific bindings were blocked using 5% milk PBST. 1:10,000 diluted mouse anti-Trx-tag primary monoclonal antibody (mAb), 1:2000 diluted goat anti-mouse IgG HRP (horseradish peroxidase) polyclonal secondary antibody (Bio-Rad, ab6789)

and ECL (enhanced chemiluminescence) HRP labeled secondary antibody detection methods (Amersham™ ECL Prime Western blotting detection reagent) were used for signal detection. Inputs were prepared by diluting the binding mixture in PBS before the addition of RBCs. BLUEstain™2 protein ladder 5-245 kDa (Gold Biotechnology, P008-500) was used as proteins size marker.

2.7. CHO-K1 Cell Culture

CHO-K1 cells (Sigma-Aldrich, 85051005) were cultured in F-12 media Ham's F-12 modified medium (Corning™ Cellgro™, Fisher Scientific, MT10080CV) supplemented with 10% HyClone fetal bovine serum (FBS, VWR, SH30071.03), 1X L-Glutamine (Invitrogen, 25030-081) and 1% penicillin-streptomycin (pen/strep antibiotics, Invitrogen, 15140-122). The cells were maintained in tissue culture petri dishes in a humidifier at 37°C with 5% CO_2. Media was changed every 2-3 days. The cells were harvested with 0.05% trypsin-EDTA (ethylenediaminetetraacetic acid) (Gibco, 25300054).

2.8. CHO-K1 Erythrocyte-binding Assay

We performed the assay as described previously (Chitnis and Miller, 1994; Jiang et al., 2011; Mayer et al., 2009; Mayer et al., 2002; Smith et al., 1998). CHO-k1 cells were re-suspended in pen/strep-free media, divided in 6-well culture plate (CytoOne, USA Scientific, CC7682-7506) containing sterile glass cover slips (Fisher Scientific, 12-546-2). 1 x 10^6 cells transfected with 4 µg pRE4 plasmid vector using Lipofectamine 2000

transfection reagent (Invitrogen, 11668-019) according to manufacturer's protocol. This vector ensures the expression of the protein on the surface of cells by substituting part of the internal region of the herpes simplex virus glycoprotein D protein (HSV gD1). The control plasmid pRE4-EBA-175 was used in CHO-K1 transfection as a RBC-binding positive control, which binds to glycophorin A expressed on the surface of RBCs, while pRE4 empty vector with green fluorescence protein (GFP) insert was used as a negative control. Control plasmids were kindly provided by Dr. Ghislaine Mayer (Mayer et al., 2004). Erythrocyte-binding assays of transfected cells with pRE4-GARP-M, along with controls were performed 24 hours following the transfection. Each coverslip was transferred to a fresh well in 6 well plate (CytoOne, USA Scientific, CC7682-7506) containing the CHO-K1 culture media and 100 µL of 10% hematocrit erythrocytes diluted in CHO-K1 culture media was added and then incubated at 37°C for 2 hours. Unbound RBCs were washed off with PBS by inverting the coverslip on small pieces of glued capillary tubes placed in the edges of each well of the 6-well plate (kindly created with the assistance of Dr. Jennifer Nwankwo of our lab). After washing, each well was filled with PBS and kept for 15 min to allow for unbound RBCs to fall off. Cells were fixed in 1% glutaraldehyde in cold PBS. Rosettes were visualized, counted, and imaged at 40X magnification objective on a Nikon Eclipse TE200 microscope and SpoT RT3 camera/Spot software (version 5.2).

Immunofluorescence assay without permeabilizing the cells was performed to confirm the transfection and extracellular expression of

proteins. Cells were fixed by 3.7% formaldehyde and stained with mouse mAb ID3 and mAb DL6 against HSVgD1epitopes located on either side of the construct at 1:10,000 dilution. These specific antibodies were kindly provided by Dr. Gary H. Cohen and Dr. Roselyn J. Eisenberg from the University of Pennsylvania. Secondary antibody, goat anti-mouse IgG with Alexa Fluor 633 conjugate, at 1:1000 dilution was used. Images were taken using 60x objective on Nikon Eclipse TE2000-E inverted microscope and MetaMorph® Microscopy Automation & Image Analysis Software (Version 7.8.8.0).

2. 9. Human RBC Aggregation Assay

We designed an *in vitro* assay slightly modified from previously published assay (Chotivanich et al., 2004) to visualize the formed unfixed human RBCs aggregates under the microscope directly without placing them on glass slides. Basically, 2 μL of 20% hematocrit of human erythrocytes was added to final volume of 500 μL of synthetic peptides solubilized in PBS or PBS alone as a negative control in a 12-well plate (CytoOne, USA Scientific, CC7682-7512) rotating for two hours on a rotator (The Belly Dancer STOVALL Life science incorporated Greensboro NC USA) at room temperature. A very low number of RBCs was used in order to visualize any rouleaux and aggregates formed clearly. Cells were observed by a Nikon Eclipse TE2000-E inverted microscope using 10x objective. Only clusters of human RBCs forming clear 3D structure were counted and considered as positive hits, as shown in the results section (Figure 3.7). Representative images were taken using MetaMorph® Microscopy Automation & Image Analysis Software (Version 7.8.8.0). Two GARP peptides synthesized from GARP-M2

region, M2K4 (Bao-Shiang Lee, Protein Research Laboratory, UIC Research Resources Center, Chicago, IL) and M2K5 (Michael Berne, Tufts University Core Facility), produced positive aggregates. M1E4R and M1G4 (Bao-Shiang Lee, Protein Research Laboratory, UIC Research Resources Center, Chicago, IL) are negative control peptides. Sequence of peptides are shown in Table 3.1.

2.10. RBC Ghosts Preparation

Untreated and enzyme-treated ghosts were prepared from 100 µL of packed human RBCs. The protocols of ghost preparation obtained from published literature (Casey et al., 1989; Dodge et al., 1963). Both groups were prepared similarly by washing and lysing RBCs multiple times with lysis buffer (cold 5 mM sodium phosphate pH 8.0, 1.0 mM EDTA pH 8.0) containing 1X protease inhibitor cocktail (Biotool, b14001) until getting whitish-colored pellets. Each wash was performed at 14,000 rcf speed for 5 minutes at 4^0C using Beckman Coulter™ Microfuge® 22R Refrigerated Microcentrifuge. Prepared ghosts were stored at -80^0C. Ghosts were incubated with 3x SDS sample buffer at 37^0C for 15 minutes and equal volumes were loaded to 12% gels for SDS-PAGE, transferred to nitrocellulose membranes and saved in distilled water at 4^0C until used for later experiments.

2.11. Blot Overlay Assay (Far Western Blotting)

The assay was performed as described previously with slight modifications (Goel et al., 2003). Basically, membranes prepared above were incubated with PBST or TBST (tris-buffered saline with Tween-20, 20 mM

Tris base, 136 mM NaCl, 1% Tween-20, pH 7.6 adjusted by 10 N HCl) for at least half an hour followed with blocking buffer (10% milk, 2% BSA in PBST/TBST) rotating overnight. The next day 1.0 µg/mL of proteins in PBST/TBST were added to the membranes and incubated overnight rotating at 4^0C. After incubation, membranes were washed with the blocking buffer five times, each time for 15 minutes at 4^0C. Primary antibody (anti-Trx mAb) at dilution of 1:10,000 in the blocking buffer was added overnight at 4^0C. Then, five washes with the blocking buffer were performed. Goat anti-mouse (GAM) alkaline phosphatase (AP) secondary antibody was mixed with the blocking buffer and added to the membranes for one hour at 4^0C according to manufacturer's protocol (Immun-Star™ GAM-AP detection kit, BioRad, 1705010). Membranes were washed four times with PBST/TBST and one time with PBS/TBS. Supersignal West Pico chemiluminescent substrate (VWR, PI34080) was added to develop the light signal.

2.12. Pull-down Assay

We followed the protocol as described previously with slight modifications (Alam et al., 2015; Casey et al., 1989; Yajima et al., 2008). Basically, untreated and chymotrypsin-treated ghosts were incubated with 1% $C_{12}E_8$ (dodecyl octaethylene glycol ether) solubilizing buffer (Protein Grade™, Calbiochem™, EMD Millipore™, Fisher Scientific, 20-553-21SET) in 5 mM sodium phosphate pH 8.0, 1.0 mM EDTA, pH 8.0 and 1X protease inhibitor cocktail for 20 min on ice, and then centrifuged at 18,000 rcf for 30 min at 4^0C. Preclearance of the solubilized ghosts was performed by incubating them with 100 µL of washed 50% Cobalt beads slurry in PBS for 1.0 hour at 4^0C, then

centrifuged at 18,000 rcf for 5 minutes at 4^0C. Around 125 µg of Trx and Trx-GARP-M2 were incubated with 50% slurry of washed-Cobalt beads in PBS for one hour rotating at 4^0C. Protein-bound-beads were then washed and centrifuged at 500 rcf for 5 minutes two times with 0.1% $C_{12}E_8$ in PBS. The precleared soluble ghosts were incubated with the washed beads bound to proteins overnight at 4^0C in PBS. The next day, the beads were washed six times with 0.1% $C_{12}E_8$ in PBS and centrifuged each time at 500 rcf for 5 min. Afterward, washed beads were mixed with 3X loading dye and loaded on two 12% gels for SDS-PAGE, one for CBB staining to detect proteins loaded to beads and the other one for Western blotting to detect band 3 binding by transferring the proteins to nitrocellulose membrane and perform Western blotting with polyclonal anti-human band 3 antibody against the cytoplasmic 43 and 41 kDa N-terminus fragments (Kindly provided by Dr. David Liu and Ms. Cathy Korsgren) (Korsgren and Cohen, 1986; Korsgren and Cohen, 1988) at 1:1000 dilution in blocking buffer (5% milk in TBST). ECL HRP conjugated secondary goat anti-rabbit IgG antibody (Bio-Rad, 170-6515) and Amersham™ ECL Prime Western blotting kit were used for signal detection methods.

2.13. Parasite Culture

P. falciparum 3D7 parasites were cultured *in vitro* in complete malaria media (CMM), which contained RPMI-1640 supplemented with 0.5% Albumax II (Thermo Fisher Scientific, 11021-037), 25 mM HEPES, 50 mg/L hypoxanthine, 50 mg/L gentamicin), and maintained at 37ºC gas chamber that consisted of 5% CO_2, 3% oxygen (O_2) and balanced by nitrogen (N_2) (Airgas, X03 NI92C2002407) as described previously (Baldwin, 2015; Schuster, 2002; Trager, 1978). Media was changed every day and fresh human RBCs were added according to the smears that were made on glass slides, fixed

with 100% methanol for 30 seconds, stained by Giemsa and used under light microscope for counting parasitemia.

2.14. Parasite Culture Supernatant

We followed the protocol as published previously (Goel et al., 2003). Briefly, *P. falciparum* 3D7 schizont-infected erythrocytes were gently separated using LS magnetic columns (Miltenyi Biotec ,130-042-401) placed in the magnetic field of a MACS® (magnetic-activated cell sorting) separator. Schizonts were kindly purified by Ms. Shreeya Hegde. Around 5 x 10^5 of purified schizonts were incubated for 20 hours in CMM at 37ºC as described above but without the addition of RBCs, in a total volume of 200 µL. The upper level of the mixture was centrifuged for 5 minutes at 500 rcf then collected supernatant was centrifuged again at 1,200 rcf for 10 minutes. The final collected culture supernatant was saved in -80^{0}C in the presence of 1X protease inhibitor cocktail. CCM without schizonts was used as a negative control. Both supernatant groups were analysed by SDS-PAGE and blotted using Pf-GARP-M anti-mouse mAb (GM-7, kindly made by Dr. Toshihiko Hanada) at 1:1000 dilution and detected by 1:2000 diluted goat anti-mouse IgG HRP polyclonal secondary antibody and Amersham™ ECL Prime Western blotting reagents.

2.15. *P. falciparum* Invasion Assay

We followed the protocol as described previously (Baldwin, 2015). Ultimately, magnetic column was used for preferential isolation of RBCs infected with schizont stage asexual parasites. Invasion assay was performed in a 96-well plate at 200 µL final volume and 2.5% hematocrit according to the above-mentioned malaria culture condition. Synchronized parasites were adjusted to 1% parasitemia. Soluble fusion proteins in PBS,

antibodies in PBS or PBS alone were added to each well at final desired concentration up to 50 μL maximum volume. Uninfected and infected wells with no proteins nor PBS added were also included as controls. Plates were incubated for 24 hours. Each well was transferred to an Eppendorf tube, centrifuged for 4 minutes at 400 rcf and washed two times with PBS. RBCs collected in the pellet were stained with 2 μM Hoechst 33342 dye in RMPI at 37ºC and kept rotating in the dark for one hour before centrifugation for 4 minutes at 400 rcf speed. The pellet was washed twice with PBS and fixed with 2% paraformaldehyde (Santa Cruz Biotechnology, Fisher Scientific, NC0238527) and 0.2% glutaraldehyde (Sigma-Aldrich, G5882) in PBS at 4^0C rotating in the dark for 45 min. Stained and fixed RBCs were washed twice with PBS, re-suspended in PBS, and kept at 4ºC in the dark until the quantification of parasitemia by BD (Becton Dickinson) LSR II flow cytometer (with the technical help kindly provided by Mr. Allen Parmelee and Mr. Stephen Kwok). The Indo blue 450/50 filter was used to count 100,000 events were counted for each sample. Summit software version 4.3 was used for analysis of data.

Chapter 3: Results

3.1. Characterization of Pf-GARP

Host-Parasite interaction of *P. falciparum* is mediated by multiple sets of protein-protein interactions between host RBC receptors and parasite surface proteins (Acharya et al., 2017; Lobo et al., 2003; Oh and Chishti, 2005; Paul et al., 2015; Rowe et al., 2009). These interactions are crucial for the efficient invasion of host RBCs by the parasites. However, our understanding of molecular interactions and the mechanism of the invasion process are still incomplete. Major parasite ligands that play a key role in RBC invasion or rosetting were first identified by detecting their binding affinity to human RBCs. These ligand-receptor interactions are critical for malaria pathogenesis (Burleigh and Sinai, 2008).

