Identification of Blood-Stage Invasins of *Plasmodium falciparum* Through Transcriptomics

NIDHI HANS, GAURAV ANAND and VIRANDER SINGH CHAUHAN*

Malaria Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110067, India

(Received on 28 June 2017; Revised on 11 September 2017; Accepted on 20 September 2017)

Malaria is one of the leading parasitic diseases in the world, despite enormous efforts to control or eradicate it. *Plasmodium falciparum* causes the most severe form of malaria and is responsible for most malaria-related deaths globally. Erythrocyte invasion by *P. falciparum* is the most fundamental step in the blood-stage life cycle of the parasite and is mediated by a series of sequential events incorporating multiple molecular interactions between parasite and erythrocyte surface proteins. The clinical symptoms of *P. falciparum* malaria are attributed to the cycles of asexual reproduction within the human erythrocytes. Understanding this complex process of invasion is extremely essential for gaining insight into the basic parasite biology which would enable the identification of novel drug targets and vaccine candidates that would efficiently block erythrocyte invasion. Advances in genome-wide transcriptomic studies have provided us with the information about the temporal expression of a cohort of genes involved in the invasion process. The availability of this information has been pivotal in the effort towards identification, characterization and functional assessment of novel proteins which have a specific role in invasion. This review aims to summarize the invasins that have been identified and characterized in the post-genomics era, utilizing the information available through *P. falciparum* transcriptome databases.

**Keywords:** Malaria; *Plasmodium falciparum*; Erythrocyte; Merozoite; Invasion-inhibitory; Antibodies; Vaccine; Invasins

Introduction

Malaria remains a major infectious disease that plagues the whole world despite extensive efforts spanning more than a century to control this disease. Currently, more than 3 billion people are at risk, with an estimated 200 million infections and more than 400,000 deaths each year (2016 World malaria report). There are several *Plasmodium* spp. that cause malaria in humans. *Plasmodium falciparum* causes the most severe form of malaria, cerebral malaria and responsible for maximum number of deaths due to malaria, particularly in Africa. *P. vivax* is the second most important cause of malaria and causes significant morbidity outside Africa with more than 50% of malaria cases in Central and South America, South and South-east Asia, and several Pacific islands. *P. malariae* and *P. ovale* also cause human malaria and represent a very minor proportion of the global malaria burden. *P. knowlesi* has emerged as an important zoonotic malaria spp., transmitted from macaques to humans by infected mosquitoes in parts of South East Asia and has been reported to cause infection in humans (Daneshvar *et al.*, 2009; Dankwa *et al.*, 2016). *Plasmodium* parasites has a complex life cycle that is completed through two hosts – the invertebrate *Anopheles* mosquito that acts as the vector through which the parasite completes its sexual cycle and the vertebrate human host in which the asexual life cycle is completed (Gaur *et al.*, 2004; Cowman and Crabb, 2006). During a blood meal, the female *Anopheles* mosquito injects invasive forms of the parasite known as sporozoites into the skin, which then migrate to the liver and finally infect hepatocytes, marking the beginning of the pre-erythrocytic stage or liver stage of the cycle. Over 7-10 days, parasites develop and divide into merozoites that are released into the...
bloodstream. An important feature of *P. vivax* is that it exhibits latency by remaining dormant in liver in the form of hypnozoites that could cause relapse in later stages of life. This does not occur with *P. falciparum*. The released merozoites infect erythrocytes initiating the erythrocytic or asexual blood stage of the parasite cycle during which the parasite grows and replicates inside them. Cycles of blood-stage replication take approximately 48 hours for *P. falciparum* and *P. vivax*, but only 24 hours for *P. knowlesi*. After the completion of one cycle infected erythrocyte undergoes egress further releasing daughter merozoites that invade new erythrocytes. *P. vivax* is the only human malarial parasite species that exhibits specific preferential invasion of reticulocytes (young immature erythrocytes) and largely invades young reticulocytes that still bear CD71 (Malleret *et al.* 2015). In addition, efficient invasion by *P. vivax* requires the Duffy antigen on reticulocytes, although invasion into Duffy-negative reticulocytes can occur (Ryan *et al.* 2006). In contrast, *P. falciparum* can efficiently invade Duffy negative erythrocytes and mature erythrocytes. Most of the clinical symptoms of malaria are attributed to asexual propagation of the parasite within human erythrocytes (Cowman and Crabb, 2006). The parasite entry into the host erythrocyte is the most critical step of its life cycle with respect to malarial pathogenesis. Invasion is a complex multi-step process which requires series of sequential interaction between parasite proteins and receptors on the surface of erythrocytes (Cowman and Crabb, 2006; Gaur and Chitnis, 2011). The parasite has a regulated mechanism of temporal expression of genes which enables the completion of such a complex life cycle within multiple hosts.

