Chapter – Two

Review of Literature
2.1 History of Malaria

Malaria is a mosquito borne infectious disease of humans and other organisms caused by eukaryotic protozoans of the genus *Plasmodium*. The disease has been and continues to be one of the highest causes of morbidity and mortality amongst all human afflictions. Four species of *Plasmodium* are capable of infecting humans (*P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*), each differing in the intensity of the diseased condition, with *P. falciparum* causing the most severe and fatal form of the malady. Documented records from Egypt, India, China and Greece indicate the presence of a debilating state with symptoms similar to what are known for malaria as early as 1000 B.C. (Miller et al., 1994). The four species of human parasites are only remotely related and are believed to have diverged before the origin of hominids (Escalante et al., 1999). The closest relative of *P. falciparum* is *P. reichenowi*, a chimpanzee parasite (Qari et al., 1996). The fact that all four species of *Plasmodium* that are parasitic to humans are genetically indistinguishable from species that infect other primates, suggests that they were acquired by lateral transfer from other hosts. *P. falciparum* diverged from *P. reichenowi* about 8 million years ago, which is consistent with the time of departure of the human lineage from apes. The other human parasites have allegedly been transferred from monkey hosts more recently (Alaya et al., 1995). The first person to visualize the organism responsible for malaria was Charles Louis Alphonse Laveran in 1880, for which he received the first Nobel Prize in malaria. By 1885 his findings had been confirmed by further studies where the development of the intracellular parasite and accumulation of the malaria pigment had been extensively recorded. The next milestone in understanding malaria biology was achieved by Ronald Ross, who correctly identified the vector *Anopheles gambiae* for *P. falciparum* transmission and studied the development of the parasite in the mosquito (Ross, 1923). Ronald Ross was awarded the second Nobel Prize for malaria in 1902 for his contributions to this field. The tissue stages of human and primate malaria parasites were discovered in the liver of rhesus monkeys.
infected by *P. cynomolgi* and liver samples of humans with *P. vivax* infection in 1948 (Garham, 1966).

### 2.2 Geographical Distribution of Malaria

Malaria in the present world is restricted to the tropical and subtropical areas at altitudes below 1,500 meters (Fig. 2.1). *P. falciparum* has a widespread distribution in Central and South America, South East Asia and Africa. Although *P. malariae* occupies the same niche, it is however, less frequent than *P. falciparum* in terms of infection. *P. ovale* is predominantly found in sub-Saharan Africa while *P. vivax* is concentrated in Central and South America, India and South East Asia (World Malaria Report, WHO, 2011). 106 countries were endemic for malaria in 2011 with a cumulative 216 million cases of reported infections. 655,000 cases resulted in death of infected persons with 60% of the deaths occurring in Nigeria, Congo, Burkina Faso, Mozambique, Cote d’Ivoire and Mali.

![Fig 2.1: World malaria endemicity map. (WHO World Malaria Report 2011).](image)

India reported 1.22 million cases of malaria in 2011 (Fig. 2.2). The species of *Plasmodium* most prevalent in India are *P. falciparum* and *P. vivax*, each accounting for approximately
50% of infections. Major species of *Anopheles* mosquito vectors in India are *Anopheles stephensi, culicifacies, fluviatilis, minimus, dirus* and *annularis*.

**Fig 2.2: Epidemiological distribution of malaria cases across India in 2011 (WHO World malaria report 2011)**

### 2.3 Clinical Manifestations and Pathophysiology of Malaria

The pathology and clinical manifestations of malaria are mainly associated with the blood stages of *Plasmodium* infection, as the tissue stages and gametocytes hardly account for any of the symptoms of the disease. The severity of the infection depends on the *Plasmodium* species, with *P. falciparum* causing the most virulent infection, augmented by poor health, nutritional and immune status of the host. All of the common symptoms of malaria are associated with malarial paroxysms. This is characterized by a cold phase where the patient experiences chills inspite of having an elevated body temperature, followed by a hot phase where the patient complains of intense heat along with nausea, headache, fatigue, dizziness etc. Next a period of intense sweating ensues during which the body temperature of the patient falls to normal. These febrile attacks last for a duration of 4-8 hours and coincide with the time period when mature schizonts burst to release invasive merozoites.
into the blood stream. Conditions that occur simultaneously with malarial paroxysms are splenomegaly, hepatomegaly and hemolytic anemia. Malarial paroxysms occur periodically, 48 hours for *P. vivax*, *P. ovale* and *P.falciparum* while it occurs every 72 hours for *P. malariae*, on account of slower growth and maturation during schizont development. The periodicity is explained by the fact that the parasite grows synchronously in the host and most schizonts rupture simultaneously. The disease caused by different species of malaria differ in their intensities, with *P. falciparum* infection being the most severe that can also have fatal consequences. The increased morbidity and mortality of *P. falciparum* infections is due in part to the high number of merozoites produced and to the fact that sequestration of infected erythrocytes protects a large proportion of parasites from being cleared by the host immune system. That *P. falciparum* infects its host more successfully can also be attributed to its ability to infect all types of erythrocytes, while *P. vivax* and *P. ovale* prefer reticulocytes and *P. malariae* only invades senescent red blood cells. Relapse of the disease may occur after primary infection with *P. vivax* and *P. ovale* on account of reactivation of hypnozoites, a dormant form of the parasite that resides in the liver (Krotoski, 1989).

The pathology associated with malaria is related to the rupture of infected erythrocytes and deposition of parasite material, metabolites, hemozoin and cellular debris. Severe untreated cases of malaria present clinical features such as neurological impairment, severe anemia, hypoglycemia, acidosis, hyperlactemia, circulatory collapse and multi organ failure (Clark and Cowden, 2003). The most notable and frequent cause of death is dysfunction of the brain in cerebral malaria, characterized by a state of consciousness ranging from stupor to coma, convulsions and unresponsiveness to pain, visual and verbal stimuli (Adams et. al., 2002). Cerebral malaria can be attributed to sequestration of infected erythrocytes in the microvasculature of the brain leading to a mechanical blockage and subsequent hypoxia. Soluble mediators released by the parasite cause metabolic effects such as hypoglycemia and acidosis. In addition, malarial antigens could lead to the release of host mediators such as TNFα and nitric oxide, which could possibly have pathological effects (Mshana et. al., 1991). Singly or in combination all of the above factors can ultimately lead to a severe fatal infection.
2.4 Life Cycle of *Plasmodium*

Apicomplexans typically follow a characteristically complex life cycle comprising of three processes: sporogony, merogony and gametogony where each of the parasitic stages has a distinct morphology and biochemistry. The *Plasmodium* species completes its life cycle by alternating between an invertebrate vector and a vertebrate host. The parasite undergoes asexual development in the vertebrate host while it completes its obligate sexual development in the mosquito vector.