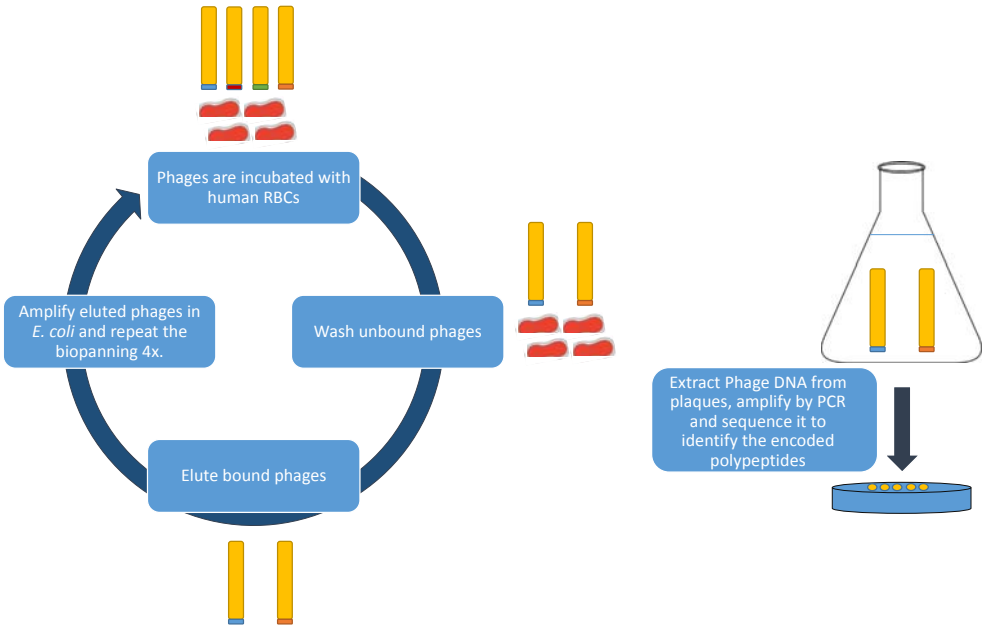
3.1.1. Isolation and Identification of Pf-GARP as an Erythrocyte-binding Protein using Independent Phage Display cDNA Library Screens

I started this project aiming to identify novel *P. falciparum* proteins that bind to host RBCs using phage display cDNA screen approach. The phage display cDNA library of *P. falciparum* 3D7 was produced using T7 phage display system (Novagen) in our lab. The library was screened based on binding to enzyme-untreated human RBCs to isolate positive cDNA clones recognizing surface RBC binding proteins. After four rounds of biopanning followed by the plaque assay, several phage clones were detected. These clones were amplified and sequenced based on promising phage inserts that showed clear bands on agarose gels with their base pair contents more than an empty phage control. Steps for the cDNA library screen are shown in Figure 3.1 (panels A and B). We elected to sequence 16 clones out of 48 clones. The BLAST analysis of cDNA sequencing results

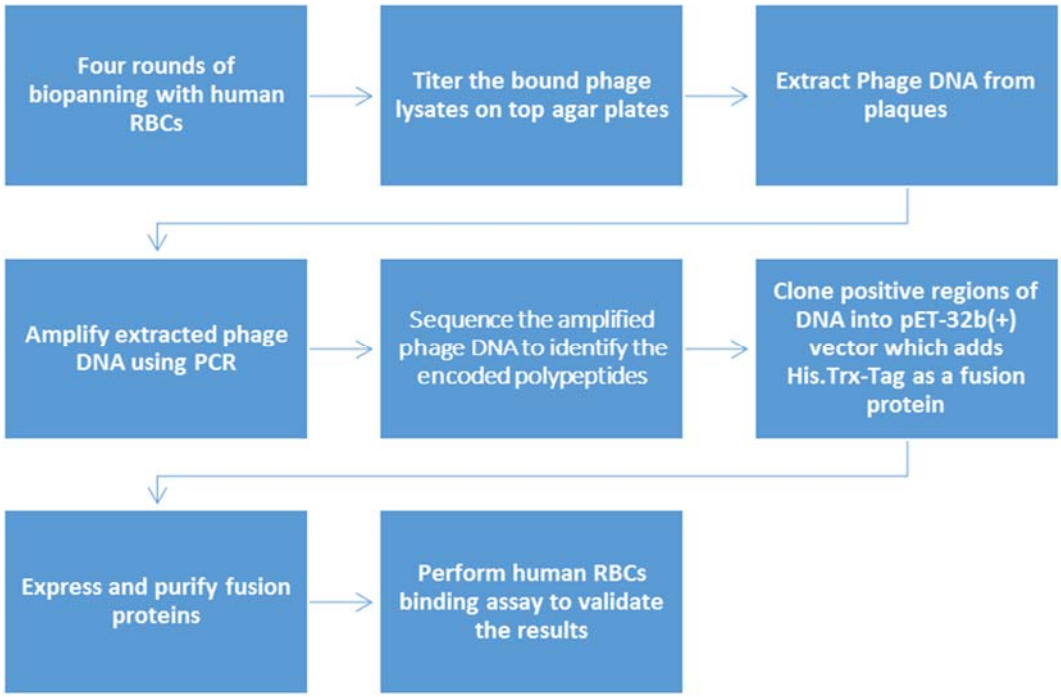
revealed several identical overlapping sequences, and some with no significant similarities to known sequences. Nucleotide and protein BLAST sequences were obtained using NCBI (the National Center for Biotechnology Information) and PlasmoDB sources (Aurrecoechea et al., 2009; Boratyn et al., 2012). LCRs were not filtered out from the results (Wootton and Federhen, 1996), because malaria genes contain many of these sequences (Brocchieri, 2001; Pizzi and Frontali, 2001; Zilversmit et al., 2010). For amino acid translation of nucleotides, we used ExPASy translate tool (Gasteiger et al., 2003). Amino acid sequences of positive clones are shown in Table 3.1.

One of the clones isolated from the screen was AA 356-552 encoding Pf-GARP, which was detected as a promising RBC binder and designated as GARP-L when expressed as a fusion protein, as explained in the next section (3.2). A smaller clone, GARP-S $_{(392\text{-}437)}$, was isolated from a separate screen using a different *P. falciparum* cDNA library made from Pf-FCR3 strain, and human RBCs that had been pre-treated with neuraminidase. We considered that independent isolation of overlapping Pf-GARP clones is a strong indication of Pf-GARP's authenticity as a RBC binding protein. We decided to pursue this finding by further validation of these intriguing results, and not to proceed with sequencing DNA of all other positive hits at this stage. However, clones expressing AA 105-257 of *P. falciparum* S (serine)-antigen protein were also found as human RBC promising selective binders. We plan to pursue this novel binding ligand in future studies.

A


Phages are incubated with human RBCs
Wash unbound phages
Elute bound phages
Amplify eluted phages in *E. coli* and repeat the biopanning 4x.
Extract Phage DNA from plaques, amplify by PCR and sequence it to identify the encoded polypeptides

B


Four rounds of biopanning with human RBCs
Titer the bound phage lysates on top agar plates
Extract Phage DNA from plaques
Amplify extracted phage DNA using PCR
Sequence the amplified phage DNA to identify the encoded polypeptides
Clone positive regions of DNA into pET-32b(+) vector which adds His.Trx-Tag as a fusion protein
Express and purify fusion proteins
Perform human RBCs binding assay to validate the results

C

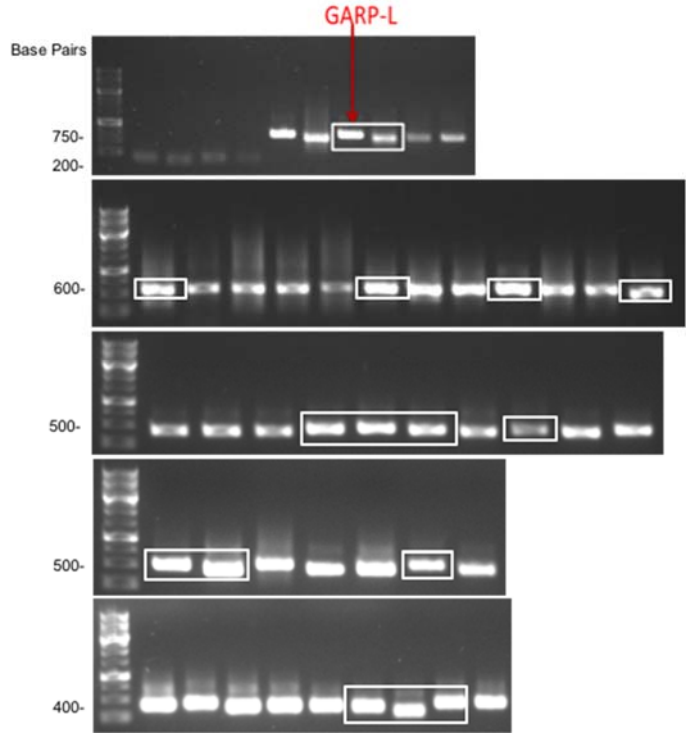


Figure 3.1. Isolation of GARP-L $_{(356\text{-}552)}$ from Phage Display cDNA Screening
A and **B**, Schematic representations of phage display library screen steps. **C**, Phage clones of the fourth round of biopanning using human RBCs. Phage inserts were amplified from independent plaques by PCR and arranged per their base pair size in 1% agarose gels containing ethidium bromide. Bands in white boxes were DNA inserts that were sequenced. DNA sequencing of the PCR product identified one of the clones as GARP-L, marked with the red arrow. Another GARP clone and three clones of S-Antigen proteins were also recognized. Other bands could not be identified by the nucleotide Blast analysis due to the lack of significant sequence similarities. Figures 3.1. A and B have been adapted from the original article "Advancement and applications of peptide phage display technology in biomedical science" by [Chien-Hsun Wu, I-Ju Liu, Ruei-Min Lu and Han-Chung Wu]. *Journal of Biomedical Science* 2016 (doi: 10.1186/s12929-016-0223-x, https://jbiomedsci.biomedcentral.com/articles/10.1186/s12929-016-0223-x). Figure 1. Changes include recreation of the figure with the addition of comprehensive biopanning steps.

Table 3.1. Selected Protein Sequences of Positive Clones Bound to Human RBCs Using the Phage cDNA *P. falciparum* 3D7 Library Screen

Sequence of proteins expressed on the surface of phages, bound selectively to human RBCs, eluted after several washes and amplified through four rounds of biopanning. FASTA sequences were obtained using NCBI database. Overlapping sequences are underlined.

Protein Name	PlasmoDB Gene ID	Protein FASTA Sequence
Glutamic acid-rich protein	PF3D7_0113000	>XP_001351053.1:357-552 glutamic acid-rich protein (garp) [*Plasmodium falciparum* 3D7] IMVPLPSPLTDVTTPEEHKEGEHKEEEHKEGE HKEGEHKEEEHKEEEHKKEEHKSKEHKSKGK KDKGKKDKGKHKKAKKEKVKKHVVKNVIE DEDKDGVEIINLEDKEACEEQHITVESRPLSQP QCKLIDEPEQLTLMDKSKVEEKNLSIQEQLIG TIGRVNVVPRRDNHKKKMAKIEEAELQKQK HVDKEEDKK >XP_001351053.1:319-437 glutamic acid-rich protein (garp) [*Plasmodium falciparum* 3D7] EKERKKKEEKEKKKKKHDKENEETMQQPDQ TSEETNNEIMVPLPSPLTDVTTPEEHKEGEHK EEEHKEGEHKEGEHKEEEHKEEEHKKEEHKS KEHKSKGKKDKGKKDKGKHKKAKKEK
S-antigen	PF3D7_1035200	>XP_001347627.1:105-257 S-antigen [*Plasmodium falciparum* 3D7] VSNGREDKVSNGGEDEVSNGGEDEVSNGRE DKVSNGGEDEVSNGREDKVSNGGEDEVSNG REDKVSNGGEDEVSNGREDKVSNGGEDEVS NGREDKVSNGREDKVSNGGEDEVSNGREDK VSNGREDKVSNGGEDEVSNGREDKVSNGGE DEV >AAA29761.1:105-165 S-antigen [*Plasmodium falciparum* 3D7] VSNGREDKVSNGGEDEVSNGGEDEVSNGRE DKVSNGGEDEVSNGREDKVSNGGEDEVSNG R

Pre-mRNA splicing factor, putative	PF3D7_0311100	>XP_001351184.1:300-425 pre-mRNA splicing factor, putative [*Plasmodium falciparum* 3D7] HDDSSSEISSHTYASRYDKERKRFRKRDINLE RHKERGYINRDKEGKHIRRDKEGKHIRRDKE EKHIRRDKEGKHIRGEQERRHQIRNKEKYRD RNDHKKPYYDENKSDRSRNSSDNNEKMRKK GK
WD (tryptophan-aspartic acid) repeat-containing protein 65, putative	PF3D7_1406500	>XP_001348235.1:1398-1506 conserved *Plasmodium* protein, unknown function [*Plasmodium falciparum* 3D7] LYNKFHKVNDVQNYDAKNVFSEYIRQKEYL ENMIEVLKDKLHKETEAFRNEKIKMMNENSL LLKEINDLKMDLNFLKSECHEAQLQNRKMKF LKKRSESKERKKEKKEK >XP_001348235.1:1446-1506 conserved *Plasmodium protein*, unknown function [*Plasmodium falciparum* 3D7] RNEKIKMMNENSLLLKEINDLKMDLNFLKSE CHEAQLQNRKMKFLKKRSESKERKKEKKEK

3.1.2. Expression and Purification of Recombinant Pf-GARP Protein and Confirmation of RBC Binding

To confirm the RBC binding of the Pf-GARP, I cloned the cDNA insert from the Pf-GARP phage clones and expressed the recombinant proteins in *E. coli*. GARP-L and GARP-S were directly amplified from the corresponding phage clones. In addition, GARP-M $_{(370\text{-}444)}$ was designed to contain the optimum and stable region of Pf-GARP based on amino acid sequence and secondary structure prediction algorithm. Figure 3.2 displays a schematic representation of all clones used in the binding assay and their AA alignments. The expression and purification of Trx-GARP-L, Trx-GARP-M, and Trx-GARP-S is shown in Figure 3.3 (left). GARP-M was found to be relatively more stable than GARP-L and GARP-S. Thus, we generated CO-GARP-M (Trx-GARP-M) fusion

protein that was used for further experiments. The observed molecular weight of GARP-M without the Trx fusion as assessed by SDS-PAGE is ~20 kDa, which is considerably higher than 9 kDa the expected molecular weight calculated by the ExPASy-ProtParam tool (Gasteiger et al., 2003; Wilkins et al., 1999). Data showing the binding of GARP-M to human RBCs are shown in Figure 3.4. Recombinant Trx-GARP proteins as well as Trx control protein were mixed with 80 μl of 25% hematocrit RBCs in the binding buffer in a total volume of 500 μL. Bound proteins were eluted from RBCs by high salt elution buffer and analyzed by Western blotting using the monoclonal anti-Trx antibody. All three Trx-GARP proteins were found to bind to RBCs in this assay. The Trx protein used as a negative control did not show any detectable binding under these conditions. Higher molecular weight bands were frequently observed with the Trx-GARP proteins in this assay, which probably represent dimers or oligomers formed by the self-assembly of Pf-GARP. However, the identity and the nature of these bands were not characterized further. Future studies will resolve the nature of these bands using mass spectrometry and biophysical characterization of Pf-GARP self-association.

