Review of Literature
1.0 Introduction:

Malaria is one of the important and most widespread parasitic diseases. Protozoan parasites of the genus, *Plasmodium* are the causative agents for malaria. Evidence of existence of malaria dates to 5\textsuperscript{th} century B.C., when Hippocrates described enlarged spleens, and tertian and quartan fevers in his writings. Thereafter, similar symptoms were described in literature of countries like India, China and Greece. Around 200 B.C, the medical literature of Romans described symptoms of malaria and open marshes as source of malaria. This gave rise to the Italian word *mal'aria* meaning bad air to describe the cause of sickness. It was only in 1880 that Charles Alphonse Laveran, a French army doctor, showed parasites in the blood smear of a patient suffering from intermittent fever. Six years after this discovery, Camillo Golgi using thin smears of fresh blood from infected patients showed multiple fission of the parasite. He also showed that beginning of fever in malaria coincided with the rupture of erythrocytes and liberation of parasites. In 1891, Dimitri Romanoswsky using heat fixed thin blood films and a combination of eosin and methylene blue stained the nucleus and cytoplasm of the parasite. Around the same time, Ronald Ross inspired by finding of Patrick Manson (1878) that mosquitoes were able to suck up juvenile stages (microfilariae) from the blood of patients suffering from elephantiasis, started working on transmission of malaria. In 1897, Ronald Ross while working in Indian Medical Service showed that parasite is transmitted through the bite of an infected *Anopheles* mosquito. Thereafter, Giovanni Grassi, an Italian scientist, described the development of three types of malaria parasites *Plasmodium falciparum*, *P. vivax* and *P. malariae* in *Anopheles* mosquito.

Currently, it is estimated that 350-500 million clinical malaria cases occur annually (Fig.1.1), with approximately 40% of the world’s
population mostly living in the world's poorest countries in Africa, Asia and Latin America being at risk of malaria. Most of these are caused by infection with *P. falciparum* and *P. vivax* (WHO report 2005). *P. falciparum* malaria is responsible for more than one million deaths per year. Approximately eighty percent of malaria deaths occur in Africa, and majority of deaths are among young children.

![Global Malaria situation](Global malaria situation, WHO report 2005).

Efforts to control malaria have been based on two strategies. Malaria could be prevented either by control of *Anopheles* mosquito vector through removal of breeding sites, use of insecticides and bed nets or by the administration of antimalarial drugs to humans. Antimalarial drugs like chloroquine, sulfadoxine-pyrimethamine have been commonly used medications for treatment of malaria. However, the extensive use of antimalarial drugs over the past few years has led to a tremendous
selection pressure on human malaria parasites to evolve mechanisms of resistance. *P. falciparum* has become resistant to chloroquine in most malaria-affected areas (Fig.1.2). Sulfadoxine-pyrimethamine has become particularly effective in areas such as Africa where chloroquine was widespread (Wernsdorfer and Kouznetsov, 1980; Peters, 1985). Recently, *Plasmodium* has developed resistance to sulfadoxine-pyrimethamine and has spread rapidly in different parts of malaria endemic regions (Fig.1.2). However, this drug is still used extensively in Africa for treatment of malaria (Landgraf et al., 1994).

![Figure 1.2. Spread of drug resistance in malaria endemic regions (WHO report 2005).](image)

Mefloquine resistance is confined to only those regions where it has been used widely like Thailand, Cambodia and Vietnam (Hastings et al., 2000). The epidemiology of resistance of *P. vivax* has not been studied in detail. Chloroquine resistant *P. vivax* has recently been reported in Papua New Guinea (Schuurkamp et al., 1992) and Myanmar (MARLAR et al., 1995). *Plasmodium sp.* have developed resistance to most antimalarial drugs,
with the exception of artemisinins. Artemisinin, derived from Chinese plant, has been widely used for treatment of multidrug resistant falciparum malaria (WHO 2001). Furthermore, 4-aminoquinoline analogs of chloroquine have been found to be effective against chloroquine-resistant and chloroquine-sensitive *P. falciparum* parasites (De et al., 1996; Ridley et al., 1996). However these derivatives still need to be tested under field conditions for their use as antimalarial drugs.