Erythrocyte invasion by *P. falciparum* merozoites is mediated by a series of sequential events incorporating multiple molecular interactions between parasite and erythrocyte surface proteins and are characterized by three phases: pre-invasion, active invasion, and echinocytosis (Fig. 1) (Gilson and Crabb 2009; Weiss *et al.*, 2015). Initial merozoite attachment with the erythrocyte appears to be mediated by the merozoite surface protein 1 (MSP1) complex which includes glycosylphosphatidylinositol (GPI)-anchored merozoite surface proteins and peripheral proteins, followed by weak deformation of the target erythrocyte membrane. Subsequent interaction with the erythrocyte is mediated by two major protein ligand families, Duffy binding-like (DBL or erythrocyte-binding-like [EBL]) protein and the reticulocyte-binding-like protein homolog (Rh or RBL) which bind with higher affinity to a range of known and unknown host receptors resulting in stronger erythrocyte deformation and merozoite reorientation. Both EBL and PfRH ligands mediate invasion through multiple alternate pathways. After merozoites have bound to erythrocytes, a small member of the PfRH family, PfRH5 binds to its receptor basigin irrespective of the activity of other PfRH ligands. Downstream to this essential PfRH5-basigin engagement, tight junction formation occurs. During this stage of invasion, AMA1 binds to another parasite protein RON2, already translocated into the erythrocyte membrane and this facilitates penetration. AMA1 is found on the surface of the merozoite and binds to RON2, which is part of a larger RON complex.

*P. falciparum* erythrocyte invasion pathways can be broadly classified into two main groups based on the utilization of sialic acid (SA) on the surface of erythrocytes (Lopaticki *et al.*, 2011): (i) SA-independent (neuraminidase-resistant) invasion pathways (Duraisingh *et al.*, 2003) and (ii) SA-dependent (neuraminidase-sensitive) invasion pathways (Persson *et al.*, 2008). Neuraminidase is a sialidase that cleaves sialic acids. Thus, if a *P. falciparum* strain is unable to invade a neuraminidase-treated erythrocyte, where sialic acid residues have been depleted then it could be termed as sialic acid
Blood-stage Invasins

Subset of genes having similar expression profiles were clustered in 15 clusters and the function of uncharacterized genes were predicted based on one or more characterized genes within the same cluster. Thus cluster of genes representing a putative function in invasion, parasite motility, cell-cycle regulation and other crucial processes were deciphered.

Another study published in the same year presented the full transcriptome data of the asexual intra-erythrocytic developmental cycle (IDC) of the parasite (Bozdech et al., 2003). P. falciparum DNA microarray using 70mer oligonucleotides was used in this study to examine the expression profiles across 1 hour time points of the 48 hour IDC of P. falciparum. This study described that 75% of the genes were activated only once during the IDC with activation correlated to time-specific processes. Moreover, this study has further described the expression induction of 262 genes predicted to be involved during erythrocyte invasion and hence presented a list of putative vaccine candidate antigens. This list was prepared by analyzing the similarity between expression profiles to seven known malaria vaccine candidates (AMA1, MSP1, MSP3, MSP5, EBA175, RAP1, and RESA1). This study also concluded an intimate relationship between transcriptional regulation and the developmental progression of this parasitic organism. Another study published in the year 2010, presented the DNA microarray-based high-resolution transcriptional profiling of P. falciparum in response to 20 growth-inhibiting compounds (Hu et al., 2010). The data was used to construct a gene interaction network that predicted the functions of 2,545 P. falciparum hypothetical proteins. This study also focused on the late stage (schizont) of the P. falciparum IDC and identified a sub-network that encompasses 416 genes likely to participate in the invasion process. In order to validate the predictions, a green fluorescent protein (GFP) tagging approach was used to define sub-cellular distribution of 42 proteins predicted to be involved in the invasion. In summary, 31 out of 42 selected proteins are associated with the structures known to be directly involved in invasion.

Taken together, this set of information has been extensively used by laboratories around the world to identify and characterize novel proteins involved in various biological processes of the parasite.
Researchers engaged in understanding the invasion process utilize two fundamental approaches to identify novel invasins. The first one involves looking for orthologues of the protein of interest across multiple *Plasmodium* species for which either functionality has been established in other *Plasmodium* species or Apicomplexans like *Toxoplasma gondii* and the presence of specific domains which could play a role during invasion. The literature has numerous examples of parasite proteins like several Rhoptry neck proteins (RONs), *P. falciparum* thrombospondin related apical merozoite protein (PfTRAMP), Apical Sushi protein which has been identified by homology searches. The second is to utilize only the expression profiling information as a useful marker to short-list invasins. The identification also takes into account the late stage expression based on the transcriptional profile of the selected gene followed by sequence-based identification to well characterized invasion associated proteins. The latter approach gained importance owing to the failure in predicting the function of *Plasmodium* proteins based on little or no similarity with proteins of other organisms. In such a scenario, the information from these transcriptome databases is very useful for evaluating the functionality of parasite proteins. Erythrocyte invasion by merozoites is the most fundamental step that ensures parasite multiplication at levels that lead to the malarial disease. Thus, it is important to identify and characterize novel blood-stage proteins (invasins) involved in erythrocyte invasion. These efforts not only augment our understanding of the basic biology of the parasite but also expand our repertoire of antigens that could induce potent invasion-inhibitory antibodies. Hence, to identify novel targets, it is important to study new parasite ligands that mediate the complex process of erythrocyte invasion.