*Gametocytogenesis and Sexual Development in Mosquito Vector:*

All of the intra-erythrocytic parasites do not proceed to invade fresh erythrocytes but differentiate into either female macrogametocytes or male microgametocytes (Talman et al., 2004). The molecular events that cause an asexual stage parasite to commit to differentiate into a sexual stage parasite, rather than propagate asexually are not known. Five distinct stages of gametocyte maturation have been documented (Sinden, 1982), of which the immature stages are rarely seen in circulation since they remain sequestered in the bone marrow and spleen (Smalley et al., 1981). When a female *Anopheles* mosquito ingests a blood meal containing gametocytes, they undergo further development into gametes in the mosquito gut. Fertilization of a macrogametocyte by a microgametocyte ensues which results in the formation of a diploid zygote. The zygote elongates into a motile ookinete that penetrates the midgut membrane and migrates to the hemocoel of the gut where it matures into an oocyst (Doolan and Hoffman, 1997). Following this, meosis occurs within the oocyst and thousands of haploid sporozoites are released that migrate to the salivary glands. Here they enter into the channels of the salivary glands and are injected into the host organism during subsequent feeding.

*Asexual Development in the Host:*

Infection in the vertebrate host is initiated when a mosquito inoculates it with sporozoites present in its saliva while taking a blood meal. Further parasitic development occurs in the liver and erythrocytes.
Chapter Two: Review of Literature

Exoerythrocytic Development:

Sporozoites injected by a mosquito migrate extensively in the skin endothelial cells before they enter the bloodstream and travel to the liver sinusoids. Sporozoite entry into hepatocytes involves a cascade of events. On entering into the liver via the blood stream they glide along the capillary endothelial surfaces until they encounter a kupffer cell (Pradel and Frevert, 2001). Once they recognize proteoglycans on the Kupffer cell surface by means of surface proteins including Circumsporozoite Protein (CSP) and Thrombospondin Related Adhesive Protein (TRAP) (Pradel et. al., 2002), they actively invade and traverse the cell in a non lysosomal vacuole (Meis et. al., 1985; Ishino et. al., 2004). Other proteins that are postulated to play a role in hepatocyte migration and invasion are Sporozoite Threonine and Arginine Rich Protein (STARP), Liver Stage antigen 1 and 3 (LSA-1 and LSA-3) and Sporozoite and Liver Stage Antigen (SALSA) (Garcia et. al., 2006). Following their exit from kupffer cells, sporozoites invade a series of hepatocytes before establishing residence in one (Mota et. al., 2001). Invasion by a sporozoite is marked by the secretion of microneme contents at the point of cell contact (Gnatt et. al., 2000). Once internalized, the sporozoite is enclosed within a parasitophorous vacuole where it develops into a young exo-erythrocytic form (Meis and Verhave, 1988). Post invasion the parasites lose their sporozoite specific organelles and grow immensely in size eventually differentiating into merozoites that can infect red blood cells (Meis and Verhave, 1988). Nutrients required for the enormous amounts of membrane and nucleic acid synthesis are acquired from reserves of glycogen and serum protein factors stored within hepatocytes (Huff, 1969). Mature merozoites are held together by the host cytoplasm membrane (Meis et. al., 1985) and are finally released in merosomes that are parasite filled vesicles which directly release merozoites into circulation while also protecting against phagocytosis (Sturm et. al., 2006)

Erythrocytic Development:

Intra erythrocytic development of the parasite starts with the invasion of RBCs by merozoites. Invasive merozoites are important with respect to the development of antimalarial strategies, since they are exposed briefly to the host defense mechanisms when
they are released into the blood stream. Merozoites are ovoid structures about 1 µm wide and 1.6 µm long with an apical prominence at one end (Langerth et. al., 1978). Ultrastructurally the merozoite is specially suited to the task of invading an RBC. It bears on its surface a thick bristly coat made of protein filaments attached to the plasma membrane that recognizes receptors on the RBC surface (Bannister et. al., 1986). Below the plasma membrane, lie two additional membranes that form the outer and inner surfaces of a flattened cistern that anchors the motor complex utilized by the merozoite during invasion (Aikawa et. al., 1967; Keeley and Soldati, 2004). The two inner membranes form the inner membrane complex which along with the plasma membrane constitutes the parasite pellicle. The apical end is characterized by an apical complex that includes membraneous vesicles which are the rhoptries, micronemes and dense granules. These discharge their contents in a progressive fashion during and after invasion. Rhoptries are pear shaped, densely staining twin bodies (~ 650X300 nm), that converge via their narrow ends called the rhoptry ducts onto the apical prominence (Bannister et. al., 2000). Micronemes are small elongated structures (~ 120X40 nM), that surround the rhoptries and discharge their contents during the early stages of invasion (Soldati et. al., 2001). Dense granules are rounded structures (~ 80 nm diameter), that lie in the apical cytoplasm and are densely packed with granular contents (Atkinson and Aikawa, 1990). Apart from the apical complex, the anterior end also houses free ribosomes, a mitochondrion and a plastid while the posterior region of the merozoite is occupied by a large nucleus. The cytoskeleton of P. falciparum is rather minimal. Three electron dense structures under the apical prominence called the polar rings anchor a longitudinally running band of sub-pellicular microtubules (Bannsiter and Mitchell, 1989) (Fig. 2.3).

Ring (1-18 hours post invasion): Post invasion the parasite transforms into a discoidal structure with a thick cytoplasmic rim where all the major organelles are present and a thin centre. Ultrastructural studies indicate the presence of an early endoplasmic reticulum-golgi system and a variably shaped nucleus apart from a mitochondrion and a plastid (Aikawa et. al., 1967). After establishing itself within the RBC, the parasite starts to feed through a dense ring called the cytosome on its surface (Slomianny, 1990). Portions of
the RBC cytosol are ingested through the cytosome and digested within vacuoles in the parasite interior.

Fig 2.3: Three-dimensional organization of a *Plasmodium falciparum* merozoite, with the pellicle partly cut away to show the internal structure (Bannister et al., 2000).

**Trophozoite (18-32 hours post invasion):** Approximately 12 hours post invasion, a ring starts to develop into a more irregularly shaped trophozoite. The trophozoite stage is essentially larger and more randomly shaped with a manifold increase in metabolic activity which distinguishes it from a ring. Concomitant with increased protein synthesis and transport, the rough endoplasmic reticulum and the golgi bodies enlarge considerably and increase in complexity (Haldar, 1998). The trophozoite is marked by the presence of hemozoin pigment. Parasites feed on the protein content of hemoglobin derived from host erythrocytes (Banerjee et al., 2002) and covert the toxic heme component into inert brown hemozoin crystals (Egan, 2002). Hemozoin initially accumulates into several small pigment vacuoles that later coalesce into a larger food vacuole (Olliaro and Goldberg, 2001). The surface area of the parasite increases considerably, and the plasma membrane in conjunction with the parasitophorous vacuolar membrane is converted into elaborate cleft like structures that penetrate deep into the erythrocyte cytoplasmod that are termed...
Maurer’s clefts (Atkinson and Aikawa, 1990). It has been proposed that these function to sort and traffic material from the erythrocyte cytoplasm into the parasite (Lauer et al., 1997). The parasite also engages in exporting proteins into the RBC cytoplasm. While some of these are secreted via the traditional secretory pathways, it is known that different trafficking routes are employed for others (Mattei et al., 1999). Some of these exported proteins form angular elevations on the surface of an infected erythrocyte called knobs that impart to it a sticky nature which aids in sequestering it within visceral blood vessels (Atkinson and Aikawa, 1990).