Epidemiological studies performed in disease endemic areas have suggested that infectious diseases remain a major selective force for evolution of humans. Similarly in case of malaria, *Plasmodium* and humans have coevolved with each other. This phenomenon was first observed in case of African population possessing sickle cell erythrocytes (Hb-S) instead of normal erythrocytes (Hb-A). Hb-S cells were resistant to malaria under conditions of low oxygen tension (Pasvol, 1980). Similarly hemoglobin C that carries a glutamate-to-lysine mutation in the β-globin chain protects West-African children against severe *P. falciparum* malaria. Recently, Fairhurst et al., have shown that parasitized CC and AC erythrocytes show reduced adhesion to endothelial monolayers expressing CD36 and ICAM-1. Moreover, parasitized CC and AC erythrocytes showed impaired rosetting interactions with non-parasitized erythrocytes, and reduced agglutination in the presence of pooled sera from malaria immune adults. Altered adherence properties of Hb-C erythrocytes relative to AA erythrocytes was linked to the reduced levels of PfEMP1 (*P. falciparum* erythrocyte membrane protein-1) or to its altered distribution in knobs. Therefore, Hb-C might protect against malaria by reducing PfEMP1 mediated adherence of parasitized erythrocytes and thereby reducing sequestration in the microvasculature (Fairhurst et al., 2005). Also, erythrocytes from humans with Melanesian ovalocytosis were resistant to invasion by *P. falciparum*. Melanesian
ovalocytosis phenotype prevalent in Papua New Guinea is caused by 27bp deletion in Band 3 present on erythrocyte surface (Jarolim et al., 1991). This mutation leads to defect in anion transport activity of Band 3. The changed transport properties of red cells might have a role in the reduced parasitemia of ovalocytic individuals (Schofield et al., 1992). Epidemiological evidence also suggests that they might also be resistant to *P. vivax* and *P. malariae* (Hadley T and Kidson C, 1983).

Furthermore, it has also been observed that certain blood group antigens could also be used as receptors by parasites for invasion into erythrocytes. Natural variations in blood group antigens present on erythrocyte surface have rendered malaria ineffective in some populations. Miller et al observed that Duffy blood group negative human erythrocytes (FyFy genotype) predominant in West Africans and African-Americans were completely resistant to infection by *P. vivax* (Miller et al., 1976). This suggests that determinants present on red cell membrane may modulate the rate of invasion by the parasite. Similarly in case of *P. falciparum*, En(a-) cells deficient in glycophorin A and S-s-U- cells deficient in glycophorin B showed resistance to invasion by some strains of *P. falciparum* merozoites (Facer, 1983). Also, Tn and wrb- cells with alterations in glycophorin A or B were resistant to invasion by some strains of *P. falciparum* (Pasvol et al., 1982).

### 1.1 Life cycle of *Plasmodium*:

The life cycle of malaria parasite is complex, with asexual reproduction taking place in mammalian host and sexual reproduction in the *Anopheles* mosquito vectors. *Plasmodium* infection in humans is initiated by the inoculation of sporozoites in the skin of the host by an *Anopheles* mosquito. Sporozoites present in the salivary ducts are injected through the bite of an infected host, usually not exceeding 10-20 in numbers.
After circulation in the blood stream, sporozoites invade hepatocytes and differentiate into exoerythrocytic schizonts. Following development over 5-15 days depending upon *Plasmodium* species, an infected hepatocyte can release approximately 40000-50000 merozoites into the bloodstream. These merozoites then immediately invade erythrocytes to initiate the erythrocytic stage of the life cycle. Inside the erythrocyte, parasites develop within a membrane-bound parasitophorous vacuole and form trophozoites. Trophozoites develop into schizonts in which parasite undergoes multiple nuclear divisions to produce next generation of merozoites. Upon maturation of the schizont, the infected erythrocyte ruptures, liberating merozoites that rapidly invade fresh erythrocytes.