Many groups have focused their attention on identifying such novel invasins (Wickramarachchi et al., 2008; Haase et al., 2008; Proellocks et al., 2009; Hinds et al., 2009; Wickramarachchi et al 2009; Chen et al., 2011; Dreyer et al., 2012; Hans et al., 2013a; Hans et al., 2013b; Knuepfer et al., 2013; Hans et al., 2015; Anand et al., 2016) (Table 1). The invasins have certain common characteristics such as up-regulated transcription during late stages of asexual life cycle, localization on the merozoite surface or in the apical organelles (micronemes, rhoptries, dense granules), presence of signal sequence at the amino-terminal, presence of transmembrane domain/GPI anchor and finally the presence of characteristic domains involved in surface adhesion or protein-protein interactions. The presence of a signal sequence is known to direct newly synthesized proteins to the endoplasmic reticulum (ER) and into the eukaryotic secretory pathway. *P. falciparum* ligand proteins that are trafficked to the apical organelles are usually routed through the ER secretory pathway. Potential parasite ligands that directly attach

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Length</th>
<th>Signal peptide</th>
<th>Transmembrane domain</th>
<th>GPI anchor</th>
<th>Localization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF3D7_0423400</td>
<td>217 aa</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Rhotry Neck</td>
<td>Wickramarachchi et al., 2008</td>
</tr>
<tr>
<td>PF3D7_0722200</td>
<td>750 aa</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Micronemes</td>
<td>Hinds et al., 2009</td>
</tr>
<tr>
<td>PF3D7_0828800</td>
<td>738 aa</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Micronemes</td>
<td>Hinds et al., 2009</td>
</tr>
<tr>
<td>PF3D7_0214900</td>
<td>950 aa</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Rhotry Neck</td>
<td>Proellocks et al., 2008</td>
</tr>
<tr>
<td>PF3D7_1035700</td>
<td>697 aa</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Merozoite surface</td>
<td>Wickramarachchi et al., 2009</td>
</tr>
<tr>
<td>PF3D7_0323400</td>
<td>1086 aa</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Micronemes</td>
<td>Chen et al., 2011</td>
</tr>
<tr>
<td>PF3D7_0423800</td>
<td>362 aa</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Micronemes</td>
<td>Dreyer et al., 2012</td>
</tr>
<tr>
<td>PF3D7_0511600</td>
<td>208 aa</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Rhotry Neck</td>
<td>Hans et al., 2013a</td>
</tr>
<tr>
<td>PF3D7_0316000</td>
<td>307 aa</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Micronemes</td>
<td>Hans et al., 2013b</td>
</tr>
<tr>
<td>PF3D7_1017100</td>
<td>310 aa</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Rhotry Neck</td>
<td>Knuepfer et al., 2014</td>
</tr>
<tr>
<td>PF3D7_1137200</td>
<td>415 aa</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Exonemes</td>
<td>Hans et al., 2015</td>
</tr>
<tr>
<td>PF3D7_1012200</td>
<td>267 aa</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Rhotry Bulb</td>
<td>Anand et al., 2016</td>
</tr>
</tbody>
</table>
with the host erythrocyte during invasion must be secured on the surface of invasive merozoite. Proteins are known to directly associate with the membrane either through hydrophobic transmembrane helices or a GPI anchor as has been shown for a number of invasive ligands. Thus, proteins that possess a transmembrane domain or GPI-anchor will be expressed on the merozoite surface with a high probability of being involved in the process of erythrocyte invasion and being a target of potent parasite neutralizing antibodies. Similarly, a number of \textit{P. falciparum} ligands that are involved in key steps such as erythrocyte attachment and junction formation have a transmembrane domain that embeds them in the parasite surface membrane. This further emphasizes the importance of transmembrane domains (Haase et al., 2008; Ito et al., 2013). The proteins identified by homology searches are not the prime focus of this review. This review largely summarizes the invasins of \textit{P. falciparum} that have been identified and characterized only using the transcriptomics information. Such studies have proved extremely useful in deciphering the function for antigens which do not have homology to known proteins.