Schizont (32-48 hours post invasion): Most of the characteristic processes that occur in trophozoites including synthesis of DNA, export of parasite proteins and ingestion of erythrocyte contents continue late into schizont development. A schizont is essentially an intra-erythrocytic parasite characterized by repetitive nuclear division. The nucleus of a P. falciparum schizont divides on an average about 4 times producing approximately 16-20 merozoites (Arnot and Gull, 1998). Nuclear division is endomitotic i.e., the segregating chromosomes remain within an intact nuclear envelope throughout the entire process (Atkinson and Aikawa, 1990). Numerous cytoplasmic changes including proliferation of rough endoplasmic reticulum and free ribosomes, multiplication of mitochondria and plastids accompany nuclear division. The next phase involves the formation of merozoite forming foci at regular intervals at the periphery of the parasite (Vickerman and Cox, 1967). Each nascent merozoite accumulates a set of rhoptries, micronemes and dense granules along with a nucleus, a mitochondrion and a plastid. Proteins are targeted to the apical organelles from the ribosomes attached to the nuclear envelope and the endoplasmic reticulum via the ER-Golgi pathway (Atkinson and Aikawa, 1990). Cytoskeletal elements are arranged below the merozoite surface and cytokinesis occurs to separate individual merozoites (Bannister et al., 2000). Finally, the erythrocytes rupture and free invasive merozoites that are released into the blood stream attach to and invade fresh RBCs.

2.5 Erythrocyte Invasion by Merozoites

The invasive zoite stages of apicomplexans are polarized motile cells that possess a typical apical complex. Invasion involves the recognition of a host cell, entry and enclosure of the
Chapter Two: Review of Literature

parasite within a parasitophorous vacuole (Fig. 2.4). The entire process is completed within a 60 second time frame to safeguard against a host mediated immune attack against highly antigenic proteins on the merozoite surface. Primary contact between the RBC and the merozoite is a low affinity reversible interaction that may occur at any point on the merozoite surface (Bannister and Dluzewski, 1990). The initial attachment has been demonstrated to be host cell specific since merozoites of *Plasmodium knowlesi* only invade erythrocytes of a susceptible species (Miller et. al., 1977). The surface coat of merozoites comprises to a large extent of glycosylphosphatidylinositol (GPI) anchored proteins and their associated partners (Sanders et. al., 2005). All GPI anchored proteins with a putative role in erythrocyte recognition however do not have similar localizations, with some being present at the apical end indicating different roles in the process of invasion (Sanders et. al., 2005). Merozoite Surface Protein 1 (MSP1) has an even distribution over the parasite periphery and may therefore play a role in primary attachment to an erythrocyte. MSP1 is essential for parasite survival and invasion which is evident from the fact that it cannot be knocked out and antibodies against it interfere with erythrocyte invasion (Singh et. al., 2003; O’Donnell et. al., 2000). Other studies have shown that it is in fact a complex of the 42KDa fragment of MSP1 and MSP9 that may be mediating interaction with the RBC surface by recognizing Band 3, an erythrocytic membrane transport glycoprotein (Goel et. al., 2003; Li et. al., 2004). Primary contact may in part also be mediated by peripheral membrane proteins that are secreted into the parasitophorous vacuole of schizonts and bind to the GPI anchored proteins on the merozoite surface (Pachecbat et. al., 2001).

Following this initial attachment the parasite undergoes a reorientation with respect to the RBC surface in order to juxtapose its apical end with the erythrocyte surface. Molecular interactions responsible for this step have not been well characterized yet. Apical Merozoite Antigen 1 (AMA1) however, has been implicated to play a direct role in this process since antibodies against this protein result in parasites that are unable to reorient (Mitchell et. al., 2004). On completion of reorientation, a typical junction is formed at the site of contact which is characterized by an electron dense thickening under the surface of the erythrocyte membrane (Aikawa et. al., 1978; Miller et. al., 1979). The formation of this junction commits the parasite to invasion and is followed by the movement of the junction
around it thereby allowing it to gain physical access to the erythrocyte interior within the parasitophorous vacuole.

The physical process of invasion involves a number of ligand receptor interactions and two protein families are the prime candidates to mediate this process. Proteins of the Duffy Binding Like (DBL) family facilitate invasion via the sialic acid dependent pathway (Singh et al., 2005). These proteins including Erythrocyte Binding Antigen (EBA) 174, 140, 181 and 1 are stored in the micronemes and bind to Glycophorins A, B and C which are the major sialylated proteins on the erythrocyte surface (Sim et al., 1994). The sialic acid independent pathway is dependent on the second group of parasite ligands which are the Reticulocyte Protein Like Homologues (RH) RH1, RH2a, RH2b, RH3, RH4 and RH5 (Stubbs et al., 2005). The receptors for most of these on the RBC surface are yet to be identified except for RH4 which has been shown to bind to Complement Receptor 1 (CR1) (Tham et al., 2010). Recently Basigin has been identified as an essential receptor for erythrocyte invasion that recognizes RH5 (Crosnier et al., 2011). The invasion processes utilizing these ligands are redundant since these proteins can be knocked out without affecting parasite viability (Cowman and Crabb, 2006). Multiple ligand receptor systems are beneficial for the fitness of a population since human erythrocyte receptors are highly polymorphic and are present in varying amounts during a cell's life span (Miller et al., 2002). Additionally, a host immune response may block a particular ligand thus generating the need for other functional pathways (Gaur et al., 2004).