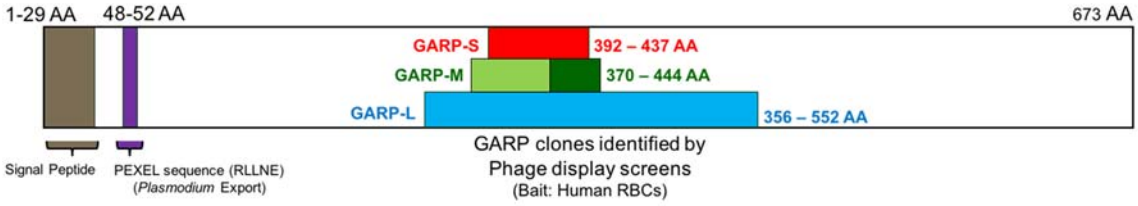
3.1.3. Mapping the RBC-binding Domain of Pf-GARP (GARP-M2) by Expressing Two Non-overlapping Recombinant Segments of Trx-GARP-M as Fusion Proteins and Detecting the RBC-binding site

GARP-M contains a series of repeat structures. To determine the core RBC binding site of Pf-GARP, and to characterize our anti-GARP monoclonal antibody (GM-7) generated against GARP-M in our lab, we cloned two non-overlapping segments termed Pf-GARP $_{370\text{-}416}$ (Trx-GARP-M1) and Pf-GARP $_{417\text{-}444}$ (Trx-GARP-M2) into

pET32 vector. The respective fusion proteins were expressed and purified as shown in Figure 3.3 (right). Both fusion proteins contain different repetitive sequences that may play an important role in Pf-GARP function. In Figure 3.5, we found that Trx-GARP-M2 could bind to human RBCs, while Trx-GARP-M1 did not bind to RBCs under the same conditions. Likely dimers of Trx-GARP-M and Trx-GARP-M1 but not Trx-GARP-M2 were observed. We used Trx-GARP-M and Trx as positive and negative controls, respectively. The GARP mAb, GM-7, recognized GARP-M1 but not GARP-M2.

Using this approach, we narrowed down the core RBC-binding region of Pf-GARP that was used for the subsequent assays to identify the RBC receptor that Pf-GARP binds. We also performed initial characterization of Pf-GARP using our Pf-GARP mAb, GM-7.

A



B

Figure 3.2. Different Regions of Pf-GARP Cloned into Trx Vector Expression System

A. Schematic representation of Pf-GARP segments. GARP-L (blue) and GARP-S (red) are two overlapping sequences obtained as human RBCs binders from independent phage display *P. falciparum* screens. GARP-M (green) is a stably expressed protein that covers GARP-S sequence with extended regions. GARP-M1 (light green) and GARP-M2 (dark green) are non-overlapping segments of GARP-M protein. GARP-M2 contains the RBC binding site that promotes RBCs aggregation (as shown in subsequent figures). **B.** Sequence alignment of GARP peptides. Pf-GARP-3D7 sequence was obtained from www.plasmodb.org (Pundir et al., 2017), while the multiple sequence alignment was generated using T-Coffee (Di Tommaso et al., 2011; Notredame et al., 2000) and BOXSHADE online tools.

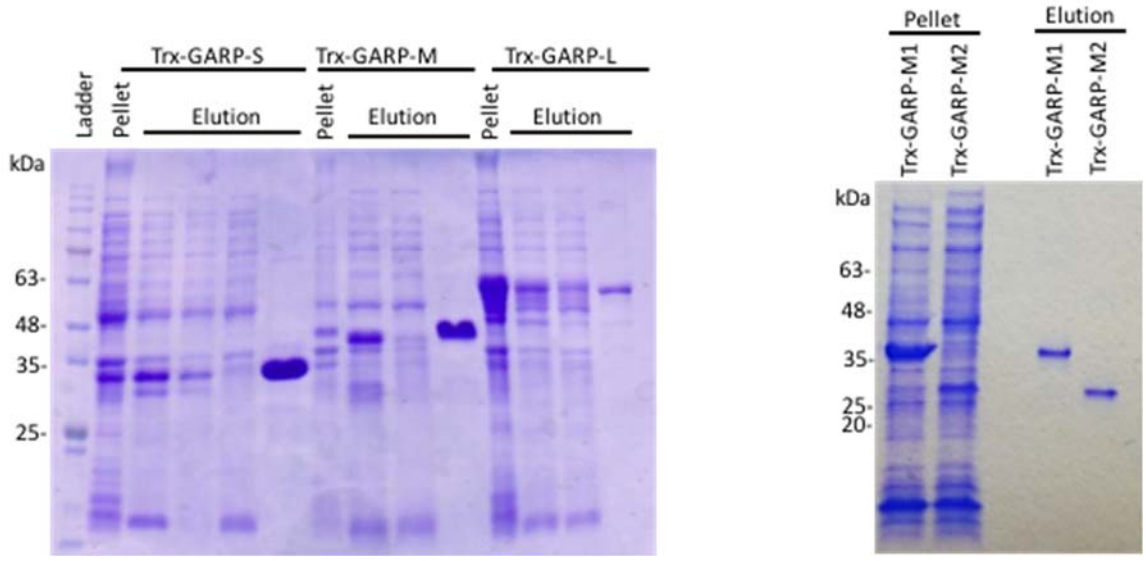


Figure 3.3. Coomassie-stained SDS-PAGE of Purified GARP Fusion Proteins
Expression and purification of Trx-GARP-M1 and Trx-GARP-M2 recombinant proteins. Crude extracts (pellet) and purified recombinant proteins (elution) were resolved by 12% SDS-PAGE followed by CBB staining. Detection of purified Trx-fusion protein bands in elution lanes by staining the 12% gel (SDS-PAGE) with 10 % CBB. Left, Trx-GARP-S, Trx-GARP-M, and Trx-GARP-L. Right, Trx-GARP-M1 and Trx-GARP-M2 (kindly purified by Mr. Christopher Schwake).

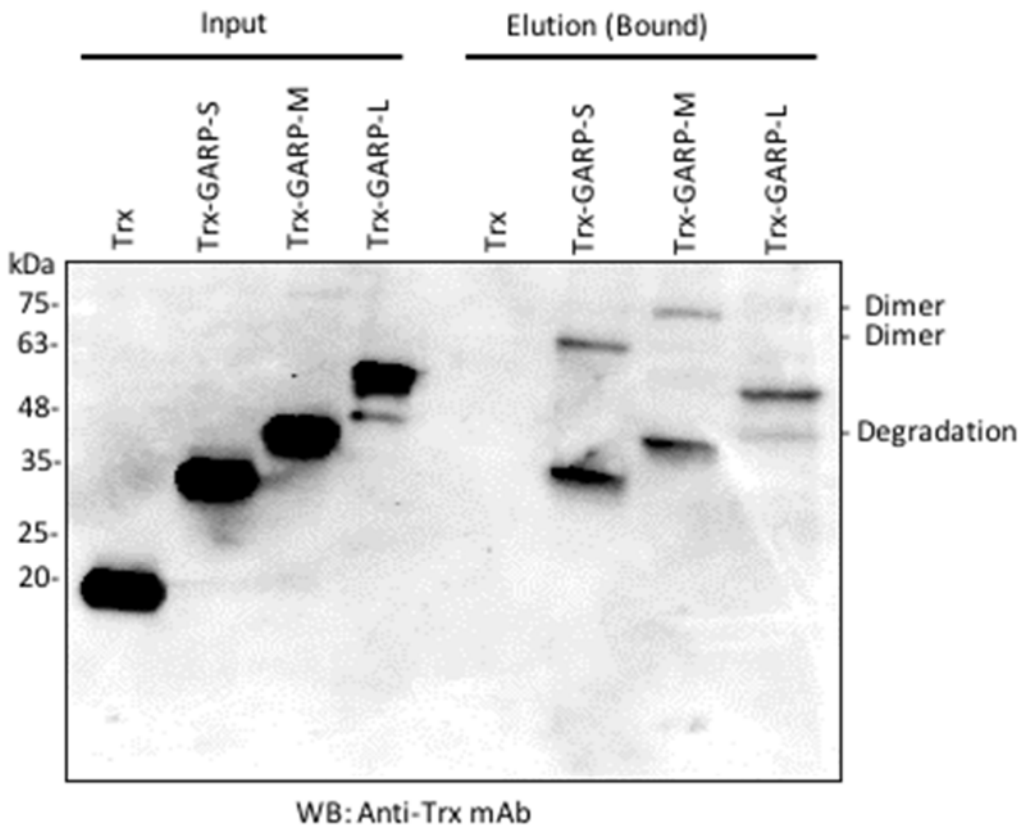


Figure 3.4. RBC-binding Assay of Untreated Human RBCs Using Different Pf-GARP Segments

All three GARP segments bind to human RBCs despite their different physicochemical properties. Lanes (**1-4**) show proteins input. TRX (**1**), Trx-GARP-S (**2**), Trx-GARP-M (**3**), Trx-GARP-L (**4**). Lanes (**5-8**) display bound protein bands in similar order of input loadings. All three fusion proteins bind to human RBCs specifically. Trx served as a negative control that does not bind human RBCs. Binding was detected using anti-Trx mouse monoclonal antibody and ECL prime western blotting detection kit. All protein concentrations were normalized at 1.0 μM. 1.5 M NaCl was used as an elution buffer.

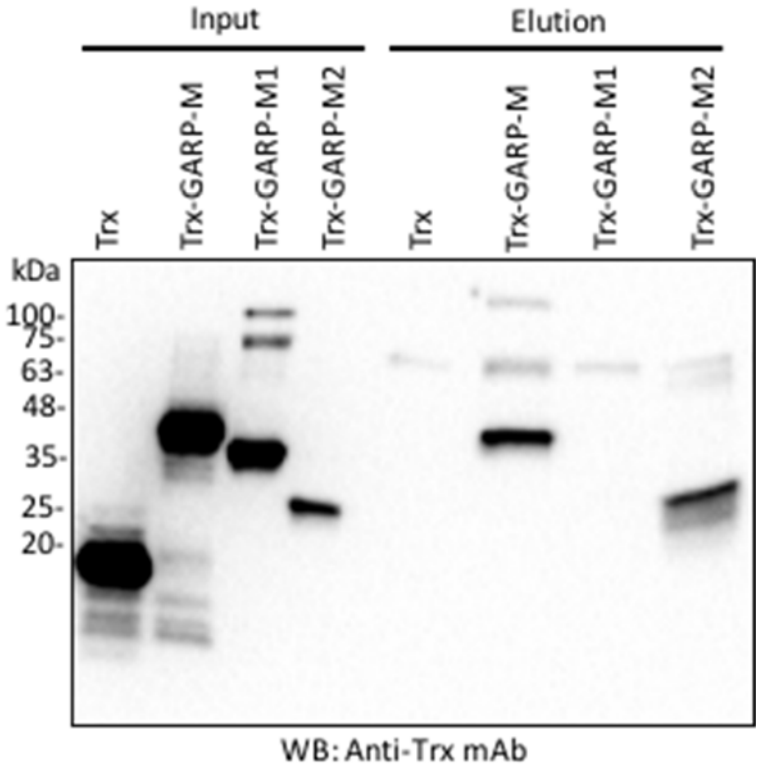


Figure 3.5. Trx-GARP-M2 Binds to Human Erythrocytes, but Trx-GARP-M1 Does Not.

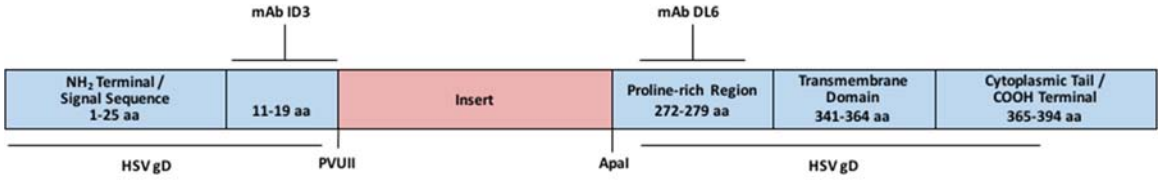
RBC binding assay of non-overlapping segments of Trx-GARP-M. Trx-GARP-M2 bound to human RBCs. Trx-GARP-M was used as a positive control. No binding was detected with Trx-GARP-M1 or the negative control Trx. All protein concentrations were normalized at 1.0 μM. 1.5 M. NaCl was used to elute RBC bound proteins. Mouse monoclonal anti-Trx antibody and ECL prime western blotting kit were used to detect the signals.

3.1.4. Expression of GARP-M on the Surface of CHO-K1 Cells and Binding of Human RBCs to GARP-M-bearing CHO-K1 cells

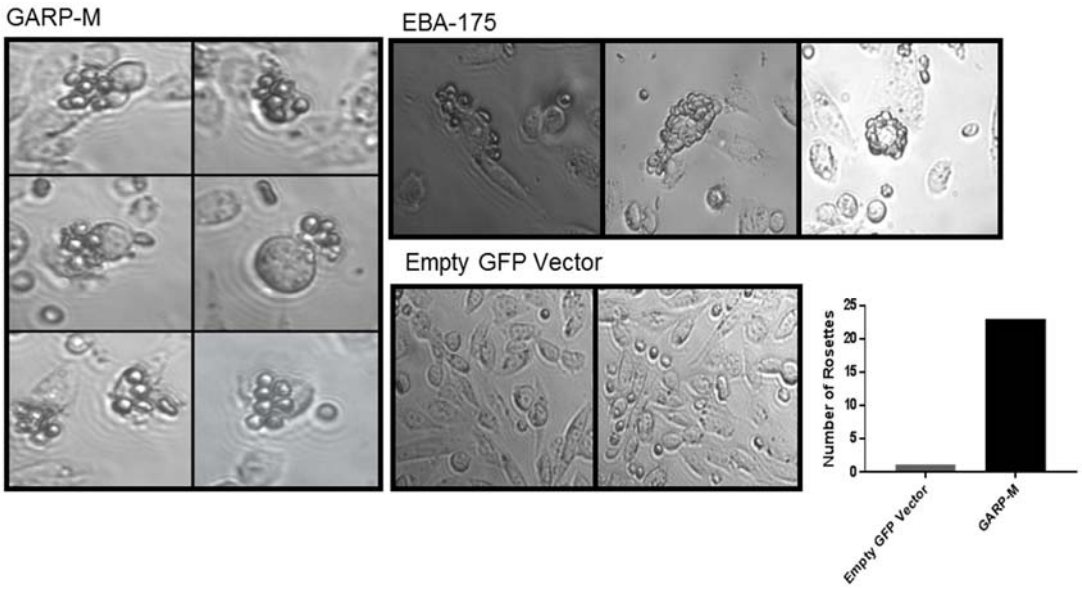
I further characterized the RBC binding activity of Pf-GARP using CHO-K1-based surface expression and RBC binding system (Chitnis and Miller, 1994; Jiang et al., 2011; Mayer et al., 2009; Mayer et al., 2002; Smith et al., 1998). The detailed schematic of the vector is shown in Figure 3.6 A (Chitnis and Miller, 1994). The stable recombinant GARP-M segment was cloned into pRE4 vector and transfected into CHO-K1 cells. Transfected CHO-K1 cells expressing GARP-M were incubated with 100 μL of 10% hematocrit human RBCs at 37^0C for two hours in 2 mL cell culture media. Following 15 minutes washing of the coverslips by inverting them in PBS to remove unbound RBCs, cells remained on the coverslips were fixed. The interaction of the transfected CHO-K1 cells with normal untreated human RBCs was identified and visualized under the microscope (Figure 3.6 B). Multiple RBCs were found to bind GARP-M-transfected cells forming rosettes. Relatively fewer number of rosettes and number of erythrocytes forming the rosettes were detected in GARP-M transfected cells than those formed by the positive control, EBA-175 (Jiang et al., 2011; Mayer et al., 2001; Mayer et al., 2004; Sim et al., 1994). However, the number of RBCs bound to GARP-M transfected CHO-K1 cells was substantially greater than the negative control with empty pRE4 vector expressing GFP only.