However, this asexual stage parasite cannot be transmitted from one human host to another. During blood stage replication, a small proportion of the parasites differentiate into gametocytes. The mosquito ingests these gametocytes during the course of biting a human host. Female and male gametocytes undergo gametogenesis in mosquito midgut to form female and male gametes. Fertilization of male and female gametes results in formation of zygotes, which then transform into motile ookinetes and cross the midgut epithelium between 24 to 48 hours post-infection. After reaching the basal side of the midgut epithelium, the parasites form protected capsules, called oocysts. During the next 10 days, a meiotic cycle followed by several rounds of mitosis produces thousand of haploid sporozoites within each oocyst. Upon maturation, which takes place 14-16 days post infection, sporozoites are released into the hemocoel. Sporozoites then migrate and invade salivary glands. The parasite cycle in the mosquito is completed when the mosquito injects infective sporozoites into the human host again (Fig 1.3).
Figure 1.3. Schematic diagram for life cycle of malaria parasite. Malaria parasite has a complex life cycle where asexual reproduction takes place in mammalian host (a) and sexual reproduction in mosquito (b) (White, 2004).

1.2 Host-parasite interactions involved during the life cycle of the parasite:

1.2.1 Sexual stage of the parasite:

*Plasmodium* completes its life cycle in two completely different hosts. Host parasite interactions are of major importance for the parasite as survival depends on invasion of host cells and survival within host cells. Invasion requires interaction of host cell receptors by parasite ligands. Parasites gain entry into host cells and thus defend against host immune responses. For survival within cell, in addition to using signaling machinery of host, parasite also uses metabolism of host cell for nutrients. Thus, a detailed understanding of host-parasite interactions
would help in designing novel strategies to interrupt the parasite life cycle and control malaria. Recently, studies have been performed to understand the differentiation of gametocytes into gametes inside the mosquito midgut. When the blood meal enters the mosquito midgut, both female and male gametocytes generate functional gametes. Male gametogenesis involves a process termed as exflagellation. Exflagellation refers to the extrusion of eight threadlike gametes from male gametocytes. This process enables male gametocytes to emerge from an adherent clump of erythrocytes to fertilize female gametes. Xanthurenic acid (XA) has been identified as the natural chemical signal produced by mosquitoes that induce exflagellation in the mosquito midgut (Bilker et al., 1998; Garcia et al., 1998). Also, Bilker et al., (2004) using a genetic approach have clearly shown that Ca\(^{2+}\) released from intracellular stores is involved in the induction of exflagellation. This calcium signal is triggered by XA, and is transduced by a member of protein kinase family. A novel calcium dependent protein kinase, CDPK4 was found to be the intracellular transducer of the Ca\(^{2+}\) signal (Bilker et al., 2003).

However, not all the *Anopheles* species of mosquitoes are permissive for *Plasmodium* parasites. Only a limited number of *Plasmodium-Anopheles* combinations allow transmission of malaria. Further within species, mosquitoes display genetic variation in their susceptibility to parasites. Refractoriness is manifested by multiple mechanisms including melanotic encapsulation of ookinetes (Collins et al., 1986). Thioesterase containing protein-1 (TEP-1) from mosquito *A. gambiae* has been shown to bind and mediate killing of ookinetes of rodent malaria parasite *P. berghei* (Levashina et al., 2001). TEP-1 binding and lysis occurs in both susceptible (S) and refractory (R) strain of mosquito as a host immune response against parasite. However in S mosquitoes, 20% of the parasites survive this response while in R mosquitoes, TEP-1 clears
almost all ookinetes crossing midgut epithelium. Furthermore, in R mosquitoes, lysis is also accompanied by melanotic encapsulation of ookinetes. Blandin et al., also showed that dsRNA knockdown of TEP-1 resulted in increase of parasite survival in S mosquitoes. TEP-1 silencing in R mosquitoes not only led to increase in parasite numbers but also completely abolished melanization of ookinetes (Blandin et al., 2004). Thus, further studies on genetics of natural population could help in understanding whether the distribution of TEP-1 alleles correlates with mosquito refractoriness in endemic regions.