Once the parasite has been engulfed by the moving junction at the interface of the merozoite and erythrocyte membranes, it is sealed within the parasitophorous vacuole in an environment that is favourable for further growth and development. Invasion by apicomplexan zoite forms is also characterized by the sequential release of the contents of the apical organelles during this process. In general, microneme contents are believed to mediate host cell recognition and binding. Proteins secreted from the rhoptries are involved in parasitophorous vacuole formation and the contents of the dense granules remodel the nascent vacuole that encloses the parasite, into a metabolically active compartment (Dubremetz, 1998).
While the exact timing of release of the apical organelles is not yet known, it may be said broadly that microneme discharge occurs before the initiation of invasion while rhoptry proteins are exocytosed during the process of invasion (Dubremetz et al., 1993; Carruthers and Sibley, 1997). Proteins sequestered in the dense granules of *T. gondii* are released after the enclosure of the parasite into the parasitophorous vacuole (Cesborn-Delauw, 1994). The physiological signals that are involved in initiating apical organelle discharge are not yet well characterized. A recent study in *P. falciparum* indicates that the low K+ concentration in the blood plasma to which a merozoite is exposed after egress causes an increase in the intra-parasitic Ca²⁺ concentration, which is the trigger for microneme discharge. Once the parasite ligands released from the micronemes interact with their receptors on the erythrocyte surface, the basal cytoplasmic Ca²⁺ concentration is restored which causes discharge of the rhoptry proteins (Singh et al., 2010).
A characteristic feature of erythrocyte invasion is the shedding of zoite surface proteins that play various roles in the process of invasion. Primary processing of these proteins involves cleavage of a large protein into multiple subunits that remain non covalently bound to each other (Carruthers and Blackman, 2005). The best documented example of primary processing of a zoite surface protein is that of MSP1, a 200 KDa protein which is catalyzed into 4 fragments (Blackman et. al., 1991). Cleavage of a protein could result in the conversion of an adhesive molecule that binds weakly, into one with a high affinity at the required time. It could also be a prerequisite for secondary processing which results in shedding of the protein from the merozoite surface (Carruthers and Blackman, 2005). Primary cleavage is followed by secondary proteolysis which ultimately culminates in the release of the protein from the parasite surface. Shedding may occur continuously during invasion as in the case of MSP1 and is referred to as shaving (O’ Donnell and Blackman, 2005). Other proteins may be capped to the posterior end before being proteolytically cleaved during the final stages of invasion (Russell et. al., 1983; King et. al. 1988). A sheddase like protein which is a metalloprotease that cleaves within areas of disorder in protein structure present at a certain distance from the membrane, is a prime candidate implicated in MSP1 and AMA1 processing (Howell et. al., 2003). Another possibility is rhomboid proteases which are intramembrane enzymes because PfAMA1 and TgMIC1 have been shown to be cleaved in a rhomboid like manner (Zhou et. al., 2004). Further evidence supporting this idea comes from the fact that many transmembrane domain containing microneme proteins contain a helix breaking GG or GA motif recognized by rhomboid proteases.

**2.6 Mechanism of Erythrocyte Invasion**

Invasion is achieved by a form of forward motion exhibited exclusively by the invasive forms of apicomplexan parasites known as gliding motility (Baum et. al., 2006). Invasive zoite forms are typically asymmetrical with a well defined anterior posterior axis where the apical end of the cell is involved in invasion. Gliding is an active form of motility that moves cells at a rate of 1-10μm/sec without any changes in cellular morphology (Daher
and Soldati-Favre, 2009). Forward movement of merozoites is mediated by movement of myosin head molecules along polymerized actin filaments (Hakansson et. al., 1999). The Myosin tail is attached to the motor complex, which in turn is in association with aldolase that connects to extracellular receptors that recognize ligands on the RBC surface. Rearward motion of the ligand receptor complex immobilized on the RBC surface, in coordination with movement of the myosin head along actin filaments results in forward propulsion of the parasite (Fig. 2.5).

The molecular machinery involved in invasion comprises of a hetero-tetrameric complex of proteins that is referred to as the glideosome. The existence of an invasion complex anchored in the pellicle of invading zoite forms of apicomplexans was first demonstrated in *Toxoplasma* (Gaskins et. al., 2004) and subsequently in the sporozoites (Bergman et. al., 2003) and merozoites (Green et. al., 2004; Baum et. al., 2004; Jones et. al., 2004) of *Plasmodium*.

![Fig 2.5: Model for the conserved apicomplexan motor responsible for gliding motility and invasion across different apicomplexan genera and lifecycle stages (Baum J et. al., 2006).](image-url)
The four components of the complex include a class XIV myosin, its associated light chain and glideosome associated protein 45 and 50. The myosin involved in invasion belongs to the unconventional Class XIV myosins and is termed Myosin A. TgMyoA is a 93 KDa protein and is considerably smaller than known myosins (Meissner et al., 2002). It contains a head region that is similar in size and amino acid composition to other myosins, with a remarkable conservation of various domains and motifs. It however, lacks a conserved phosphorylatable or negatively charged residue that is essential for the mechano-chemical activities of myosins (Bement and Mooseker, 1995), suggesting that TgMyoA activity is differently regulated. PfMyoA retains a threonine at the relevant site suggestive of regulation by a heavy chain kinase. A novel feature of TgMyoA is that it completely lacks the neck region that typically contains IQ motifs which are important for modulating myosin activity (Heintzelman and Schwartzman, 1997). The tail region of TgMyoA is extremely short and lacks any homology with known myosins, except for the presence of a basic residue that is involved in binding to phospholipids and thereby mediating membrane-protein interactions (Mooseker and Cheney, 1997; Sellers et al., 1996). PfMyoA is similar to TgMyoA with respect to its size and domain architecture and like the latter lacks the regulatory neck region. Both proteins instead possess a degenerate IQ motif at the C terminus that binds to a calmodulin like protein called TgMLC1 (*Toxoplasma gondii* Myosin Light Chain homologue 1) or PfMTIP (*Plasmodium falciparum* Myosin Tail Interacting Protein 1). MyoA from both genera bind their regulatory light chains by utilizing their C termini since deletion of this region leads to abrogation of this interaction (Bergman et al., 2003, Herm-Gotz et al., 2002). The fact that PfMTIP and PfMyoA interact via the partial IQ motif was reinforced by the demonstration that the C terminal tail peptide of PfMyoA can abolish this association in vivo (Bosch et al., 2006; Green et al., 2006). The light chain homologues themselves are calcium independent modulators of myosin activity; since they lack the canonical calcium binding domains that are present in myosin light chains and calmodulin like proteins to which they show a distant similarity (Heintzelman, 1997; Heintzelman and Schwartzman, 2003). Furthermore, the interaction between PfMTIP and PfMyoA is not modulated by calcium (Green et al., 2006).
The crystal structure of PfMTIP, comprising residues E60 to Q204 complexed with a PyMyoA tail peptide corresponding to residues S803 to A817 revealed a compact structure. The hinge region connecting the N and C terminal domains of PfMTIP stabilizes the structure via formation of a salt bridge by utilizing a glutamic acid residue. The N terminal domain of PfMTIP forms 9 hydrophobic and hydrophilic interactions with MyoA. The C terminal domain undergoes a major conformational change to create a hydrophobic pocket that accepts hydrophobic side chains from the MyoA tail region. A key lysine residue that occupies the 7th position in the IQ motif in the MyoA tail plays a crucial role in the formation of the compact complex, since it forms associations with residues on the PyMyoA tail as well as on PfMTIP. The 7th position of a conventional IQ motif is typically occupied by a glycine and therefore the PfMTIP-PyMyoA structure appears to be unique (Bosch et al., 2007). A basic RKK motif near the degenerate IQ motif of PfMyoA is essential for its interaction with PfMTIP since its mutation to AAA abolishes the interaction. Of the three residues, the interaction between the two proteins is supported most robustly by the initial arginine, followed by the lysine in the 2nd position and least of all by the terminal lysine. The Q residue in the IQ motif "IQxxxRGxxxR" also plays a vital role in this interaction. Conventionally, the Q loop motif interacts with an IQ motif present on interacting protein partners. The Q loop of PfMTIP consists of WGD with the ultimate D residue playing the most crucial role in maintaining this interaction (Jones et al., 2004). MyosinA and its regulatory light chain are expressed in the invasive stages and colocalize in the inner membrane complex of Toxoplasma and Plasmodium (Bergman et al., 2003; Herm-Gotz et al., 2002).