**Invasins of \textit{Plasmodium falciparum}**

\textit{Plasmodium falciparum} Apical Asparagine Rich Protein (PfAARP) (PF3D7_0423400)

PfAARP is a 217 amino acid (aa) long protein that contains a putative N-terminal signal sequence, C-terminal transmembrane domain, asparagine repeats in the C-terminal half of the protein and a conserved polyproline stretch just before the transmembrane region. PfAARP orthologues were also identified in \textit{P. berghei} (PBANKA_0523800), \textit{P. chabaudi} (PCHAS_0524000), \textit{P. vivax} strain Sal-1 (PVX_090210) and \textit{P. yoelii yoelii} strain 17XNL (PY06454). PfAARP was also found to be conserved among different \textit{P. falciparum} strains and field isolates, suggesting that PfAARP is a conserved protein. PfAARP has maximal expression during the schizont stage of parasite consistent with the transcription data. PfAARP is localized in rhoptry neck of the merozoites. PfAARP N-terminal region (amino acid 20–107) binds to human erythrocytes in a sialic acid dependent manner (Wickramarachchi et al., 2008). Antibodies targeting PfAARP ecto-domain exhibited invasion inhibitory activity and provided synergistic effects in growth inhibitory assays in combination with antibodies to other parasite proteins validating its important role in erythrocyte invasion (Pandey et al., 2013; Kalra et al., 2016). PfAARP was recognized by human serum samples from malaria-endemic regions, suggesting that naturally acquired human antibodies were raised against PfAARP. Presence of anti-PfAARP antibodies in human immune sera from endemic regions and conservation of PfAARP gene across different \textit{P. falciparum} strains and field isolates suggest that this antigen is important for parasite survival, exposed to the human immune system during \textit{P. falciparum} infections and may induce effective host-immune response against the parasite. Recently it was reported that immunization with recombinant PfAARP induces long-lasting humoral and cellular response in mice. Thus PfAARP definitely needs further investigation to be included as a multi-component blood-stage vaccine candidate.

\textbf{Plasmodium falciparum Rhopty-Associated Leucine Zipper-Like Protein 1 (RALP1) (PF3D7_0722200)}

RALP1 was identified by genome wide analysis of the available gene expression profiles and primary sequence data for candidate genes involved during invasion (Haase et al., 2008; Ito et al., 2013). RALP1 is a 750 amino acid protein, possesses an N-terminal signal peptide and a leucine zipper-like domain, a structural feature that facilitates protein-protein interaction. RALP1 is conserved in \textit{Plasmodium} spp. and its orthologues were also identified in \textit{P. berghei} (PBANKA_0619700), \textit{P. chabaudi} (PCHAS_0621400), \textit{P. vivax} (PVX_096245) and \textit{P. yoelii yoelii} (PY07382). RALP1 is expressed during schizont stage of the intra-erythrocytic cycle and is apically localized in the rhoptry neck of merozoites. RALP1 is conserved amongst field isolates and refractory to gene deletion studies, suggesting that RALP1 might play an important role in invasion. RALP1 binds to human erythrocytes through its C-terminal region and is conserved amongst field isolates and refractory to gene deletion studies, suggesting that RALP1 might play an important role in invasion. RALP1 binds to human erythrocytes through its C-terminal region and is conserved amongst field isolates and refractory to gene deletion studies, suggesting that RALP1 might play an important role in invasion. RALP1 binds to human erythrocytes through its C-terminal region and is conserved amongst field isolates and refractory to gene deletion studies, suggesting that RALP1 might play an important role in invasion. RALP1 binds to human erythrocytes through its C-terminal region and is conserved amongst field isolates and refractory to gene deletion studies, suggesting that RALP1 might play an important role in invasion. RALP1 binds to human erythrocytes through its C-terminal region and is conserved amongst field isolates and refractory to gene deletion studies, suggesting that RALP1 might play an important role in invasion.
is a structural component of the rhoptries and/or part of a densely packed rhoptry matrix and its function might be in the development, maintenance, or discharge of the rhoptries, aiding the process of invasion.

**GPI-anchored Micronemal Antigen (GAMA) (PF3D7_0828800)**

GAMA was identified using a bio-informatics approach to define candidate proteins that may be components of the basic invasion machinery that is conserved between merozoites and sporozoites (Hinds *et al*., 2009), using proteome data for both merozoites and sporozoites (Florens *et al*., 2002). The transcriptome data also confirmed its expression during the later stages of the asexual blood cycle, with maximal expression during 40 to 48 hours post-invasion. GAMA is a 738-amino-acid protein (with a predicted molecular mass of ~83 kDa). GAMA has a signal peptide (residues 1 to 24), a long asparagine-rich region (residues 356 to 485), and a transmembrane domain (residues 715 to 738) encompassing a GPI anchor (Hinds *et al*., 2009). GAMA is conserved across *Plasmodium* species and its orthologues were also identified in *P. vivax* (PVX_088910), *P. knowlesi* (PKNH_1322900), and *P. yoelii* (PY07130), suggesting that it may perform a genus-specific function. GAMA localizes to the micronemes of *P. falciparum* schizonts and merozoites and displayed cap-like staining on the surface in free merozoites (Arumugam *et al*., 2011). GAMA possesses an erythrocyte binding epitope in the C-terminal region and it binds to the surface of erythrocyte in a chymotrypsin-sensitive but neuraminidase and trypsin resistant manner. Growth inhibition assays revealed that anti-GAMA antibodies inhibited *P. falciparum* invasion in a dose-dependent manner, plays a role in the sialic acid (SA)-independent invasion pathway and in combination with PfEBA-175 antibodies exhibited a significantly higher level of invasion inhibition. GAMA could be detected by the sera samples from malaria infected individuals, residing in malaria endemic areas. GAMA has also been shown to be refractory to gene knockout studies.