The extracellular expression of the proteins cloned into pRE4 vector on the surface of transfected CHO-K1 cells was confirmed by immunofluorescence assays using mouse anti-ID3 mAb (Figure 3.6 C).

A



B



C

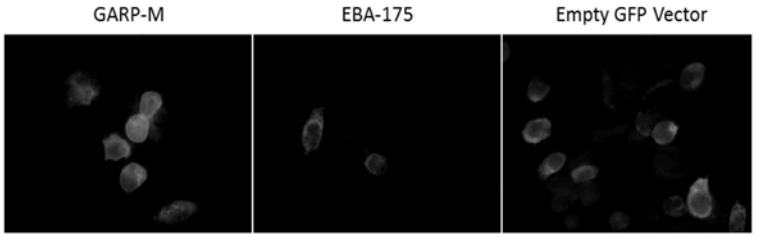


Figure 3.6. Expression of Different Malaria Proteins on the Surface of CHO-K1 Cells

A. Malaria protein inserted into pRE4 vector, a chimeric construct of HSV gD1, was used for expressed on the surface of mammalian cells. All proteins used in the assay were cloned into pRE4 vector. **B** and **C**, CHO-K1 Erythrocyte-binding Assay. After performing the RBC binding assay with CHO-K1 cells, some rosettes were formed with GARP-M (**B**, left). Multiple rosettes were formed with CHO-K1 cells that were transfected with the positive control pRE4-EBA-175 (B, upper right), while no rosettes

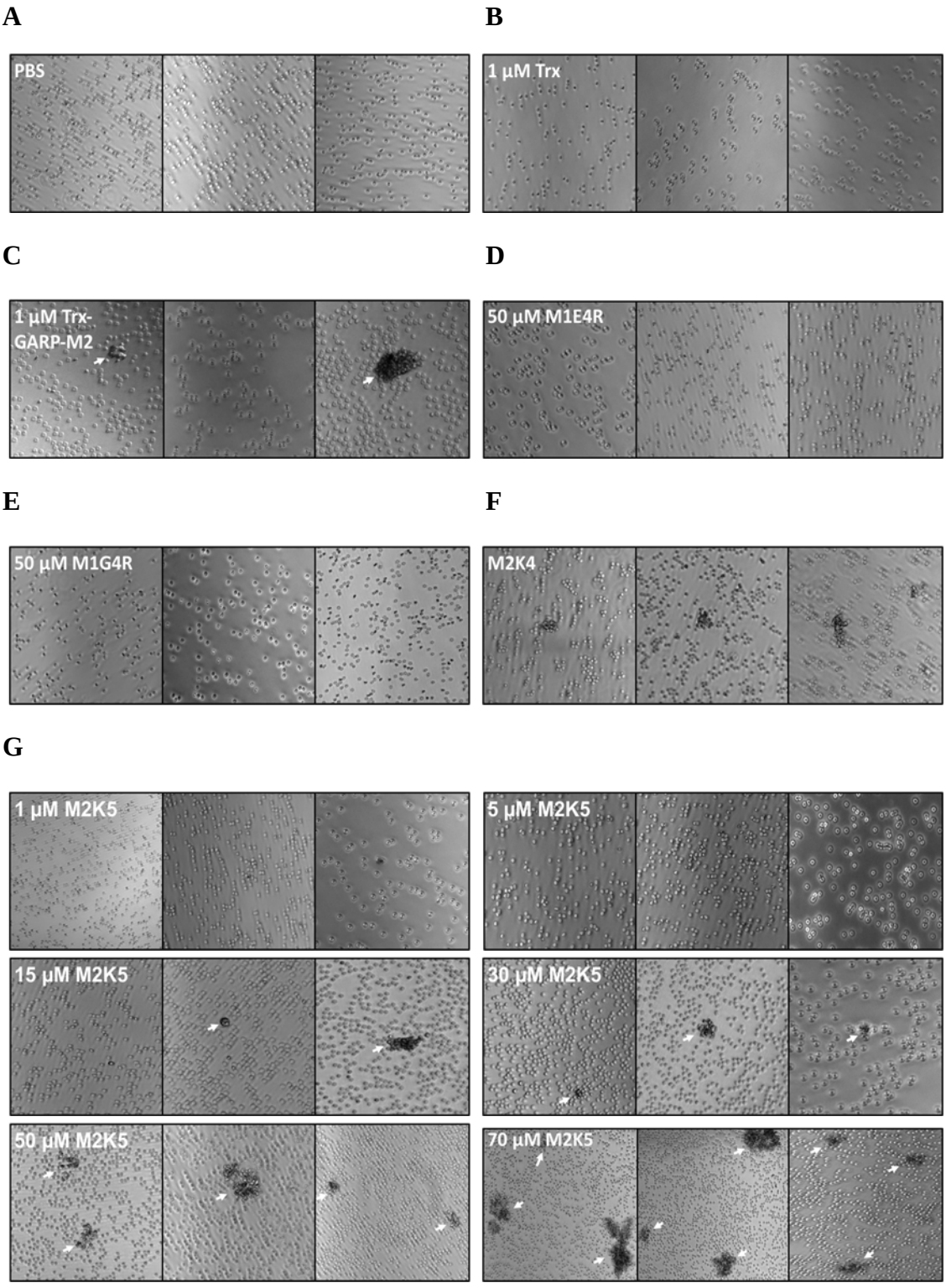
were seen in cells transfected with the negative control pRE4 empty vector (**B,** lower right). This experiment was repeated two times. Similar results were obtained. A representative number of rosette count is shown in the bar graph. Immunofluorescence assay of CHO-K1 cells that were transfected with GARP-M, EBA-175 and the empty vector confirmed surface expression of the respective proteins using mouse anti ID3 mAb and goat anti-mouse IgG secondary antibody with Alexa Fluor 633 conjugate (**C**). Figure 3.6 A has been adapted from Chitnis, C. E., & Miller, L. H. (1994). Identification of the erythrocyte binding domains of Plasmodium vivax and Plasmodium knowlesi proteins involved in erythrocyte invasion. *J Exp Med, 180*(2), 497-506. Figure 1. Changes include recreation of the pRE4 vector map that includes detailed amino acid numbers of different regions of the construct.

3.1.5. Aggregation of Human RBCs Induced by the Addition of Pf-GARP Synthetic Peptides

Since we observed clusters of RBCs formed on the GARP-M expressing CHO-K1 cells, I hypothesized that Pf-GARP has the intrinsic ability to link RBCs to form clusters. This phenomenon is directly relevant to the pathology of malaria since rosette formation has been correlated with occlusion of microvasculature in the brain (Ho et al., 1991; Kaul et al., 1991; Naumann et al., 1991; Wahlgren et al., 1989). To test this hypothesis, I designed a series of peptides from GARP-M (Table 3.2). Two peptides represent the sequence within GARP-M2 region while others were designed for repetitive sequences found in GARP-M1. Peptides were chemically synthesized and their ability to crosslink RBCs was tested *in vitro*.

The experimental approach we designed was slightly modified from a previously published assay (Chotivanich et al., 2004). Enzyme-untreated human RBCs (2 μL of 20% hematocrit) were incubated with Pf-GARP peptides, M2K4 and M2K5, in 500 μL of binding buffer, PBS. Live unfixed RBC aggregates were formed and unambiguously

detected under the microscope following two hours of gentle rotation at room temperature. There was no need to fix the aggregates or transfer the mixture on glass slides for visualization purposes. Figure 3.7 shows representative photographs of the RBC aggregates visualized under the microscope. We used M2K5 as an optimum RBCs aggregation-stimulating peptide for further experiments, and found that it caused aggregation of RBCs in a dose dependent manner with an optimal concentration of 50 μM (Figure 3.7, panel G). M2K4 formed RBC aggregates as well (Figure 3.7, panel F). Very few aggregates were formed with lower concentrations of M2K5 (1.0 - 30 μM) (Figure 3.7, panel G) or with 1.0 μM of Trx-GARP-M2 (Figure 3.7, panel C). No aggregates formed in the PBS group (Figure 3.7, panel A) or by the negative control peptides M1E4R and M1G4R (Figure 3.7, panels D and E) and by Trx protein (Figure 3.7, panel B).


A
PBS
B
1 µM Trx
C
1 µM Trx-GARP-M2
D
50 µM M1E4R
E
50 µM M1G4R
F
M2K4
G
1 µM M2K5
5 µM M2K5
15 µM M2K5
30 µM M2K5
50 µM M2K5
70 µM M2K5

H

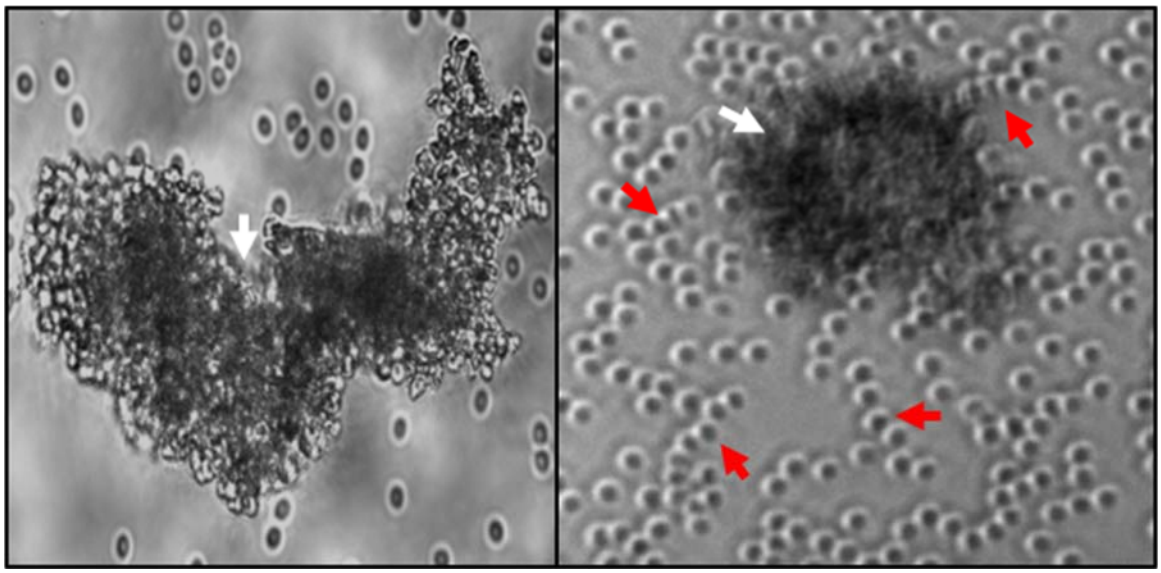


Figure 3.7. *In vitro* human RBC aggregates induced by Pf-GARP synthetic peptides. **A**, M2K5 peptide promotes RBC aggregation in a dose dependent manner. Multiple and larger RBC aggregates were clearly detected by 50 μM and 70 μM concentrations (pointed by white arrows, panel **G**). Similar effect was seen with M_2K_4 (concentration not determined, panel **F**). Very few aggregates were detected by Trx-GARP-M2 (panel **C**). No aggregates detected in the negative control groups; PBS, Trx, M1E4R peptide and M1G4R peptide (panels **A**, **B**, **D** and **E**). **H**, Magnification of RBC aggregates (white arrows) and rouleaux structures (red arrows) formed. This experiment was repeated three times. Comparable results were obtained each time.

Table 3.2. Sequence of Synthetic Peptides Used in the Study.
M2K4 and M2K5 are part of GARP-M2 which contain the encoding amino acid sequences 417-436 and 417-441 of Pf-GARP, respectively. M1E4R and M1G4R are considered as negative controls which contain multiple repeats of Pf-GARP.

Peptide Name	Peptide Sequence
M2K4	Biotin-SGSG-KGKKDKGKKDKGKHKKAKKE
M2K5	Biotin-*KGKKDKGKKDKGKHKKAKKEKVKKH
M1E4R	Biotin-SG-EEHKEEEHKEEEHKEEEHKE
M1G4R	Biotin-SG-GEHKEGEHKEGEHKEGEHKE
	*Aminohexanoic acid linker

3.1.6. Detection of Pf-GARP in the *P. falciparum* Supernatant

If Pf-GARP protein plays a functional role in RBC binding and RBC clustering, it would be expected that Pf-GARP protein is found in the extracellular environment; i.e., plasma in the *in vivo* situation and the culture supernatant under the *in vitro* condition. Upon rupture of iRBCs, all exported malaria proteins are expected to be released from the iRBCs. However, some of them are secreted out even before their rupture (Soni et al., 2016). I tested if Pf-GARP protein was present in the culture supernatant of *P. falciparum* 3D7 culture using our GM-7 mAb that detects GARP-M1 region of the Pf-GARP as shown in Figure 3.2 (panel A). The GM-7 mAb recognizes a 48 kDa antigen in the parasite lysate (data not shown). This 48 kDa band probably represents a processed form of Pf-GARP protein *in vivo*, since the expected molecular weight (MW) of full length Pf-

GARP protein is much larger (calculated MW ~80 kDa (Aurrecoechea et al., 2009)). We employed a slightly modified assay to obtain parasite supernatant (Goel et al., 2003). Schizonts were purified by magnetic columns. 5 μL of human RBCs alone and ~ 5 x 10^5 schizont-iRBCs (without the addition of extra uRBCs) were incubated for ~20 hours in CMM at 37^0C according to the malaria culture protocol. Supernatant was collected and clarified by multiple centrifugations. Clear obtained supernatant was mixed with protease inhibitor cocktail, and tested by Western blotting to detect Pf-GARP. It was stored at -80^0C when necessary.