Studies in Toxoplasma gondii have revealed that the C terminal domain of MLC1 interacts with MyoA while its N terminal domain interacts with the C terminus of GAP45. TgGAP45 thus recruits MLC1 and MyoA to the IMC possibly via interactions with the fixed membrane anchor of the glideosome TgGAP50. Additionally, two putative palmitoylations at the C terminus of TgGAP45 have been shown to be crucial in mediating the association of GAP45 with the IMC. Acyl modifications at the N terminus of TgGAP45 which include a myristoylation and a palmitoylation have been shown to be essential for its association with the plasma membrane. Both interactions mediated via the N and the C termini of
TgGAP45 have been proposed to play a vital role in maintaining parasite pellicle cohesion (Frenal et. al., 2010).

The glideosome is composed of two novel proteins which have no significant similarity to any known proteins. These have been named glideosome associated proteins on account of the fact that they are part of the glideosome complex. Both GAP45 and GAP50 were first identified in *Toxoplasma gondii* (Gaskins et. al., 2006). Sequence analysis of GAP45 suggests the presence of an N terminal coil coil domain and a C terminal globular domain. The protein has been demonstrated to localize at the inner membrane complex surface facing the plasma membrane (Gaskins et. al., 2004). TgGAP45 runs at an aberrant molecular weight of 45 KDa instead of the expected 27 KDa possibly on account of an extended configuration assumed by the coil coil region of the protein or due to a high content of charged residues that may affect its motility. PfGAP45, similar to TgGAP45 localizes to the merozoite periphery in blood stage parasites and is associated with the IMC (Baum et. al., 2006; Jones et. al., 2005). The protein characteristically migrates as a doublet which may be indicative of post translational modifications.

Although TgGAP50 does not show similarity with any other protein of known function, it has well conserved orthologues in *Eimeria* and *Plasmodium*. The protein localizes to the inner membrane complex, associates with the parasite pellicle and is the likely candidate that anchors the glideosome in the IMC (Gaskins et. al., 2004; Jones et. al., 2006). PfGAP50 is associated with the endoplasmic reticulum from late ring to mid trophozoite stage and is seen arranged as punctate structures at the parasite periphery during maturation (Yeoman et. al., 2011). The sequence of TgGAP50 indicates putative N and C terminal transmembrane domains (Gaskins et. al., 2004). The N terminal transmembrane domain of PfGAP50 is a signal sequence, which is cleaved resulting in the protein being anchored within the membrane via the C terminal transmembrane domain, with a large N terminal soluble domain in the lumen of the IMC and a short C terminal tail facing the plasma membrane (Bosch et. al., 2012). The C terminal tail is involved in attachment to the glideosome complex, an interaction that is probably strengthened by lipid modifications on PfGAP45 (Gaskins et. al., 2004; Bosch et. al., 2012). GAP50 from both *Toxoplasma* and *Plasmodium* share about 20-25% identity with tartarate resistant purple acid phosphatases
Chapter Two: Review of Literature

(Gaskins et al., 2004). A report has suggested that PfGAP50 possesses phosphatase activity that may play a role in the process of invasion or elsewhere in parasite development (Muller et al., 2010). The recombinant protein however, does not show catalytic activity (Yeoman et al., 2011) and sequence analysis of the protein has revealed that most of the catalytic residues are not conserved in GAP50. The PfGAP50 crystal structure indicates the presence of a metal binding site and a large conserved domain in the N terminal soluble region that may interact with unknown proteins in the lumen of the IMC. The identity of the metal ion that occupies the above mentioned site is not known but it may stabilize the protein structure (Bosch et al., 2012).

Expression of some of the proteins that form the glideosome such as PfMyoA, PfMTIP and PfGAP45 occurs late in the intra-erythrocytic development of parasites consistent with a role in invasion (Baum et al., 2006; Jones et al., 2006; Green et al. 2006). PfGAP50 expression is initiated earlier which may be essential for proper processing and insertion of the protein into the IMC (Baum et al., 2006; Jones et al., 2006). Other proteins that play key roles in aiding invasion are PfActin which powers motion of the parasite in conjunction with PfMyoA. PfActin expression is also initiated much earlier in the parasite life cycle indicative of functions that are independent of invasion (Jones et al., 2006). The glideosome complex is attached to the extracellular ligands via PfAldolase which is expressed throughout the intra-erythrocytic development of the parasite as it plays a role in glycolysis apart from invasion (Baum et al., 2006). The formation of a protoglideosome consisting of MyoA, MTIP and GAP45 that associates before final assembly of the glideosome via interaction with GAPSO has been suggested in Toxoplasma gondii (Gaskins et al., 2004). In contrast, a pre-complex of PfGAP45 and PfGAP50 has been proposed in Plasmodium falciparum which binds to PfMyoA and PfMTIP (Jones et al., 2009).