**Plasmodium falciparum Duffy binding like merozoite surface protein (PfDBLMSP1) (PF3D7_1035700)**

Initially, this Duffy binding-like domain containing protein was identified using transcriptome analysis of the intra-erythrocytic developmental cycle (IDC) of *P. falciparum* and the available proteome data (Le Roch *et al*., 2003; Bozdech *et al*., 2003). PfDBLMSP1 is a 697 amino acid long protein with a putative N-terminal hydrophobic signal sequence. It contains a central Duffy binding-like (DBL) domain (159-419 aa) and a secreted polymorphic antigen associated with merozoites (SPAM) domain (543-696 aa) at the C-terminus (Wickramarachchi *et al*., 2009). Recently, a new MSP3 multi-gene family was predicted, and PfMSPDBL1 is one of the members of this family (Singh *et al*., 2009). The expression of PfDBLMSP1 is strongly up-regulated during the late schizont stage of parasite. PfDBLMSP1 is localized on the surface of merozoite. The C-terminal region of the protein contains erythrocyte binding domain and the binding was sensitive to neuraminidase and trypsin treatment. The antibodies generated against the protein interfere with its binding to the erythrocyte. The DBL and SPAM domains were found to be the targets of natural immune response against natural exposure to *P. falciparum*. PfDBLMSP1 has polymorphic regions within the DBL-SPAM domain region. The study suggested that PfDBLMSP might...
play a role in the initial attachment of merozoite with the erythrocyte during the process of invasion. A recent study reports that the antibodies to PfDBLMSP1 inhibit erythrocyte invasion of parasite in vitro in a dose dependent manner (Sakamoto et al., 2012). PfDBLMSP1 is also part of the larger MSP1 complex (Lin et al., 2014). Taken together, these reports suggested that PfDBLMSP1 is a novel blood-stage malaria vaccine candidate.

**Plasmodium falciparum PfRH5 Interacting Protein (PfRipr) (PF3D7_0323400)**

Mature full-length *P. falciparum* RH5 interacting protein (PfRipr) has a molecular weight of 123 kDa with 10 epidermal growth factor-like domains and 87 cysteine residues distributed along the protein (Chen et al., 2011). An EGF domain has 6 cysteine residues and the position of each is relatively conserved in the ten EGF-like domains of PfRipr (Chen et al., 2011). In mature schizont stages, PfRipr undergoes processing towards its central region to yield two polypeptides that associate and form a complex with PfRH5. The PfRipr protein is localized in the apical end of the merozoites in micronemes. Antibodies to PfRipr modestly inhibit merozoite attachment and invasion into human red blood cells. PfRipr could not be genetically disrupted, suggesting an essential role of the protein. (Chen et al., 2011). PfRipr is one of the components of the essential PfRH5 adhesion complex. Determination of antibody levels in human sera collected from malaria-exposed individuals against PfRipr is currently being studied to find any possible association with clinical malaria and naturally acquired immunity. Further analysis of polymorphism, genetic diversity and conservation in different worldwide *P. falciparum* strains and field isolates would also give valuable information on the utility of this probable vaccine candidate (Ntege et al., 2016).

**Cysteine-rich Protective Antigen (CyRPA) (PF3D7_ 0423800)**

CyRPA was identified by analyzing the genome-wide transcriptome and proteomic information generated since 2002 and it is also clustered into an interaction network implicated in merozoite invasion (Le Roch et al., 2003; Bozdech et al., 2003; Hu et al., 2010). CyRPA is a 362 amino acid long protein having an N-terminal signal peptide and 12 cysteine residues (Dreyer et al., 2012). CyRPA is a conserved merozoite protein and attempts for its genetic disruption were not successful suggesting its essential role in parasite invasion (Dreyer et al., 2012; Reddy et al., 2015). CyRPA is highly conserved (single polymorphism among 18 *P. falciparum* strains) and is not under immune pressure (Dreyer et al., 2012). CyRPA was shown to be expressed in schizont stage of the parasite by western blotting and immunofluorescence assay using the monoclonal antibodies (mAbs) generated against it (Dreyer et al., 2010; Dreyer et al., 2012). CyRPA is localized to micronemes in schizont stage parasites and is present at the apical surface of an invading merozoite during invasion. CyRPA-specific mAbs substantially inhibited parasite growth in vitro as well as in a *P. falciparum* animal model based on NOD-scid IL2Rγ (null) mice engrafted with human erythrocytes (Dreyer et al., 2012). Antibodies against bacterially expressed CyRPA full-length protein display potent, strain-transcending invasion inhibition against multiple *P. falciparum* strains (Reddy et al., 2015). It has been shown that CyRPA and PfRipr form a complex with PfRH5 and PfRH5 binds to basigin on the erythrocyte surface (Reddy et al., 2015). Recently, P113 was identified to be another component of the PfRipr/ PfRH5/CyRPA complex (Galaway et al., 2017).