We confirmed native Pf-GARP expression and release in the culture supernatant, however, the precise release time was not determined at this stage and would require a timed collection of samples overnight using more sensitive quantitative assays. The GM-7 mAb, raised against the middle region of Pf-GARP protein, detected two released peptides specific for Pf-GARP with approximate molecular weights of ~48 kDa and ~38 kDa, as shown in Figure 3.8. No corresponding bands were detected in the uRBCs negative control group.

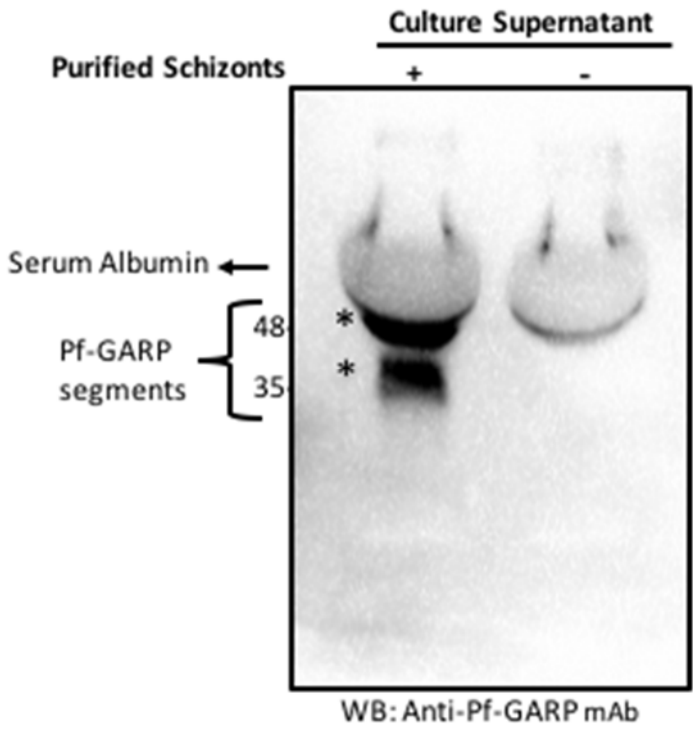


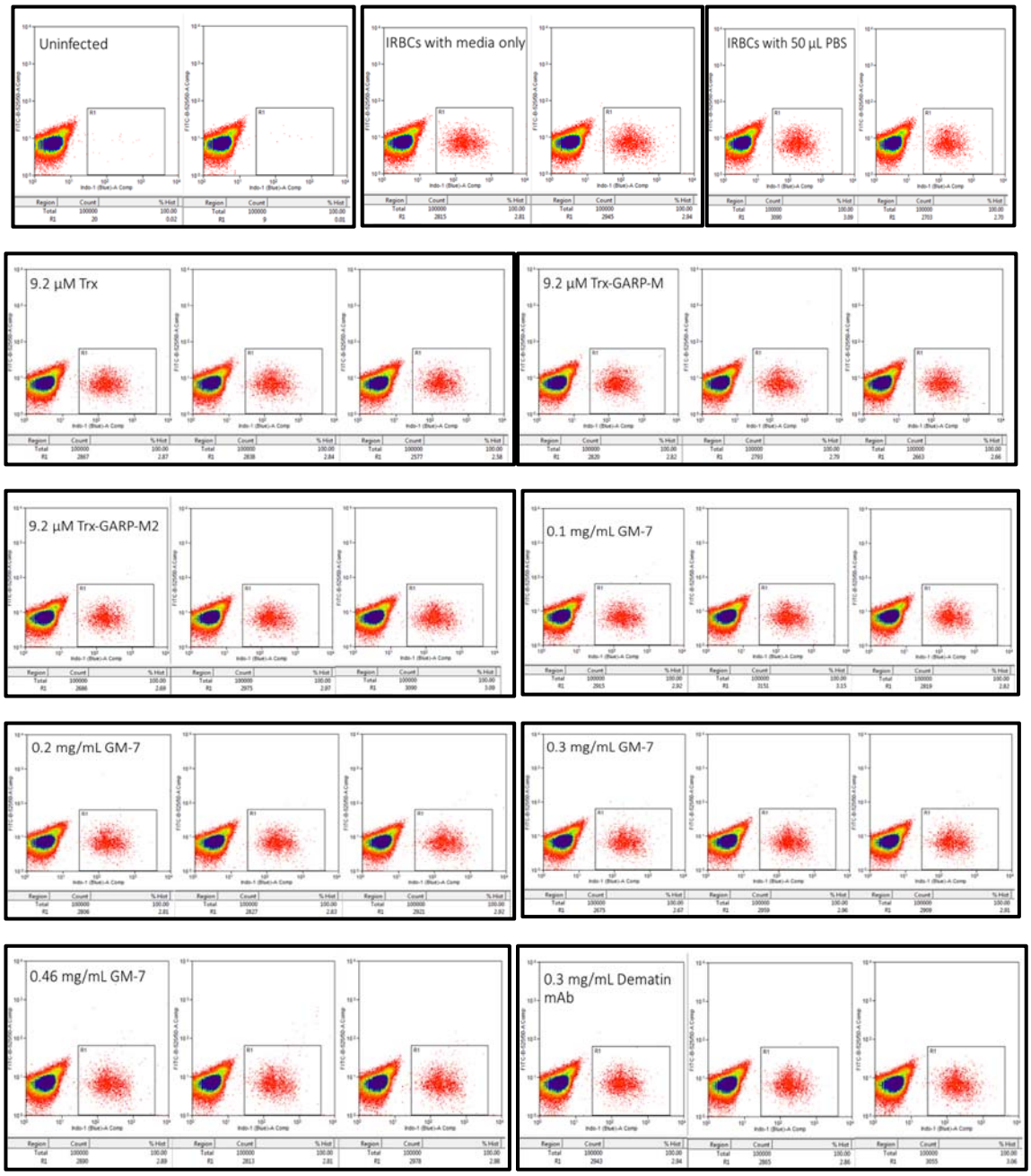
Figure 3.8. Secretion of Pf-GARP in the *P. falciparum* Supernatant
Secretion of Pf-GARP was detected within 20 hours following the addition of magnetic purified schizonts to the CMM. Western blotting detected two bands in the *P. falciparum* 3D7 supernatant that likely originated from the GARP-M segment of Pf-GARP. The lower 38 kDa could be a proteolyzed product of 48 kDa polypeptide.

3.1.7. Low Concentrations of Recombinant Pf-GARP Segments Did Not Interfere with *P. falciparum* Invasion in Human RBCs

A major interest of ours as well as others for studying the RBC binding proteins of *P. falciparum* is their potential role in the RBC invasion process by malaria parasites. I tested whether Pf-GARP plays a role in invasion by using Pf-GARP recombinant proteins as competitive inhibitors. I also tested the effect of GM-7 mAb for the inhibition of invasion. Invasion assays of *P. falciparum* 3D7 were performed using uRBCs preincubated with GARP-M and GARP-M2 proteins as well as GM-7 mAb (specific amounts added are shown in Figure 3.9 panels A and B) for two hours at 37^0C. 1% of magnetically purified schizonts were added to 2.5% hematocrit of uRBCs in a total volume of 200 μL. After 24 hours of incubation under standard malaria culture condition, the culture pellet was stained and fixed. Percentage of parasitemia was calculated using flow cytometry as described in section 2.14. The parasitemia increased from 1% to 3% in all treatment groups without significant differences between them than the control groups, CCM and PBS (Figure 3.9 B). Dematin mAb was also included as a negative control for GM-7 mAb.

The GARP-M proteins and GM-7 mAb did not significantly inhibit the invasion efficiency under these conditions. At this stage, we can speculate several possibilities for this observation: 1) Higher concentration of recombinant proteins is required to block the invasion, 2) GM-7 mAb is not sufficiently concentrated to block the Pf-GARP function and/or 3) GARP-M is not involved in the invasion process.

A


Uninfected
IRBCs with media only
IRBCs with 50 µL PBS
9.2 µM Trx
9.2 µM Trx-GARP-M
9.2 µM Trx-GARP-M2
0.1 mg/mL GM-7
0.2 mg/mL GM-7
0.3 mg/mL GM-7
0.46 mg/mL GM-7
0.3 mg/mL Dematin mAb

B

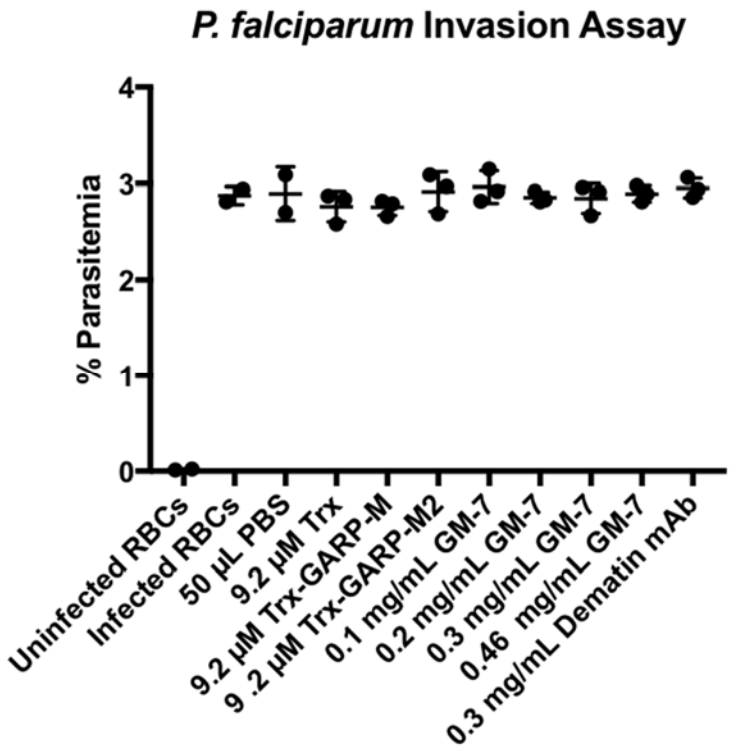


Figure 3.9. Recombinant Pf-GARP proteins or Pf-GARP mAb did not interfere with *P. falciparum* invasion.
Flow cytometric analysis of parasitemia in the absence and presence of different treatments. One-way ANOVA (Analysis of variance) was performed between iRBC groups. No significant difference was detected. This experiment was repeated three times. Similar results were obtained.

3.2. Identification of Host RBC Receptor that Binds to Pf-GARP

3.2.1. Receptor Sensitivity of Human Erythrocytes to Enzymes with Known Specificity

Certain surface proteins of the RBCs are known to be sensitive to the proteases and glycosidase enzymes treatment (Dahr et al., 1987; Steck et al., 1971). For instance, trypsin is known to digest glycophorin A, chymotrypsin cleaves band 3 (Howard et al., 1982), and neuraminidase removes sialic acid residues from glycoproteins (Doinel et al., 1978; Luner et al., 1975). Such enzyme treated-RBCs were used historically to characterize the receptor molecules on RBCs, and evaluate changes in receptor sensitivity toward certain parasite ligands (Burleigh and Sinai, 2008; Mayer et al., 2004; Perkins, 1981). Likewise, using mutant erythrocytes or antibodies that target specific receptors can determine the specificity of ligand binding. Therefore, enzyme treatment of RBCs is generally considered as the first step in detecting potential host receptors on erythrocytes (Burleigh and Sinai, 2008). Several enzymes including neuraminidase (5 Units/ml), trypsin (1.0 mg/mL), and chymotrypsin (1.0 mg/mL) were used to treat human RBCs prior to their use in the RBC-binding assay. Enzymes were incubated with 80 μL of 25% hematocrit RBCs for one hour rotating at 37^0C. RBCs were extensively washed and then treated for 20 minutes rotating at room temperature with trypsin/chymotrypsin inhibitor, and followed by a wash with PBS in case for neuraminidase treatment. RBCs were washed again and used for RBC-binding studies by incubating them with 1.0 μM of Trx-GARP-M or Trx for two hours rotating at room temperature. Mixture was separated on silicone oil. Unbound proteins were detected in the binding supernatant before the washing and elution steps to eliminate any possibility of remaining traces of

chymotrypsin remained after the erythrocyte treatment that could induce cleavage of Trx-GARP-M. Unbound protein amounts were equal in all groups (Figure 3.10, right panel). The remaining pellet of RBCs was washed extensively. Bound proteins were eluted by high salt buffer and detected by Western blotting using Trx mAb. The binding affinity of Trx-GARP-M was decreased with chymotrypsin-treated human erythrocytes (Figure 3.10). Experiments with lower amounts of chymotrypsin and trypsin (0.5 mg/ml) were performed, and a similar reduction in the binding sensitivity was also detected with chymotrypsin pre-treated RBCs (data are not shown).