Recently other proteins that are associated with the glideosome complex have been identified. Three families of six pass transmembrane proteins called the Glideosome Associated Proteins with Multiple membrane spans (GAPM1, GAPM2 and GAPM3) have been identified. Plasmodium falciparum expresses one protein from each group which associate with the motor complex and have an IMC localization. These proteins form homo oligomeric complexes within the IMC while also interacting with the cytoskeletal alveolins.
and may therefore function as tethers that anchor the motor complex (Bullen et al., 2009). TgGAP40 is a polytopic protein that resides in the IMC and associates with the MyoA-MLC1-GAP45-GAP50 complex. The protein is highly conserved in apicomplexans and is expressed in the invasive forms (Frenal et al., 2010). More recently a 15 KDa calmodulin like protein called Essential Light Chain 1 (ELC1) was found to colocalize and co-immunoprecipitate with GAP45 and MLC1 in Toxoplasma gondii and has been predicted to regulate the motor complex in a Ca\textsuperscript{2+} dependent fashion (Nebl et al., 2011). Post translational modifications have been reported to occur on various proteins that comprise the glideosome complex. GAP45 is phosphorylated in both Toxoplasma and Plasmodium. TgGAP45 is phosphorylated at at least seven residues including serine 163 and serine 167 in the region between the coil coil domain and the globular domain. Phosphorylation at these sites inhibits the ability of TgGAP45 to interact with TgGAP50 suggesting that dephosphorylation of the protein is essential to stabilize the motor complex (Gilk et al., 2009). Phosphorylation at these sites does not affect invasion capacity, parasite viability or targeting of TgGAP45 to the IMC. Another study has identified seven phosphorylation sites on TgGAP45 in the region between the coil coil domain and the globular domain. Three of these sites are calcium dependent, two of which are conserved across apicomplexans (Nebl et al., 2011). PfGAP45 has been shown to be an in vitro substrate of Plasmodium falciparum Calcium Dependent Protein Kinase 1 (PfCDPK1) (Green et al., 2008; Kato et al., 2008). PfGAP45 has a glycine at the 2\textsuperscript{nd} position and a cysteine at the 5\textsuperscript{th} position, which are predicted to be putative myristoylation and palmitoylation sites respectively. It is cotranslationally N myristoylated with a further palmitoylation occurring at the time of motor complex formation. It is possible that the second acylation event increases the affinity of the complex for the IMC membrane (Rees-Channer et al., 2006).

PfMTIP is phosphorylated in vitro by PfCDPK1 (Green et al., 2008; Kato et al., 2008) at Serine 47 and Serine 51 (Green et al., 2008). TgMLC1 is phosphorylated in a calcium independent manner at serine 55 in its N terminal domain as well as in a calcium dependent fashion at threonine 98 and serine 132 in its C terminal region, none of which are conserved across apicomplexans (Nebl et al., 2011). A post translational modification in the N terminus of TgMLC1 the nature of which has not been identified has been shown to
inhibit the myosin motor activity (Heaslip et. al., 2010). Motility of *Toxoplasma gondii* tachyzoites has also been shown to be regulated by a lysine methyltransferase that plays a role in egress and host cell invasion (Heaslip et. al., 2011). TgMyoA is phosphorylated at serine 21, a site that is conserved in all apicomplexans, which lies in the head region of the molecule involved in binding to actin (Nebl et. al., 2010).

An independent study has identified 8 phosphorylation sites on PfGAP45, 22 phosphorylation sites on TgGAP45, 1 phosphorylation site each on PfMyoA and PfMTIP and 14 phosphorylation sites on TgMyoA and TgMLC1 by mass spectrometric analysis of *P. falciparum* schizonts and tachyzoites of *T. gondii* (Treeck et. al., 2011).

TgGAP50 is glycosylated at three residues, two of which are well conserved in apicomplexans and have a higher impact on protein function. The modifications result in the addition of immature and truncated oligo-mannose sugars to the protein. These are essential for it to be correctly trafficked from the endoplasmic reticulum to the IMC membranes, and for maintaining the integrity of the motor complex possibly via glycan-glycan or glycan-protein interactions (Fauquenoy et. al., 2008).

### 2.7 Phosphoinositide Signaling in Eukaryotic Organisms:

Phosphoinositides are differentially phosphorylated forms of phosphatidylinositol (Fig. 2.6). These molecules form a group of secondary messengers that can be modified at the inositol head group to form membrane targeting signals intracellularly. Seven differentially phosphorylated phosphoinositides (at positions 3, 4 and 5 in the inositol ring) have been identified, that play roles in signal transduction pathways involved in cytoskeletal organization, intracellular trafficking, transcription regulation etc. (Paolo and Camilli, 2006). Since each of the lipid molecules (including DAG a catabolic product of PI-(3, 4, 5)-P3) are bioactive, since they are capable of recruiting proteins that contain domains capable of binding to them to various locations.
Fig 2.6: An illustration of the seven known PIs, and kinases (blue), phosphatases (violet) and phospholipases (green) involved in their metabolism (Rusten and Stenmark, 2006).

While rare phosphoinositides (those phosphorylated at the 3’ position) bind to a small subset of domains, phosphoinositides that are more abundant such as PI-(4,5)-P2 bind to a wider array of domains. Phosphoinositides are created by the action of phosphoinositide kinases and dephosphorylated by lipid phosphatases (Sigal et al., 2007). These lipid kinases are broadly classified into three families; Phosphoinositide-3-kinases (PI3Ks) that phosphorylate a variety of phosphoinositides at the 3’ position, Phosphoinositide-4-kinases (PI4Ks) that catalyzes the conversion of PI to PI-(4)-P by transferring a phosphate group to the 4’ position and Phosphoinositide-5-kinases (PI5Ks) that convert PI-(4)-P to PI-(4,5)-P2 (Carpenter and Cantley, 1990). Phosphotidyl inositol itself is synthesized from CDP-diacylglycerol and myo-inositol by the enzyme phosphatidyl inositol synthase (Paulus and Kennedy, 1960).

The binding domains are diverse in structure and in their interactions with their target. Some of the characterized phosphoinositide recognition modules are the PH domain (Harlan et al., 1994), the FYVE domain (Gillooly et al., 2000), the PX domain (Ellson et al., 2002) and the ENTH domain (De camilli et al., 2002). PI-(3,4,5)-P3 binding PH domains display strong and specific interactions with the head group mediated by hydrogen
bonding. PI-(4,5)-P2 interacts electrostatically with short basic stretches of amino acids. FYVE and PX domains recognize a specific ligand i.e. PI-(3)-P but bind only weakly, an association that is strengthened further by inserting hydrophobic side chains into the lipid bilayer or increasing their avidity via oligomerization (Cullen et al., 2001).

PH domains are modules that share structural homology with a domain capable of binding to phosphorylated inositides originally identified in pleckstrin, a downstream target of PKC in platelets. These domains encompass regions of about 120 amino acids. The domain core is a 7 stranded β-sheeted sandwich. One end of the open structure is closed off by a C terminal α-helix while the other open end involved in phosphoinositide binding is occupied by three interstrand loops (Hyvonen et al., 1995). Although PH domains in general are known to bind to a range of phosphoinositides with varying degrees of specificity and strength, there are examples of rare PH domains that bind strongly and specifically to a few select phosphoinositides. These include the PH domain of PLCδ that binds to PI-(4,5)-P2 (Garcia et al., 1995) and that of PKB that binds to PI-(3,4)-P2 and PI-(3,4,5)-P3 (Frech et al., 1997).

There is limited information available about Phosphoinositide metabolism and signalling in *Plasmodium*. Phosphatidylinositol-3-Kinase trafficking and function has been studied in *P. falciparum* (Vaid, Ranjan et al., 2010). It has also been shown that PI-(3)-P localizes in the food vacuole and apicoplast membrane (Tawk et al., 2010). Binding to the endoplasmic reticulum PI-(3)-P has been postulated to traffic proteins to the host cell (Bhattacharjee et al., 2012). Further, a PI-(3)-P binding protein which interacts with it via a FYVE domain has been shown to be targeted to the food vacuole of *P. falciparum* (McIntosh et al., 2007).