**Plasmodium falciparum Apical Rhotry Neck Protein (PfARNP) (PF3D7_0511600)**

PfARNP is a rhoptry neck protein which has been shown to be present at the tight junction during invasion (Hans et al., 2013a). It is 208 amino acid long protein harboring a well defined signal peptide and a transmembrane domain. The protein is conserved amongst *Plasmodium* species. The expression of PfARNP showed sharp induction during late asexual stage of the parasite. PfARNP is present in the rhoptry neck of free merozoites and was found to localize at the tight junction during invasion. Interspecies conservation of the protein, late stage expression, localization in apical organelle, presence of protein at tight junction and in parasite culture supernatant indicates that it could play a role in erythrocyte invasion (Hans et al., 2013a).

**Plasmodium falciparum Merozoite Associated Antigen (PfMA) (PF3D7_0316000)**

PfMA is a 307 amino acid protein (~ 37.1 kDa) that contains an N-terminal stretch of hydrophobic
residues, a C-terminal single transmembrane domain and a short cytoplasmic tail (Hans et al., 2013b). PfMA homologues were identified in *P. vivax* (PVX_095435, *P. berghei* (PBANKA_041380), *P. chabaudi* (PCHAS_041470), *P. yoelii* (PY03459) and *P. cynomolgi* (PCYB_083370). It harbors a weak signal peptide and a well-defined transmembrane domain. Expression of PfMA was shown to be transcriptionally and translationally up-regulated during the schizont stage of parasite. PfMA was also shown to be expressed in several other *P. falciparum* strains. The study reported that PfMA is localized to the micronemes of mature schizonts and free merozoites. Native and recombinant PfMA was shown to bind to human erythrocytes in a sialic acid independent, chymotrypsin and trypsin-resistant manner. The antibodies generated against the ectodomain of PfMA inhibited the binding of native PfMA protein to erythrocyte in a dose-dependent manner. The antibodies generated against PfMA also inhibited *P. falciparum* erythrocyte invasion.

**Rhoptry Neck Protein 12 (RON 12) (PF3D7_1017100)**

RON12 was identified by using the criteria similar to identification of GAMA (Hinds et al., 2009; Knuepfer et al., 2014). RON12 is a 310 amino acid long protein and orthologues in other *Plasmodium* species such as *P. vivax* (PVX_001725), *P. knowlesi* (PKH_060120), *P. berghei* (PBANKA_050140) and *P. yoelii* (PY00202) are highly conserved. RON12 has a predicted signal peptide but no transmembrane domain and it is expressed during the schizont stage of parasite. Immunofluorescence studies and immuno-electron microscopy indicated that RON12 localizes in the rhoptry neck of merozoites. Knuepfer et al., 2014 looked into the localization of RON12 at the time of invasion and established that RON12 remains mainly within the rhoptry neck until the invasion has almost completed before being relocated to the parasitophorous vacuole (PV). RON12 also associates with the progressing moving junction, suggestive of a potential role within this structure. The study also reported that RON12 could be knocked out in *P. falciparum* and *P. berghei*. The parasite however exhibited growth retardation compared with wild-type parasites. This study described an invasin which is localized to rhoptry neck and involved in the establishment of PV of the intracellular parasite and detection at the moving junction (MJ) in a small number of invasion events also indicates that it might also play a direct role in host cell invasion.

**Plasmodium falciparum Apical Exonemal Protein (PfAEP) (PF3D7_1137200)**

The gene encoding PfAEP is located on chromosome 11 as a single exon. PfAEP protein is 415 amino acid long with a predicted molecular weight of 50.2 kDa. The encoded protein is predicted to contain a weak N-terminal signal peptide sequence (1-22). PfAEP homologues were identified in other *Plasmodium* species. The orthologs of this gene was identified in *Toxoplasma, Babesia, Kluyveromyces* and *Neospora*. Like other invasion related proteins, PfAEP expression is upregulated at schizont stage of the intra-erythrocytic parasite. PfAEP is localized in the apical secretory organelle exornemes (Hans et al., 2015; Agarwal et al., 2012). Inter-species conservation of the protein, schizont stage expression and apical localization in exornemes indicates that it could play a role in erythrocyte egress or invasion (Hans et al., 2015).