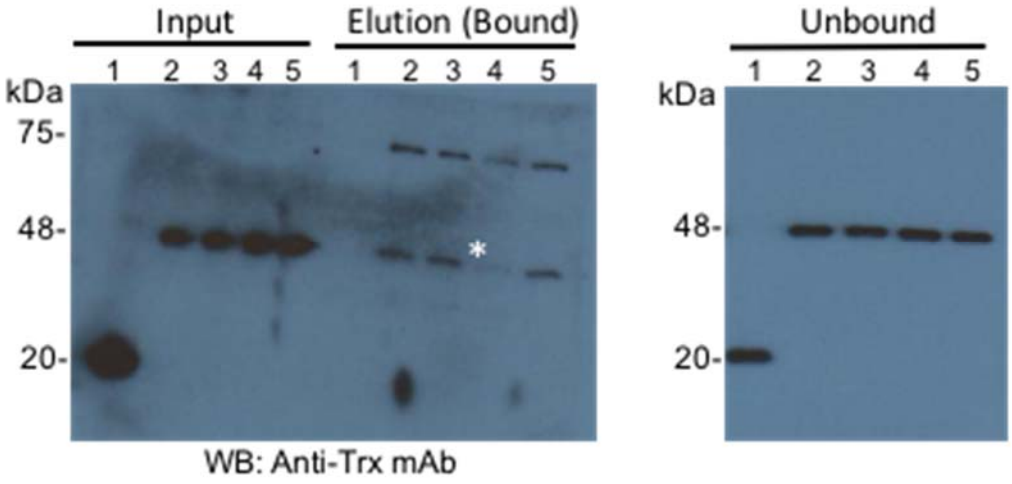


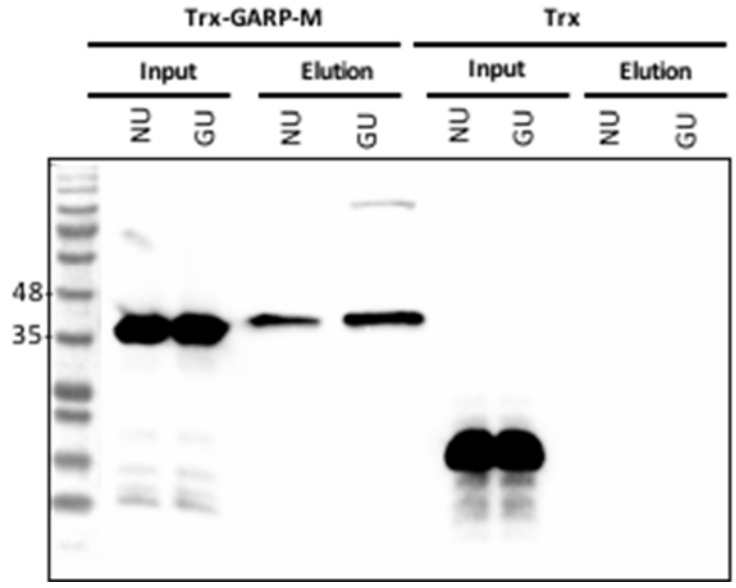
Figure 3.10. Trx-GARP-M Binding is Sensitive to Chymotrypsin.
RBC-binding assay of enzyme-treated RBCs. **Left**, input and RBCs bound (elution) proteins are shown. Lanes **1** and **2** are Trx and Trx-GARP-M in untreated human RBCs group, respectively. Lanes **3-5** correspond to Trx-GARP-M in the groups of RBCs treated with 5.0 Units/mL neuraminidase, 1.0 mg/mL chymotrypsin and 1.0 mg/mL Trypsin. **Right**, unbound excess Trx and Trx-GARP-M is shown at equivalent amounts in all groups recovered from the binding assays.

3.2.2. Glycophorin B is Not the Chymotrypsin-sensitive Receptor of Human RBCs for Pf-GARP

Glycophorin B is a well-known chymotrypsin-sensitive receptor on human RBCs (Reid, 2009; Reid and Storry, 2001). Using our established RBC-binding assay, we tested the binding ability of Trx-GARP-M towards glycophorin B-null erythrocytes that lack the antigens carried on the GPB receptor (S-s-U-) (Reid, 2009; Reid and Storry, 2001) for several reasons: 1)- Pf-GARP binding to RBCs was affected by pre-treatment of human RBCs with chymotrypsin, 2)- Chymotrypsin is known to cleave the extracellular region of the type I transmembrane glycophorin B protein (Reid and Storry, 2001) besides band 3 cleavage (Howard et al., 1982), and 3)- Unlike band 3 null erythrocytes, glycophorin B-null erythrocytes are stable and therefore suitable for the binding studies. 80 μL of 25% hematocrit of normal and glycophorin B-null RBCs were incubated with 1 μM of Trx-GARP-M or Trx for two hours at room temperature in the binding buffer. RBCs were washed several times and proteins were eluted in 40 μL of elution buffer. Bound proteins were blotted and detected using Trx mAb. We found that Trx-GARP-M could bind to the S-s-U- human erythrocytes. In fact, the binding was even more efficient as compared to normal RBCs (Figure 3.11 panel A). This was a surprising finding since we expected a complete or marked inhibition of Trx-GARP-M binding to glycophorin B-null RBCs. Unpredictably, we found a further enhancement of Trx-GARP-M binding after pre-treatment of the GYPB-null S-s-U- erythrocytes with chymotrypsin as compared to untreated GYPB-null S-s-U- erythrocytes (Figure 3.11 B). These observations suggested that the surface accessibility of the putative RBC receptor for Pf-GARP could be

influenced by the orientation and conformational stability of the receptor in the presence and absence of glycophorin B.

A



B

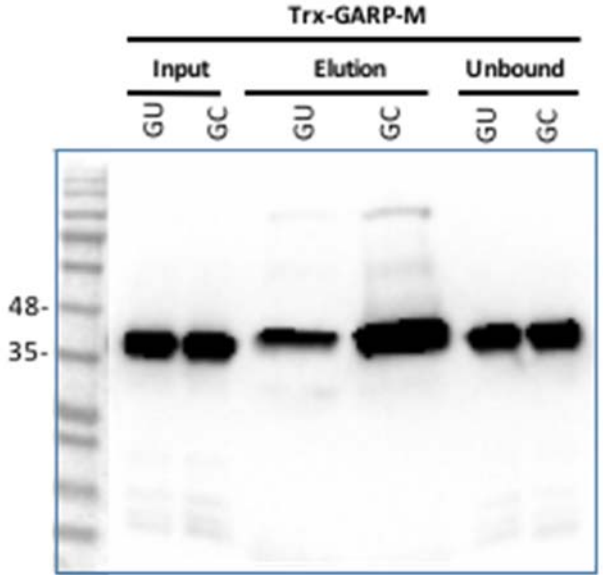


Figure 3.11. Trx-GARP-M Binds to Normal and Glycophorin B-null Human Erythrocytes.
A, Binding of Trx-GARP-M towards glycophorin B-null human RBCs. Proteins at 1.0 μM concentration were incubated with human RBCs in PBS containing 1.0 mg/ml BSA for two hours at room temperature. Unbound proteins were washed three times. Bound proteins were eluted by 1.0 M NaCl and detected by mouse monoclonal anti-Trx antibody and ECL prime western blotting detection kit. Normal human RBCs were used as positive control for Trx-GARP-M binding. No binding was detected with the negative control, Trx. **B**, Enhanced binding of Trx-GARP-M towards chymotrypsin-treated glycophorin B-null human RBCs. Abbreviations used: NU, normal untreated; GU, glycophorin B-null untreated and GC; glycophorin B-null chymotrypsin-treated human RBCs.

3.2.3. Phage Display Screens of Human Reticulocyte cDNA Libraries with Pf-GARP-M

Reverse phage display library screens were performed to identify the human RBC receptor that binds to Pf-GARP. The cDNA phage-display random and oligo dT primed human reticulocyte libraries were generated by the Chishti lab and used for the screens. 10 μg/mL of Trx-GARP-M and His-GARP-M recombinant proteins were coated on Immulon microtiter 96 well plates (Thermo Fisher Scientific), washed, blocked with blocking buffer, and used as bait for cDNA screens. Phage cDNA library was incubated in each well for 30 minutes and then washed extensively. Bound phages were eluted by high salt buffer and amplified through four rounds of incubation and biopanning. Plaque assays were performed, and DNA sequence results of amplified PCR products from positive hits were analyzed. Multiple screens of both cDNA phage display human reticulocyte libraries yielded multiple potential hits, and up to 83 clones were sequenced. No specific interactions or any RBC proteins/receptors as positive clones were found in these screens. A summary of the clones is shown in Table 3.2.

One major limitation of reticulocyte-derived phage cDNA libraries is the hyper abundance of globin clones in such screens. Therefore, it was not feasible to identify specific positive binders to the recombinant Pf-GARP using this methodology.

Table 3.3. Phage Display Screens of Random and Oligo dT Primed Human Reticulocyte Libraries with GARP-M
Both Trx-GARP-M and His-GARP-M were used as baits for the screens. This table shows a summary of the frequent clones obtained. No specific RBC membrane proteins were identified as host receptors for Pf-GARP.

Summary of phage cDNA clones obtained after the biopanning with Trx-GARP-M
Homo sapiens zinc finger CCCH-type containing 6 (ZC3H6)
Homo sapiens hemoglobin alpha 1 (HBA1)
Homo sapiens hemoglobin alpha 2 (HBA2)
Homo sapiens hemoglobin beta (HBB)
Homo sapiens ribosomal protein L13a
Homo sapiens ribosomal protein L19
Multiple short sequences with no significant similarities

3.2.4. Identification of Putative RBC Pf-GARP Binders using Blot Overlay Assay

Because we were not successful with the screening cDNA phage display human reticulocyte libraries to identify Pf-GARP erythrocyte receptor, I decided to use the blot overlay assay (far Western blotting) as an alternative approach to identify and characterize the RBC receptor (Wu et al., 2007). This method has yielded successful results in the malaria field previously (Dankwa et al., 2016; Goel et al., 2003; Lanzillotti

and Coetzer, 2006). 100 μL of 100% hematocrit RBCs were used to prepare RBC membrane proteins (ghosts) by hypotonic lysis in the presence of protease inhibitor cocktail. Similar amounts of ghosts per group were loaded to each lane of the SDS-PAGE gel and transferred on the nitrocellulose membranes. Four different preparations of RBC ghosts were made: normal untreated (NU), normal chymotrypsin-treated (NC), S-s-U-untreated (GU), and chymotrypsin-treated S-s-U- as shown in Figure 3 (panels A and B) by the CBB and Ponceau S stains. Membranes were blocked extensively by the blocking buffer overnight at 4^0C. 1 μg/mL Trx-GARP-M2 was incubated with the membranes at 4^0C overnight to detect the protein-protein interactions using PBST and TBST buffers. Membranes were washed extensively, incubated with Trx mAb, and incubated with the secondary antibody. Signals of bound proteins were detected by the addition of chemiluminescent substrate. Several bound proteins were detected by the anti Trx mAb. The respective size of promising positive bands detected with Trx-GARP-M2 incubation using TBST buffer were: ~48 kDa in all four groups, ~60 kDa in the chymotrypsin-treated groups of both normal and GYPB-null human erythrocytes, and ~100-135 kDa mainly with the GYPB-null groups (Figure 3 panel D). A similar pattern of binding was observed with Trx-GARP-M2 with the use of PBST binding buffer instead of TBST (Figure 3 panel F), to exclude differences in the binding ability using different chemiluminescent substrates. The Trx-GARP-M showed comparable results (Figure 3 panel C). No binding was detected when the negative control Trx was incubated with RBC ghosts (Figure 3 panel E). The identification of the 60 kDa band with chymotrypsin-treated groups made us consider the chymotryptic fragment of band 3 as a strong receptor candidate for Pf-GARP that must be evaluated further by other means. As

shown in Figure 3 (panel G), we performed western blotting using a polyclonal anti-band 3 antibody. The ~100-135 kDa and ~60 kDa bands were detected, indicating that these bands correspond to band 3 and its fragments. The ~48 kDa band was only weakly detected in the Western blots suggesting that this band might be a degradation product of band 3 or an unknown receptor, thus raising the possibility that Pf-GARP might bind to multiple receptors in human RBCs.

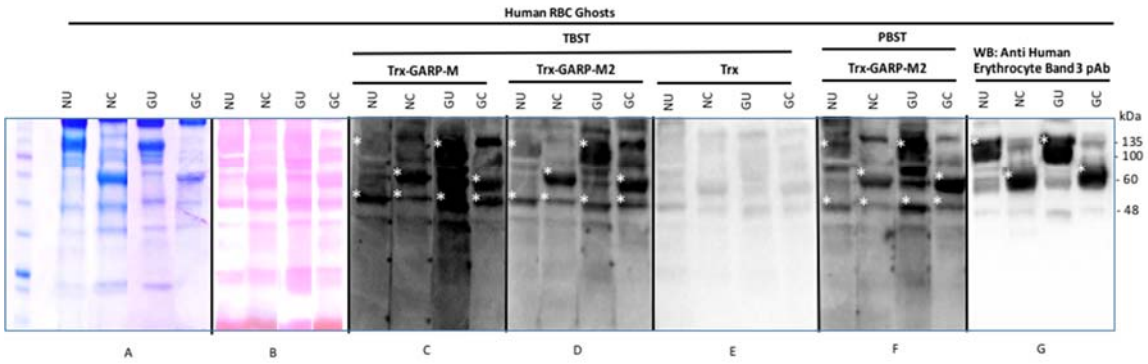


Figure 3.12. Blot Overlay Assay on Immobilized Human RBC Ghosts Using Trx-GARP-M2 and Trx-GARP-M as detectors

Human RBC ghosts were prepared from 100 µL of packed NU, normal untreated, NC, normal chymotrypsin-treated, GU, Glycophorin B-null untreated and GC, Glycophorin B-null chymotrypsin-treated human erythrocytes. **A**, Ghosts were analyzed by CBB-stained 12% SDS-PAGE. **B**, Ghosts blotted on nitrocellulose membranes were visualized by Ponceau S stain. **C-F**, Blot overlay results after incubation with 1.0 µg/ml of Trx-GARP-M (**C**), Trx-GARP-M2 (**D**) or Trx (**E**) using TBST, and Trx-GARP-M2 using PBST (**F**). Anti-Trx monoclonal antibody and Immun-Star goat anti-mouse Alkaline Phosphatase secondary antibody chemiluminescence kit were used for binding and signal detection. **G**, Western blotting using rabbit polyclonal anti-band 3 antibody against the entire N-terminal fragment of human RBC protein. Anti-rabbit IGG HRP secondary antibody and ECL prime kit were used for signal detection.

3.2.5. Human Erythrocyte Band 3 is the Host Receptor for Pf-GARP as Identified by the Pull-down Assay using Recombinant Trx-GARP-M2

Since band 3 appears to be a promising host receptor from the blot overlay assays, consistent with reduced Trx-GARP-M binding to chymotrypsin-treated normal RBCs, I decided to perform pull-down experiments to confirm whether band 3 is as a positive binder for Pf-GARP fusion protein attached to the beads (Alam et al., 2015; Rathore et al., 2017; Yajima et al., 2008). The Trx-GARP-M2 protein was incubated and immobilized on cobalt beads for one hour at 4^0C. The detergent-solubilized RBC ghosts' proteins were prepared by treatment of RBC ghosts with 1% $C_{12}E_8$ detergent in the hypotonic buffer on ice. Solubilized human RBC membrane-bound proteins, in their native state, were obtained by centrifugation of solubilized ghosts. The solubilized ghosts were first precleared for one hour by incubation with Cobalt beads alone to eliminate any possibility for non-specific binding of membrane proteins to the beads. The pre-cleared solubilized ghosts were incubated with Trx-GARP-M2-bound Cobalt beads overnight at 4^0C. Proteins attached to the beads were extensively washed before subjecting to Western blotting using rabbit an anti-human band 3 polyclonal antibody raised against the cytoplasmic N-terminus domain of band 3. The Trx-bound Cobalt beads were used as negative control in parallel with Trx-GARP-M2 beads. Western blotting results confirmed that band 3 and its chymotryptic fragment are specific host binding proteins for Pf-GARP using Trx-GARP-M2 bound to Cobalt beads and not by Trx-bound beads (Figure 4 panel C). Normal and chymotrypsin-treated solubilized ghosts by the non-ionic detergent, $C_{12}E_8$, were used as lysate input to normalize the loading controls (Figure 4 panel D). It is to be noted that degradation products originating from non-ionic

detergents, mainly their ether contents, are known to cause band 3 irreversible aggregation and degradation due to the activation of RBC proteases (Casey and Reithmeier, 1991; Golovtchenko-Matsumoto et al., 1982; Mandal et al., 2003; Pappert and Schubert, 1983; Rinalducci et al., 2012; Tarone et al., 1979). High molecular weight oligomers and ~70 kDa band were detected specifically as human band 3 fragments in both input samples, while the 60 kDa band was detected exclusively in the chymotrypsin-treated group. However, no band-3 oligomers were captured by the Trx-GARP-M2 under the same conditions.