1.2.2 Asexual stage of the parasite:
Following injection into the human host, parasite needs to invade hepatocytes and erythrocytes, which requires specific receptor-ligand interactions.

1.2.2.1 Hepatocyte invasion by sporozoites:
Malaria infection in humans is initiated when *Plasmodium* sporozoites are injected into a host during the bite of an infected mosquito. Sporozoite is a banana shaped cell ranging from 9-16.5μm in length and 0.4μm to 2.7μm in width, depending upon species (Sinden, 1978). It is covered by pellicle consisting of an outer sporozoite membrane and two inner sporozoite membranes (Meszoely et al., 1989). The nucleus surrounded by a nuclear envelope, is located centrally in the sporozoite. One or two mitochondria remain associated with the nucleus. Mitochondria of sporozoites possess cristae, which are absent in the mitochondria of the asexual stage parasite. These mitochondrial cristae are likely to be associated with the presence of Krebs cycle and oxidative metabolism within sporozoites (Mack and Vanderberg, 1978). The anterior half of the sporozoite consists of two classes of electron dense tubules called rhoptries and micronemes. Rhoptries are paired elongate
structures while micronemes are smaller and appear more convoluted than rhoptries. Circumsporozoite protein (CS) is released from micronemes of mature salivary gland sporozoites and translocates along the surface of sporozoite (Stewart and Vanderberg, 1991). A monoclonal antibody against CS protein of \textit{P. berghei} inhibited motility and thus invasion of sporozoites into HepG2 cells (Stewart et al., 1986). This suggests that CS expressed on surface of sporozoites helps in gliding motility of sporozoites for invasion into hepatocytes. CS protein consists of two conserved motifs: Region I and Region II (Fig. 1.4A). Tewari et al., replaced the endogenous CS gene of \textit{P. berghei} with that of \textit{P. falciparum} CS and also with CS lacking either region I or II. Region I deficient sporozoites showed no impairment of motility or infectivity in either mosquito or vertebrate host. However, disruption of region II completely abolished gliding motility of sporozoites and thus invasion of mosquito salivary glands and hepatocytes (Tewari et al., 2002). This further confirms the role of CS protein in gliding motility of sporozoites. Furthermore, deletion of CS gene in \textit{P. berghei} resulted in parasites, which were unable to form sporozoites within oocysts, suggesting a structural role for CS (Menard et al., 1997). CS protein besides being involved in morphogenesis and gliding motility also mediates hepatocyte recognition and entry by sporozoites. The specific interaction between the CS protein and heparin sulfate protein glycoconjugates expressed on the basolateral surface of hepatocytes (Cerami et al., 1992; Frevert et al., 1993, 1996) and the observation that intravenously injected CS protein is selectively targeted to the liver (Cerami et al., 1994; Sinnis et al., 1994), have suggested an important role of this sporozoite surface protein in sporozoite recognition of hepatocytes. Similarly, another protein, Thrombospondin Related Adhesive Protein (TRAP) located in micronemes and on the surface of salivary gland sporozoites is required for gliding motility of the sporozoite. \textit{P. berghei} parasite disrupted for TRAP produced sporozoites, which could neither glide nor invade host
cells (Sultan et al., 1997). TRAP consists of motifs or domains, which are important for cell-cell, cell-matrix and matrix-matrix interactions. Presence of A-domain and RGD motif in TRAP (Fig.1.4B) allows this protein to mimic a variety of host molecules such as thrombospondin, VonWillebrand factor, and integrins and to bind to their natural receptors. Recombinant PfTRAP constructs expressed in *E. coli* bound sulfogalactosylcerebroside (sulfatides), a human hepatocyte-derived cell line (HepG2) (Muller et al., 1993), and the basolateral cell membrane of human hepatocytes in the space of disse (Robson et al., 1995). Thus, TRAP and CS are both involved in gliding motility and recognition of hepatocytes, suggesting that TRAP and CS are functionally coupled with each other for successful invasion of hepatocyte by sporozoites (Tewari et al., 2002).