### 2.8 Calcium Signaling in *Plasmodium falciparum*:

Calcium is a crucial secondary messenger that modulates various processes during parasite development (Garcia, 1999; Billker et al., 2009). It has been shown to regulate cytoskeletal dynamics in the parasite (Wasserman et al., 1990), apical organelle secretion (Singh et al., 2010), egress from erythrocytes (Dvorin et al., 2010), gametogenesis (Billker et al., 2004),

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25
erythrocyte invasion (Wasserman and Chaparo, 1996) and gliding motility (Siden-Kiamos et. al., 2006). Intracellular parasites are present in a microenvironment with a calcium concentration of ~40 μm, 100-1000 times higher than that in the parasite and the RBC cytosol where it is in the nm range (Adovelande et. al., 1998; Gazarini et. al., 2003). The higher calcium concentration in the parasitophorous vacuole could be accounted for by i) extracellular calcium that is trapped in the vacuolar space during invasion, ii) a Ca^{2+}-ATPase located in the vacuolar membrane that supplies Ca^{2+} to the vacuole or iii) the diffusion of Ca^{2+} from the extracellular medium into the vacuole through specialized membranous structures that extend from the vacuolar membrane to the underside of the erythrocyte membrane (Gazarini et. al., 2003). Numerous proteins have been characterized in Plasmodium species that are involved in handling and sensing calcium fluctuations in the parasite including the calcium dependent protein kinases, Ca^{2+}-ATPases, and calmodulin (Garcia, 1999).

Calcium homeostasis is maintained by sequestering Ca^{2+} within intracellular organelles. The largest reservoir of calcium in eukaryotic cells is usually the endoplasmic reticulum where its concentration can reach as high as a few millimolars (Koch, 1990). Calcium release in P. falciparum from the endoplasmic reticulum is sensitive to the Phospholipase C inhibitor U73122, thus indicating that PLC mediated catalysis of PI-(4,5)-P2 into IP3 and DAG is responsible for mobilizing intracellular calcium (Beraldo et. al., 2005). Calcium release from the endoplasmic reticulum has also been postulated to occur via ryanodine receptors on its membrane by using inhibitors such as dantrolene and 8Br-cADP Ribose (Jones et. al., 2009). Although pharmacological studies indicate the presence of IP3 and RyR channels there is no bio-informatic or molecular evidence for their existence in protozoan parasites (Moreno and Docampo, 2003). The influx of calcium into the endoplasmic reticulum is facilitated by the sarco-endoplasmic reticulum Ca^{2+}-ATPases. One such SERCA channel has been described in Plasmodium falciparum (Kimura et. al., 1993). The mitochondria of Plasmodium falciparum has also been shown to be capable of reversibly accumulating excess intracellular Ca^{2+} and may play a role in maintaining calcium homeostasis (Gazarini et. al., 2003). Acidocalcisomes are acidic compartments that are characterized by their acidic nature and high content of pyrophosphate, polyphosphate,
calcium, magnesium and other elements (Docampo and Moreno, 2001). A Ca\(^{2+}/H^+\) ATPase has been postulated to be required for transporting Ca\(^{2+}\) into these organelles. A gene encoding a similar ATPase has been found in the genome of *Plasmodium falciparum* (Moreno and Docampo, 2003) although the cytological nature of this acidic pool in *Plasmodium falciparum* is not known (Garcia, 1998).

### 2.9 Protein Kinases in *Plasmodium falciparum*:

*Plasmodium falciparum* follows a very complex life cycle comprising of many stages and is completed in two different hosts. This would require stringent regulation of various parasite activities that are essential for parasite life cycle progression and propagation. Phosphorylation by protein kinases is one of the key regulating mechanisms by which various processes are controlled in eukaryotic cells. Kinase domains are conserved stretches of amino acids approximately 250 to 300 amino acids in length (Hanks et al., 1988). The kinase domain is further subdivided into 12 sub domains that contain characteristic patterns of conserved residues. The highly conserved nature of kinase domains results in them folding into a similar 3-dimensional core structure, which transfers phosphates via a common mechanism while maintaining functional specificities (Cheek et al., 2002). Phylogenetically eukaryotic protein kinases have been classified into 4 groups; the AGC group, the CaMK group, the CMGC group and the conventional protein tyrosine kinase group. Later three new groups of eukaryotic protein kinases were established namely, the casein kinases, the STE kinase group and the tyrosine kinase like group (Hanks, 2003). Classification of kinases into these superfamilies has been carried out on the basis of their sequence homology and substrate specificities. Proteins that fall out of these families are referred to as the other protein kinases (Hanks and Hunter, 1995).

The internal architecture of a kinase consists of two structurally and functionally distinct lobes. The amino terminal lobe comprising sub domains I-IV is responsible for binding and orienting the nucleotide phosphate donor in a complex with a divalent ion. The carboxy terminal lobe comprising the rest of the kinase domain is involved in binding to the peptide substrate and initiating phosphotransfer (Taylor and Kornev, 2011).
The *Plasmodium falciparum* genome codes for 86-99 protein kinases that have been classified into the above mentioned kinase families (Ward et. al., 2004; Srinivasan et. al.) (Fig. 2.7). A significant deviation of the malarial kinome with respect to the kinomes of higher eukaryotic organisms is the complete absence of any member that can be classified into the STE group that includes enzymes participating in MAP kinase pathways and tyrosine kinase superfamilies. In addition, several kinases do not cluster with any defined group of protein kinases and have been termed orphan kinases. Four “composite kinases” have been identified that share characteristics of two kinase superfamilies and may represent common ancestors to families that subsequently diverged (Ward et. al., 2004). Other differences between the plasmodial kinome and that of metazoans is the presence of a group of calcium dependent protein kinases, a feature characteristic of plant kinomes and the fact that though most of these proteins cluster into established kinase groups, it is difficult to identify clear orthologues in mammalian systems (Doerig et. al., 2008).

A recent study aimed at analyzing the essentiality of various kinases in *Plasmodium* by generating knockouts of these proteins has revealed that 36 plasmodium kinases are essential for the asexual life cycle of the parasite while 12 are non essential and an additional 14 are probably dispensable. A phospho proteomic analysis performed on schizont stage parasites in the same study identified 1177 phosphorylation sites on 650 parasite proteins (Solyakov et. al., 2011). A similar phospho proteomic study identified 8,463 phosphorylation sites on 1,673 proteins (Treeck et. al., 2011).