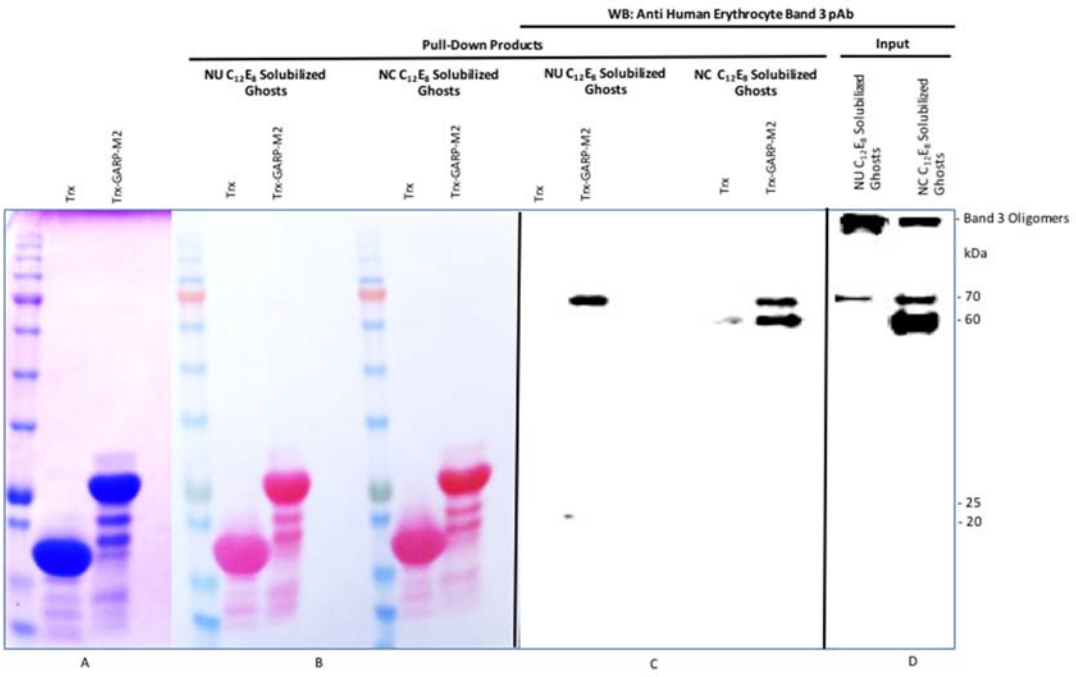


Figure 3.13. Trx-GARP-M2 Pull-down Assay Using Detergent Solubilized RBC Membrane Proteins

A, CBB stained 12 % gel (SDS-PAGE) showing Trx and Trx-GARP-M2 attached to Cobalt beads. **B** and **C**, Trx and Trx-GARP-M2 attached to Cobalt beads were incubated with $C_{12}E_8$ solubilized normal untreated and chymotrypsin-treated ghosts. Bound proteins were blotted on nitrocellulose membrane. **B**, Ponceau S stain of the beads-bound proteins. **C**, Anti-band 3 Western blotting. The 70 kDa band specifically associated with Trx-GARP-M2 in untreated RBCs (left panel). Both 60 kDa and 70 kDa bands were

detected in the chymotrypsin treated RBCs (right panel). No binding of band-3 towards Trx-bound beads was detected. **D**, Western blot of $C_{12}E_8$ solubilized normal and chymotrypsin-treated ghosts as inputs. Western blotting of Trx-GARP-M2 and Trx proteins alone did not reveal any non-specific signal detected by band-3 antibody (data not shown). Abbreviations used; NU, normal untreated and NC, normal chymotrypsin-treated human RBCs.

In summary, our results suggest that *P. falciparum* utilizes Pf-GARP as a potential virulence factor to exacerbate the severe complications of malaria infection. These pathophysiological changes involving potentiation of erythrocyte aggregation and adhesion pathways are likely to be mediated via the recognition of host RBC band 3 by Pf-GARP.

Chapter 4: Discussion and Future Directions

Previous Pf-GARP knockout experiments have shown that it is not essential for parasite survival, however, its gene disruption causes substantial reduction in the adhesion characteristics of iRBC (Maier et al., 2008). Our phage display cDNA library screens revealed positive Pf-GARP-human RBC interactions using two independent cDNA libraries of different *P. falciparum* strains, and this strong ligand-receptor interaction was confirmed by biochemical studies. Based on these observations, we hypothesize that Pf-GARP plays a key role in promoting the cytoadherence and rosetting virulence effects of malaria. These investigations will contribute to our understanding of the role of Pf-GARP in malaria pathogenesis.

4.1. Pf-GARP is a RBC-binding Ligand

Phage display library screening has been employed to identify human RBC binding proteins of *P. falciparum* (Lauterbach et al., 2003). It is a powerful technique to identify novel ligand-receptor interactions. Using this method, researchers have identified novel ligand-receptor interactions and selected RBC positive binders from millions of *P. falciparum* cDNA clones. Some of these ligand-receptor interactions are critical for parasite life cycle (Burleigh and Sinai, 2008). For example, a specific region of MSP-1, a strong malaria vaccine candidate (Barry and Arnott, 2014; Epp et al., 2003; Goodman et al., 2010; Kauth et al., 2003), was found to bind human RBC glycophorin A (GPA) by screening a phage display cDNA library prepared from *P. falciparum* FCR3 strain (Baldwin, 2015; Baldwin et al., 2015). Similarly, we have shown that a specific segment of parasite EBL-1 ligand binds to human glycophorin B by using a phage display screen (Li et al., 2012). Here we report the first identification and mapping of the core-binding

site in Pf-GARP that directly binds to human RBCs using multiple phage display screens from different cDNA libraries of *P. falciparum.*

Because of intriguing findings supporting an interaction between Pf-GARP with human RBCs, we decided not to sequence and characterize all potential cDNA clones and instead focus on the novel Pf-GARP ligand. Nonetheless, it is relevant to mention at least one more ligand here. The S-antigen, which was also identified in our phage display screens for binding to human RBCs, has been reviewed and characterized extensively by Wahlgren & Perlmann (Wahlgren and Perlmann, 2003). S-antigen is a polymorphic exported protein to the PVM and cytoplasm of iRBCs (Bickle and Coppel, 1992). It is believed to be a peripheral merozoite surface protein secreted or released from iRBCs during the late-schizont stage rupture and attached to MSP-1 on merozoite surface (Babon et al., 2007; Cowman et al., 2012; Li et al., 2004; Perkins and Rocco, 1990). A monoclonal antibody against one S-antigen isoform inhibited the invasion of parent *P. falciparum* parasite isolates *in vitro* (Wahlgren and Perlmann, 2003).

Both GARP and S-antigen proteins share low-complexity regions (LCRs) in their structures (Aurrecoechea et al., 2009; Brocchieri, 2001; Davies et al., 2016; Maier et al., 2008). The low-complexity elements are sequences with few amino acid repeats that have no stable three-dimensional structures (Brocchieri, 2001). Although they are abundant in more than 90% of chromosome 2 and 3 loci, their function is still unclear. Several LCRs of Pf-GARP-3D7, as reported in PlasmoDB (Aurrecoechea et al., 2009), are located between the following AA (70-77, 118-166, 262-334, 372-444 and 545-660). A recent study characterized the translocation ability of different repeated segments of Pf-GARP to the RBC membrane (Davies et al., 2016). All segments were cloned in expression

plasmids where the GFP is tagged at the C-terminus and fused with N-terminus segments required for proper export (Pf-GARP$_{119\text{-}163}$, Pf-GARP$_{253\text{-}340}$, Pf-GARP$_{372\text{-}446}$ and Pf-GARP$_{535\text{-}673}$). The first three out of the four segments were translocated from erythrocyte cytosol to periphery in iRBCs. GARP-M2, the RBC-binding region of Pf-GARP identified in this Thesis, overlaps with the third segment. In a different study, expression of Pf-GARP on the surface of iRBCs was detected by polyclonal antibodies against AA (410-673) (Raj et al., 2016). However, this localization did not differentiate whether the Pf-GARP is associated on the inner side of the membrane or bound to the outer surface of iRBCs. Future studies using immunogold labeling approaches may be required to precisely determine the localization of Pf-GARP in infected erythrocytes (Davies et al., 2016).

Recombinant protein expression methods and direct RBC-binding assays are valuable tools for identifying the stable RBC-binding domain of Pf-GARP. In this study, multiple overlapping Pf-GARP segments were identified. GARP-S and GARP-L clones encode overlapping peptide sequences of Pf-GARP. Since GARP-L is very susceptible to degradation, we designed GARP-M as a synthetic gene that encodes codon optimized sequence larger than GARP-S but smaller than GARP-L. The codon optimization is often required to reduce the AT-rich content found in most *Plasmodium* genes so that the transcription, translation, and expression of proteins in bacterial and mammalian systems become more efficient (Narum et al., 2001; Yadava and Ockenhouse, 2003).

To further characterize the specificity of Pf-GARP interaction with RBCs, we determined the core RBC-binding site of Pf-GARP that was designated as GARP-M2. Other binding domains in full-length Pf-GARP are not excluded, but clearly, we could

not detect any RBC binding activity of GARP-M1 using our RBC-binding assays. Overall, the RBC-binding site of Pf-GARP has to be mostly corresponded to the overlapping region between GARP-M2 and GARP-S, specifically within AA (417-437), which corresponds to this peptide (KGKKDKGKKDKGKHKKAKKEK).

Surface expression of GARP-M segment on CHO-K1 cells further validated the ability of Pf-GARP to form rosettes with human erythrocytes. This approach has been used in several previous studies to characterize the RBC-binding ability of proteins (Mayer et al., 2004; Michon et al., 2001).

4.2. *P. falciparum* Exports Extracellular Secretory Antigens

It is known that iRBCs secrete antigens. Several proteins have been detected in the culture supernatant of un-ruptured infected erythrocytes during asexual stages. These proteins vary in their isoelectric points and some other properties such as signal peptide segments, transmembrane regions, and/or PEXEL motifs (Malhotra, 2009). Based on GARP strong interaction with human RBCs, we tested for its presence in the parasite supernatant. Our lab has recently generated a mouse monoclonal antibody against GARP-M region. This antibody is currently under characterization for specificity and localization. Using this antibody, we detected two bands of ~48 kDa and ~38 kDa in the supernatant of magnetically-purified 3D7 *P. falciparum* schizonts. This result suggests that full-length Pf-GARP undergoes multiple cleavage processes in addition to the first expected cleavage at the PEXEL sequence. This observation further indicates that GARP-M might encode the core sequence of functional Pf-GARP that mediates its binding to human RBCs.

4.3. Pf-GARP is Not a Direct Merozoite-invasion Ligand

We tested the role of Pf-GARP in merozoite invasion using the GM-7 monoclonal antibody directed against the Pf-GARP-M1 repeats as described above. A direct role of the monoclonal GM-7 in inhibiting *P. falciparum* invasion was not detected by the addition of up to ~0.5 mg/mL antibody concentration. These initial studies showing lack of a direct role of Pf-GARP in merozoite invasion could be a consequence of the high amounts of Pf-GARP present in the parasite culture consistent with the presence of nearly one million copies of band 3 present on human erythrocyte (McPherson et al., 1993; Wrong et al., 2002). The high abundance of the ligand-receptor complex may require relatively higher concentrations of the antibody to block invasion. Another reason could be that the antibody is directed against the GARP-M1 region, which did not show RBC binding activity in our RBC-binding assays. In fact, our preliminary results showed that invasion efficiency of *P. falciparum* was modestly increased with the pre-incubation of human RBCs with high concentration (up to 200 μM) of His-GARP-M (data not shown). A detailed evaluation of the role of Pf-GARP in merozoite invasion process will be required before making a definitive conclusion about its status as an invasion ligand.

4.4. Pf-GARP Induces RBC Aggregation

Malaria culture supernatant, which contains many secreted proteins and toxic by-products from rupturing of iRBCs, can induce RBC aggregation and formation of rheological structures (Balaji and Trivedi, 2013; Dondorp et al., 2000). The precise mechanism of this phenomenon remains poorly understood. One possible explanation includes detection of methemoglobin as a major toxic substance that induces irreversible RBC aggregation through

the production of free radicals. The free radicals generate oxidative stress environment inside RBCs and trigger surface exposure of phosphatidylserine, which then leads to increased RBC stickiness (Balaji and Trivedi, 2013). Moreover, this aggregation effect is not limited to iRBCs since hemoglobin released from both ruptured uRBCs and iRBCs is ultimately oxidized (Balaji and Trivedi, 2013). Our finding showing the presence of Pf-GARP in the parasite supernatant (Figure 3.8) may offer an alternate mechanism for the RBC aggregation phenomenon. Specifically, we wanted to address the question if Pf-GARP can promote RBC aggregation and clustering. In the assay we have optimized for this study, the potential contribution of coagulation factors, plasma proteins, and platelets-mediated agglutination factors is excluded. We detected a direct effect of synthetic GARP-M2 peptides on the formation and assembly of uRBC clusters. The GARP-M2 peptides promoted the formation of multiple clusters of un-fixed cells that were clearly visualized by microscopy. Since Pf-GARP is a malaria parasite derived protein, this observation suggests a novel role of Pf-GARP in malaria induced host cell aggregation. It is noteworthy that Pf-GARP expression is limited to *P. falciparum* and very closely related species, but not in *P. vivax, ovale, and malariae* (Liu et al., 2010; Maier et al., 2008; Otto et al., 2014; Sundararaman et al., 2016; Triglia et al., 1988), thus implying a functional role of Pf-GARP as a pathogenic protein that causes severe malaria.