**Plasmodium falciparum Rhoptry Associated Adhesin (PfRA) (PF3D7_1012200)**

The PfRA native parasite protein comprises of 267 amino acids with a putative N-terminal signal peptide (amino acid 1-22). PfRA orthologues were also identified in *P. vivax* (PVX_094830), *P. knowlesi* (PKNH_0812000), *P. yoelii* (PY03476) and *P. chabaudi* (PCHAS_1211300). Like other invasion related proteins, PfRA is expressed only at the schizont stage of the intra-erythrocytic parasite across multiple *P. falciparum* strains. PfRA is localized in the apical organelle, rhoptry and undergo translocation to the merozoite surface during erythrocyte invasion. PfRA exhibits erythrocyte binding activity and acts as a ligand engaging with sialic acids on the erythrocyte surface, thus mediating invasion through the sialic acid dependent pathway. PfRA antibodies specifically blocked the erythrocyte binding of both native and recombinant PfRA. PfRA antibodies inhibited erythrocyte invasion and in combination with antibodies against other parasite ligands produced an additive inhibitory effect, thus validating its important role in erythrocyte invasion (Anand et al., 2016).
based search includes rhoptry neck proteins (RONs). PfRON2, PfRON4 and PfRON5 were identified in *Plasmodium* by homology to ones known in *Toxoplasma gondii*, TgRON4, TgRON2 and TgRON5 (Alexander *et al*., 2006; Cao *et al*., 2009; Collins *et al*., 2009; Morenhaan *et al*., 2009). The RONs have been shown to be part of moving/tight junction complex in association with micronemal protein AMA-1 which commits the merozoite entry into the erythrocyte. RON1 which is also denoted as apical sushi protein (ASP) was also identified by homology search to detect sushi domains within *Plasmodium* proteins. Sushi domains are known to be site of protein-protein interaction for immune complement mediators. ASP has been shown to be localized in the rhoptries and released sequentially during the invasion process (O’Keeffe *et al*., 2005; Srivastava *et al*., 2010; Zuccala *et al*., 2012). Several other proteins identified based on homology searches contain the conserved thrombospondin structural repeat motif (TSR). *P. falciparum* TRAMP (PfTRAMP) contains a conserved thrombospondin structural repeat (TSR) in the ecto-domain and plays a critical role in erythrocyte invasion (Thompson *et al*., 2004; Siddiqui *et al*., 2013). In totality, this approach has also been successful in identification and characterization of novel invasins.

**Conclusion**

The emergence of drug-resistant parasites and insecticide resistant mosquitoes has complicated the battle against malaria, enforcing an urgent need for a malaria vaccine (Chauhan *et al*., 2010). Malaria vaccine development has been hindered by the enormous complexity of the parasite life cycle, widespread antigenic polymorphisms and inadequate knowledge of host pathogen interaction. However, there are strong indicators that a malaria vaccine may be possible. Passive transfer of antibodies from malaria protected individuals to naïve individuals conferred protection against challenge. Further, naturally acquired antibodies against merozoite surface proteins and invasion ligands have been correlated with protection from severe and clinical malaria studies from malaria endemic regions. The most advanced malaria vaccine targeting sporozoites, known as RTS,S has completed phase three trials in African children and demonstrated only modest efficacy of 29-36% (when a booster dose was given, and varying by age group) (RTSS Clinical Trials Partnership 2015). Till date, seven *P. falciparum* merozoite antigens have been assessed in human vaccine trials. These include AMA1 (Sagara *et al*., 2009), EBA175 (El Sahly *et al*., 2010), MSP1 (Ogutu *et al*., 2009), MSP2 (Genton *et al*., 2003), MSP3 (Essen *et al*., 2009; Audrun *et al*., 2005; Sirima *et al*., 2009; Druilhe *et al*., 2005), Glutamate-rich protein (GLURP) (Essen *et al*., 2009; Hermsen *et al*., 2007) and Serine repeat antigen 5 (SERA5) (Horii *et al*., 2010). AMA1 is known to play an important role in erythrocyte invasion. AMA1 is the most widely studied candidate and has been tested in 23 human trials on its own, and in nine studies in combination with other antigens. Phase-I studies have shown the vaccine to be safe and immunogenic. However, very little to no efficacy has been reported in the Phase II trials conducted so far (Ouattara *et al*., 2010; Thera *et al*., 2011). In Phase II studies, the vaccine recipients had reduced parasite growth rates but vaccine failed to protect malaria naïve participants whilst undergoing sporozoite challenge. *P. falciparum* MSP1 based vaccines have yet to demonstrate clear efficacy in humans. AMA1 and MSP1 have been the leading candidates for blood stage malaria vaccine development, but a high frequency of antigenic polymorphisms are believed to be responsible for their failure to induce protection in field efficacy trials (Crompton *et al*., 2010). *P. falciparum* reticulocyte binding-like homologous protein 5 (PfRH5) is emerging as a promising vaccine candidate and PfRH5-based vaccines are now progressing into clinical trials (Douglas *et al*., 2015).

Current efforts on the development of an effective malaria blood-stage vaccine are based on few parasite antigens and taking into consideration their current field efficacy, there is an urgent need to identify and validate novel vaccine candidates. It is apparent that our knowledge on the complete repertoire of parasite ligands that mediate erythrocyte invasion through multiple pathways is limited and even the precise function of known ligands is poorly understood. This clearly poses a major challenge in the development of effective malaria blood-stage vaccine.