Both of these studies have revealed that protein phosphorylation is a major regulatory mechanism employed by the parasite since housekeeping proteins as well as proteins involved in parasite specific activities such as cytoadherence and host cell invasion are phosphorylated. Surprisingly tyrosine residues are phosphorylated in the parasite inspite of no known tyrosine kinase till date. Further, *Plasmodium* uses phosphorylation motifs that are distinct from *Toxoplasma* and higher eukaryotes which could probably be accounted for by the high AT content of its genome (Solyakov et. al., 2011; Treeck et. al., 2011).
Chapter Two: Review of Literature

Fig 2.7: The *P. falciparum* kinome. Phylogenetic tree and classification of putative eukaryotic protein kinases of *P. falciparum*. *PlasmoDB* gene id is shown in red and representative members of major subgroups from humans are depicted in black (Ward et al., 2004).

*Plasmodium* kinases, PfPKB and PfCDPK1 have been shown to play vital roles in erythrocyte invasion. PfCDPK1 is one of seven calcium dependent protein kinases present in *Plasmodium falciparum*. Calcium dependent protein kinases contain a kinase domain as well as a calmodulin like domain and are typically found in plants and alveolates (Zhang and Choi, 2001). Of the seven CDPKs in *Plasmodium falciparum* only PfCDPK1 is essential for asexual development of the parasite. PfCDPKs 3, 4 and 6 do not play a role in the blood stages however, PfCDPK4 is known to be essential for cell cycle progression in the
Chapter Two: Review of Literature

microgametocyte and for transmission of the parasite to the mosquito (Billker et. al., 2004). PfCDPKs 2, 5 and 7 have not been characterized yet (Kugelstadt et. al., 2011).

Calcium dependent protein kinases typically consist of N acylation sites followed by a kinase domain which is separated from a C terminal calmodulin domain by a junctional domain. An activation mechanism has been worked out for PfCDPK4 which may be extended to other PfCDPKs (Fig 2.8). The junctional domain of PfCDPK4 consists of an N terminal auto-inhibitory motif and a C terminal calmodulin domain interacting region. In the absence of calcium, the auto-inhibitory region occupies the catalytic site of the enzyme thus keeping it inactive. Binding of calcium to the calmodulin domain promotes the interaction between the same and the C terminal region of the junctional domain. This interaction may cause a physical constraint on the association between the catalytic site and the auto-inhibitory region thus freeing the catalytic site and rendering the kinase active (Ranjan et. al., 2009).

Fig 2.8: A. Schematic diagram illustrating Calcium Dependent Protein Kinase domain architecture as exemplified by PfCDPK4. B. A model for calcium-mediated regulation of PfCDPK4 by its JD and CLD (Ranjan et. al., 2009).
PfCDPK1 is an essential enzyme (Kato et al., 2008) and its expression starts about 36 hours post invasion. The protein is localized around the periphery of mature merozoites as well as in the membrane surrounding the parasite food vacuole (Green et al., 2008). The enzyme can phosphorylate PfGAP45 and PfMTIP in vitro (Green et al., 2008; Kato et al., 2008; Winter et al., 2010). PfRKIP has also been demonstrated to be an in vitro substrate of PfCDPK1. Further it has been proposed to be a regulator of PfCDPK1 activity, since phosphorylation of PfRKIP increases PfCDPK1 auto phosphorylation, while decreasing its ability to phosphorylate downstream substrates in vitro (Kugelstadt et al., 2007). PfCDPK1 is targeted to the membrane (Green et al., 2008) which is determined by a triple motif including dual acyl modification sites (a myristoylation and a palmitoylation) as well as a cluster of basic amino acids (Moskes et al., 2004). It has been implicated in parasite development during schizogony (Kato et al., 2008) and merozoite invasion (Green et al., 2008) using pharmacological compounds.

PfPKB is a serine threonine protein kinase and has been classified with the AGC family of kinases (Ward et al., 2004). PfPKB is a 446 amino acid long protein which shares 71% homology with the kinase domain of human protein kinase B (Kumar et al., 2004). The domain architecture of PKB from higher eukaryotes is organized into an N terminal Pleckstrin homology (PH) domain, a C terminal hydrophobic motif and an intervening catalytic domain (Song et al., 2005). PfPKB is distinct from mammalian PKB since the N terminal PH domain is replaced by a regulatory region (Kumar et al., 2004; Vaid et al., 2006). Activation of PKB in mammalian systems is dependent on events that are mediated by PI3-Kinase (James et al., 1996). PI-(3,4,5)-P3 and PI-(4,5)-P2 that are generated by the activity of PI-3 kinase recruit inactive cytosolic PKB to the membrane, bringing it into approximation with PDK1 (Andjelkovic et al., 1997). Once in the vicinity of PDK1, PKB undergoes phosphorylation at two residues, T308 in the kinase T-loop and S473 in the C terminal hydrophobic motif (Alessi et al., 1996). Phosphorylation at T308 is mediated by PDK1 (Alessi et al., 1997), whereas the secondary phosphorylation is carried out by the mammalian target of rapamycin (mTOR) and its associated protein rictor (Sarbassov et al., 2005). Following activation PKB acts on various downstream targets including anti and pro apoptotic proteins as well as enzymes involved in glycogen synthesis (Hanada et al., 2004).
PfPKB is catalytically activated by phosphorylation at serine 271 which is complementary to threonine 308 of PKB from higher eukaryotes (Kumar et. al., 2004). Maximal activation of the enzyme however requires an additional phosphorylation at serine 474 in its C terminal hydrophobic motif. The N terminal region of PfPKB is regulatory in nature and is capable of physically interacting with the catalytic domain and maintaining it in an inactive state. A 21 amino acid calmodulin binding domain (CBD) was identified in the NTR of PfPKB. Interaction of Ca\textsuperscript{2+}/CaM complex with the CBD frees the catalytic domain allowing it to undergo autophosphorylation resulting in its activation (Vaid et. al., 2006) (Fig. 2.9).

![Diagram of PfPKB domain architecture and activation mechanism.](image)

**Fig 2.9:** A. Schematic representation of PfPKB domain architecture. B. Activation mechanism of PfPKB by Ca\textsuperscript{2+}/CaM binding to CBD followed by autophosphorylation of Ser-271 in the activation loop (Vaid et. al., 2006).

PfPKB is expressed in schizonts and merozoites and localizes at the apical tip of merozoites (Kumar et. al., 2004). The enzyme plays a pivotal role in erythrocyte invasion which has been demonstrated by the use of two strategies: A443654, an ATP competitive compound, inhibits PfPKB by preventing the binding of ATP to its catalytic domain and blocks
Chapter Two: Review of Literature

erthocyte invasion by merozoites. Similar results were obtained by the use of peptide inhibitors that span the region of the CBD that interacts with the substrate binding site. Based on these and other findings it has been inferred that the Ca²⁺/CaM – PfPKB pathway may be involved in red blood cell invasion (Kumar et. al., 2004; Vaid et. al., 2008).