4.5. Band 3 is a Host Receptor for Pf-GARP

Published evidence has established that different enzymes can be used to test the changes in RBC receptor sensitivity toward certain malaria ligands (Burleigh and Sinai, 2008). Binding sensitivity of polypeptides/ligands to specific enzyme-treated RBCs is a reasonable approach to narrow down potential host receptors for a specific ligand, and then use more direct techniques to establish their identity (Howard et al., 1982). Several malaria ligand-receptor interactions have been characterized using enzyme-treated RBCs, as summarized in a recent review (Tham et al., 2012).

Consistent with this methodology, our results showed that binding of Trx-GARP-M, the most stable Pf-GARP fusion protein, towards intact human RBCs, was substantially decreased upon chymotrypsin pre-treatment of the cells. Chymotrypsin is known to cleave specific RBC membrane proteins such as band 3 and glycophorin B (Dahr et al., 1987; Groves and Tanner, 1992). This decreased binding of Trx-GARP-M was not observed when human RBCs were pre-treated with trypsin, another protease that is known to cleave RBC glycophorin A and glycophorin C (Dahr et al., 1987). In addition, Trx-GARP-M binding to human RBCs was not affected when RBCs were treated with neuraminidase, an enzyme that removes the sialic acids from multiple cell surface proteins (Luner et al., 1975). This observation is consistent with previous screens performed by the Chishti lab when GARP-S was identified in the phage display library screen using neuraminidase-

treated RBCs. Thus, our overall objective was to characterize and identify the putative host (RBC) receptor that binds to Pf-GARP.

Based on our observations, we excluded glycophorin B as a possible host receptor for Pf-GARP using glycophorin B-null human erythrocytes in the RBC binding assays. Interestingly, we found that Pf-GARP in fact binds more efficiently to glycophorin B null S-s-U- cells, and treatment of these genetically-null RBCs with chymotrypsin further enhanced Pf-GARP's binding to the RBCs. Although unexpected, such increase in binding sensitivity to mutant RBCs including S-s-U- cells had been detected with the malaria proteins previously (Lobo et al., 2003). These findings may originate either from conformational changes of receptors or from the loss or compensation of other blood antigens on the cell surface that may affect access of the malaria ligand toward its host receptor (Lobo et al., 2003). Alternatively, these observations suggest that Pf-GARP may bind to other RBC receptors in a setting when the ligand binding is not completely inhibited. It is known that chymotrypsin cleaves band 3 at two closely spaced sites located in its third extracellular loop thus leaving the multi-pass membrane protein still attached and functional (Reithmeier et al., 2016). Based on these observations, erythrocyte band 3 emerged as a strong candidate receptor for Pf-GARP.

To test the possibility that band 3 is a potential receptor, we first decided to map the RBC binding site within Pf-GARP. The core-binding site

was mapped to GARP-M2, which was then used as a specific detector protein in the blot overlay and pull-down assays, which are powerful methods in detecting protein-protein interactions (Hall, 2015; Louche et al., 2017; Wu et al., 2007). For example, binding of *P. knowlesi* invasion ligands, PkDBPβ and PkDBγ, was substantially decreased to RBC ghosts prepared from neuraminidase-treated macaque RBCs using the blot overlay assay (Dankwa et al., 2016). Additionally, band 3 was recently identified as a potential receptor for PvTRAg38 (*P. vivax* tryptophan-rich antigen) (Alam et al., 2015).

Ultimately, our pull-down assay identified human erythrocyte band 3 as a host receptor for Pf-GARP, and demonstrated that the 60 kDa chymotryptic fragment of band 3 contains the specific binding site. The 60 kDa fragment retains the first three extracellular segments of band 3. Our finding showing a direct interaction between Pf-GARP and host band 3 is consistent with a previous observation that multiple *P. falciparum* polypeptides interact strongly with human band 3 (Jones and Edmundson, 1991). In summary, our results define the core RBC-binding site of Pf-GARP, a potential specific parasite ligand, and its host receptor band 3 to mediate pathogenesis at the blood stage of malaria infection.

4.6. Proposed Models of Enhanced Rosetting, RBC Aggregation, Platelet-mediated Clumping and Cytoadherence through Pf-GARP, Band 3, and CD36 Interactions

In a series of studies, Sherman and colleagues have shown an intriguing role of modified band 3 as a malaria adhesion receptor on iRBCs by an unknown mechanism,

suggesting clustering and oxidative environment as potential mediators (Sherman et al., 2003; Shimo et al., 2015; Tokumasu et al., 2005; Winograd et al., 2005). It was observed that band 3 promoted the adhesion of iRBCs, specifically through its third ectodomain. Band 3 monoclonal antibodies and synthetic peptides corresponding to AA (537-547), (546-553, termed pfalhesin), and (824-829) were able to block adhesion of *P. falciparum* iRBCs *in vitro* while other regions of band 3 and scrambled peptides did not (Crandall and Sherman, 1996; Sherman, 2016; Sherman et al., 1995; Sherman et al., 2003). Moreover, a large number of late stage *P. falciparum* iRBCs were detected in the blood of *Aotus* and *Saimiri* monkeys 24 hours following the injection of band 3 synthetic peptides, indicating their adhesive/sequestration inhibitory effect (Crandall et al., 1993; Sherman, 2016; Sherman et al., 2003). In fact, specific regions of modified band 3 were found to bind specific receptors. DIDS-binding region (DBR) correspond to first band 3 synthetic peptide binds CD36. DIDS (4,4'-diisothiocyanostilbene-2,2'- disulfonic acid) is an irreversible inhibitor of the anion transport (Hsu and Morrison, 1983). Pfalhesin binds to both CD36 and TSP (thrombospondin) (Sherman, 2016). The TSP is a secreted glycoprotein by multiple cell types and interacts with multiple molecules and cells (Lahav, 1993).

CD36 is expressed in multiple tissues and has been shown to be involved in adhesion of malaria iRBCs (Chilongola et al., 2009). A recent study has also confirmed the expression of CD36 on the surface of RBCs (Deitch et al., 2014) after several decades of contradicting observations (Crandall and Sherman, 1996; Handunnetti et al., 1992). Interestingly, it was shown that CD36 protein exists in all RBCs but its surface expression can be enhanced under certain circumstances of stress such as trauma-

hemorrhagic shock (Deitch et al., 2014). Like malaria, the RBCs also become less deformable in trauma-hemorrhagic shock (Deitch et al., 2014). Thus, it is possible that Pf-GARP binds to band 3, modifies it, and mediates its binding to CD36 while MSP-1 can bind to AA (720-761) of band 3 to mediate invasion (Salinas and Tolia, 2016). Another possible scenario is that Pf-GARP binds to band 3 and forms a stable sticky complex, which in turn interacts with other receptors. This phenomenon could be further potentiated if Pf-GARP can perhaps through binding to other band 3 receptors on different RBCs assuming that Pf-GARP has the ability to self-associate. In fact, self-associated Pf-GARP fusion proteins were detected in our RBC-binding assays.

Recently, a *P. vivax* ligand, PvTRAg38, has been shown to bind to AA (424-462), (538-570), and (807-860) regions of human band 3 to promote the growth of parasites (Alam et al., 2015; Alam et al., 2016). The PvTRAg38 ligand is lysine-rich and contains 3 lysine residues, which is similar to the lysine content of GARP-M2 showing a tandem repeat of kxxxxkxxk. The GARP-M2 contains two of these repeats and Pf-GARP has a total of ten of these repeats as shown in Table 4.1. We identified GARP-M2 as a RBC binding site but other sites might also bind to band 3 or to other host receptors. This model is consistent with a recent demonstration that PvTRAg38 can also bind to host basigin on the surface of RBCs by using a different region that promotes parasite growth as well (Rathore et al., 2017).

Table 4.1. Tandem Lysine Repeats "kxxxxkxxk" in Pf-GARP
Pf-GARP contains multiple overlapping lysine repeats. GARP-M2, the RBC-binding region of Pf-GARP, has two repeats. Other repeats are possible RBC-binding regions. GARP-M1 does not contain any lysine tandem repeats.

AA Position	Sequence
119-127	**K**KKDK**K**EK**K**
127-134	**K**HKKD**K**KE**K**
283-291	**K**KQEE**K**EK**K**
290-298	**K**KKQE**K**ER**K**
298-306	**K**KQEK**K**ER**K**
320-328	**K**ERKK**K**EE**K**
417-425	**K**GKKD**K**GK**K**
427-435	**K**GKHK**K**AK**K**
432-440	**K**AKKE**K**VK**K**

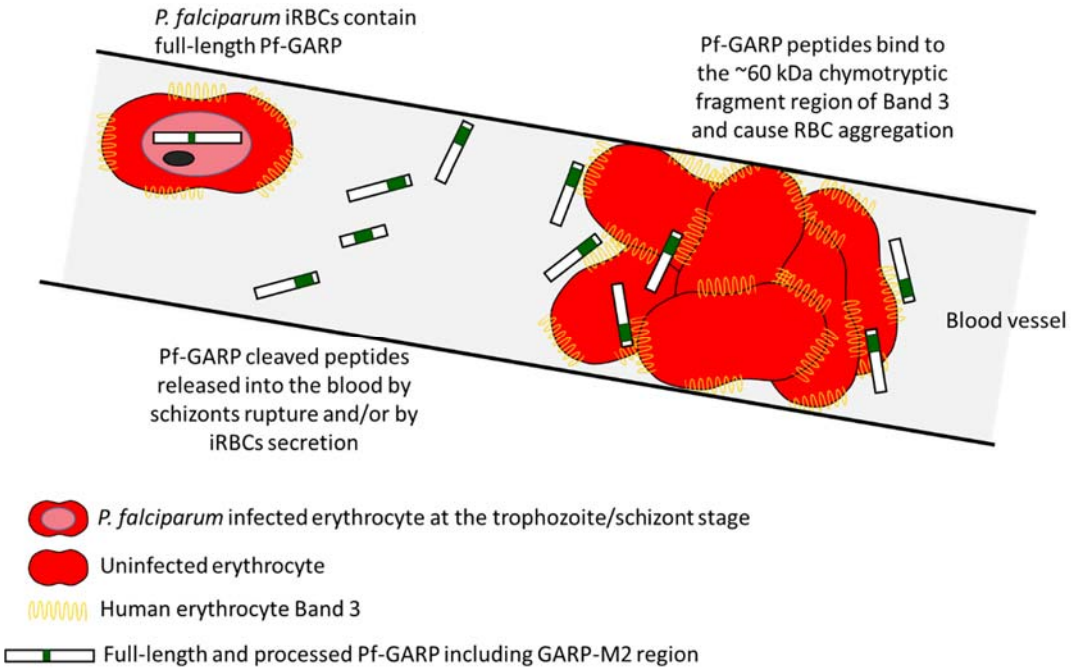


Figure 4.1. Proposed Model of a Functional Role of Pf-GARP in Severe *P. falciparum* Infection

RBC aggregation induced by Pf-GARP released to the blood stream and bound to human band 3 protein on the surface of erythrocytes. Aggregation triggers blood vessel obstruction and organ damage, e.g., cerebral hypoxia.

4.7. Future Directions

In this study, I identified Pf-GARP as a secreted antigen that binds to host band 3 on the surface of human RBCs. Following the addition of specific Pf-GARP peptides to human erythrocytes, frequent RBC aggregates were observed by microscopy. The reported findings may underlie the mechanism that is essential for the cytoadherence and rosetting phenomena directly relevant for the development of severe malaria. The cytoadherence and rosetting properties are known to be mediated by proteins expressed on the surface of iRBCs (Craig et al., 2012; Sherman et al., 2003). There are very few studies that have investigated the role of secreted antigens as promoters of cerebral malaria (Pal et al., 2016).

Although we have identified GARP-M2 as the RBC-binding domain of Pf-GARP, the precise binding site has to be determined in future studies. Furthermore, we have shown that the chymotrypsin cleaved band 3, likely at its AA Tyr 555 (Reithmeier et al., 2016), has the ability to bind GARP-M, thus eliminating a direct role for the glycosylation site of band 3 in this interaction (Jay, 1986; Reithmeier et al., 2016). An obvious next step would be to map the exact ectodomain sequence of band 3 that is involved in the RBC-binding to Pf-GARP, and confirm whether it is involved in the RBC aggregation phenomena or potential role of other adhesion receptors in this process. Binding studies with synthetic non-overlapping and mutated peptides can used to further characterize the ligand-receptor interaction (Alam et al., 2016). Functional effects of Pf-GARP derived peptides on adhesion and rosetting inhibition studies as well as their potential hemolytic ability can be tested and characterized in future studies. Sub-cellular localization of Pf-GARP in iRBCs using our monoclonal antibody and electron

microscopy approaches will further shed light on possible function(s) of Pf-GARP and whether it interacts with other host and parasite proteins within the iRBC. This model is consistent with the formation of multi-protein clusters by several malaria proteins required for pathogenesis (Baldwin et al., 2015; Lin et al., 2016).

Previous studies on malaria pathogenesis have identified vesicles released from iRBCs into extracellular space (Akers et al., 2013; Trelka et al., 2000). These vesicles are not limited to iRBCs (de Vooght et al., 2013; Zecher et al., 2014), but their formation and contents are greatly enhanced in malaria infection (Babatunde et al., 2018). These vesicles are formed either from the endosomal network, designated as exosomes (~ 30 – 100 nm) or from the budding of the extracellular membrane and are called macrovesicles (~ 50 – 1,000 nm) (Akers et al., 2013; de Vooght et al., 2013; Sampaio et al., 2017). Recent studies have shown a functional role of these microvesicles and exosomes in cell-cell communication by transferring genetic material and proteins in promoting growth and development of parasite sexual stages (Babatunde et al., 2018; Barteneva et al., 2013; de Vooght et al., 2013; Mantel et al., 2013; Ratajczak and Ratajczak, 2016; Regev-Rudzki et al., 2013; Sampaio et al., 2017). The RBC proteins, mainly band 3, has been detected as a key component of these vesicles as well as many malaria proteins (Abdi et al., 2017; Mantel et al., 2013). Importantly, Pf-GARP was also detected as one of proteins that is transported by exosomes and microvesicles isolated from the supernatant of cultured *P. falciparum* strain harvested from malaria patients in Kenya (Abdi et al., 2017). These findings are consistent with our results that Pf-GARP is a secreted antigen. In fact, Pf-GARP could be a major component of these microvesicles, a key issue to be resolved by future studies. These vesicles contain multiple proteins that have been shown

to bind RBCs, e.g., EBA-181 (Mantel et al., 2013; Mayer et al., 2004), and have the tendency to bind and stick to the surface of RBCs. Therefore, the secreted vesicles are likely required to play a functional role in cellular adhesion. Hence, a future task would entail the functional characterization of secreted microvesicles from malaria iRBCs, and interrogation of the function of each component vis-à-vis invasion, growth, and adhesion events at the blood stage of malaria infection.

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