Despite the tremendous research efforts since the publication of the *P. falciparum* genome sequence (Gardner *et al*., 2002), approximately, 60% of the
proteins encoded by \emph{P. falciparum} genes have no defined function associated with them and bear little or no similarity with proteins of other organisms (Le Roch \emph{et al.}, 2003; Bozdech \emph{et al.}, 2003; Hu \emph{et al.}, 2010). In the absence of tools for high-throughput gene knockdown strategies such as RNA interference (RNAi), the identification and validation of essential genes of the parasite remains a major bottleneck (Visvesvara and Garcia 2002). The identification of essential genes in the parasite has been very challenging due to an exceptionally AT-rich nuclear genome with an unusually low frequency of homologous recombination, which makes the genome refractory to genetic manipulation (Balu 2007). Also, \textit{in vitro} cultivation is a delicate process that is still mainly restricted to the blood stage of the parasite (Augagneur \emph{et al.}, 2012) and the limitation of using primates as animal models makes the \textit{in vivo} assessment of essential genes limited and expensive. However, with the recent advancements in research, it seems that cost-effective gene knockdown strategies of \emph{P. falciparum} genes may be feasible at a high-throughput scale (Pino \emph{et al.}, 2012).

In the past decades, high throughput mass-spectrometry based proteomic studies have been explored for the identification of essential proteins expressed in different stages of parasite development. Approximately, 2,400 proteins from sporozoites, trophozoites, merozoites and gametocytes have been identified using multi-dimensional protein identification technology (MudPIT) (Florens \emph{et al.}, 2002). The Leiden Malaria Group provided a mass spectrometry-based merozoite proteome that is currently available at the \emph{Plasmodium} database (http://plasmodb.org) and the data produced are reliable and the technology is able to identify proteins of low abundance level from every stage of the life-cycle including gametocytogenesis (Silvestrini \emph{et al.}, 2010), oocysts, oocyst-derived sporozoites, salivary gland sporozoites (Lasonder \emph{et al.}, 2008), liver stages (Taran \emph{et al.}, 2008), as well as late/rupturing schizonts (Bowyer \emph{et al.}, 2011). In addition, features such as the presence of phosphorylated serine and threonine residues has been detected by mass spectrometry methods and the first phospho-proteome of blood stage parasites has been described recently (Lasonder \emph{et al.}, 2008; Treeck \emph{et al.}, 2011).

The development of high-density deoxyribonucleic acid (DNA) microarrays of open reading frames (ORFs) facilitated the study of the functional roles of individual genes and their relationship to other genes. Le Roch \emph{et al.}, 2003 and Bozdech \emph{et al.}, 2003 were the first to use DNA chips to analyze gene expression in \emph{P. falciparum} across the entire asexual blood stage life cycle of the parasite. Their study concluded that expression profiles can be used to empirically assign functions to un-characterized proteins encoded by the \emph{P. falciparum} genome. Thus, resources obtained from the complete genome sequence of \emph{P. falciparum} and the existing information from the \emph{P. falciparum} transcriptomics and proteomics of different stages makes it more realistic to use genome wide approaches to identify novel invasion-related proteins.

Thus, in order to get a complete understanding of biology of the blood stage of the parasite, identification and characterization of novel \emph{P. falciparum} blood-stage proteins involved in erythrocyte invasion becomes inevitable. The transcriptome data for \emph{P. falciparum} has proven to be instrumental in the identification of new antigens which are involved in the process of invasion. It is noteworthy that none of the above mentioned invasins had a domain homology to known proteins which could have helped in predicting their function. This exercise of continuous identification of new antigens will surely enrich our understanding of basic parasite biology and would definitely help in translating the findings for the development of novel malaria intervention strategies.

\textbf{Acknowledgments}

We apologize to the researchers whose work could not be cited here. We thank Shalini Agarwal for help with the merozoite figure. Work on erythrocyte invasion in our laboratories has been supported by the Program Support Grant (CEIB), Department of Biotechnology (DBT), Government of India. G.A is the recipient of Senior Research Fellowship of the University grants commission (UGC), Government of India.
References


Douglas A D, Baldeviano G C, Lucas C M, Lugo-Roman L A, Crosnier C, Bartholdson S J et al. (2015) A PfRh5-based vaccine is efficacious against Heterologous strain blood-stage \textit{Plasmodium falciparum} infection in aotus monkeys \textit{Cell Host Microbe} \textbf{17} 130-9


Gaud D and Chitnis C E (2011) Molecular interactions and signaling mechanisms during erythrocyte invasion by malaria parasites Curr Opin Microbiol 14 422-428


Pino P, Sebastian S, Kim E A, Bush E, Brochet M, Volkmann K et al. (2012) A tetracycline repressible transactivator system to study essential genes in malaria parasites Cell Host Microbe 12 824-34


Treeck M, Sanders J L, Elias J E and Boothroyd J C (2011) The phosphoproteomes of Plasmodium falciparum and Toxoplasma gondii reveal unusual adaptations within and beyond the parasites’ boundaries Cell Host Microbe 10 410-9

parasites *Clin Microbiol Rev* **15** 327-8