**Figure 1.4. Schematic representation of sporozoite surface proteins.**

A. *Circumsporozoite surface protein* (CS) has several conserved features: a central repeat region and two conserved regions region I and II. The first 20 residues of CS have the features of eukaryotic signal sequence and the C-terminal region contains a canonical GPI-anchor addition site. B. *Thrombospondin Related Adhesive Protein* (TRAP) contains adhesive domains such as A-domain, TRM and RGD motif. In addition, TRAP possesses a proline rich region, varying in length and number of repeats in different *Plasmodium* species and a transmembrane region and a highly conserved cytoplasmic tail.
Sporozoites enter hepatocytes and are transformed into exoerythrocytic schizonts over a period of 50hrs post inoculation. Meis et al., for the first time studied the natural transformation of intrahepatocytic sporozoites into exoerythrocytic forms in vivo (Meis et al., 1983). Recently, Frevert et al., monitored passage of red or green fluorescent sporozoites in vivo using intravital microscopy. Digital recordings showed that sporozoites entering a liver lobule abruptly adhere to the sinusoidal cell layer. They glide along the sinusoid, with or against the blood stream, to a Kupffer cell, and penetrating through a Kupffer cell traverse across the space of Disse. Once inside the liver parenchyma, sporozoites move rapidly for many minutes, traversing several hepatocytes and finally invading a hepatocyte with a parasitophorous vacuole (Frevert et al., 2005; Mota et al., 2001). This transmigration of sporozoites into hepatocytes makes sporozoites competent for infection, by activating the secretion of products that are involved in the formation of vacuole (Mota et al., 2002). However, breaching of plasma membranes leads to wounding of hepatocytes. Hepatocyte wounding by sporozoite migration induces the secretion of hepatocyte growth factor (HGF), which renders hepatocytes susceptible to infection. Infection depends on activation of the HGF receptor, MET. The malaria parasite uses MET as a mediator of signals that make the host cell susceptible to infection. HGF/MET signaling induces rearrangements of the host-cell actin cytoskeleton that are required for the early development of the parasites within hepatocytes (Carrolo et al., 2003).

After establishing a successful infection in the hepatocyte, sporozoites transform into exoerythrocytic schizonts. Using expression profiling, Matuschewski et al., and Kaiser et al., identified genes specifically expressed in preerythrocytic stages (Matuschewski et al., 2002, Kaiser et al., 2004). uis3 and uis4 (up-regulated in infective sporozoite stage 3 and
4) are two genes, which are expressed exclusively in infective sporozoites and developing liver stages. uis3 has been shown to be essential for liver stage development of sporozoites. uis3 deficient sporozoites infect hepatocytes but are unable to establish blood stage infections in vitro. Immunization with uis3 deficient sporozoites confers complete protection against infectious sporozoite challenge in a rodent malaria model (Mueller et al., 2005). Similar results were obtained with uis4-deficient sporozoites (Mueller et al., 2005). These two observations support the fact that genetically attenuated whole organism malaria vaccine could be feasible for prevention of malaria.

1.2.2.2 Erythrocyte invasion:

All the clinical symptoms and pathogenic manifestations associated with malaria are caused by the erythrocytic phase of the Plasmodium life cycle. This phase is initiated when thousands of invasive merozoites are released into blood stream from mature schizonts infecting hepatocytes. The merozoites invade erythrocytes, develop into trophozoites and multiply, forming schizonts. After rupture of these erythrocytes, merozoites are released from erythrocytes. These merozoites then further invade fresh erythrocytes.

Plasmodium merozoites are small, ovoid cells that are 1.5-2μm long and 1.0-2.0μm wide. Merozoites are normally broader at the posterior end than at the anterior apical pole. Plasmodium merozoites possess the basic machinery of a eukaryotic cell like nucleus, chromosomes, mitochondria, endoplasmic reticulum, Golgi network and ribosomes. Merozoites have several organelles and structural features which are specialized for efficient invasion into erythrocytes. The differentiation of merozoite is marked by the presence of apical organelle complex. The apical end of the merozoite has a truncated, cone shaped projection demarcated by the polar rings. This triplet of polar rings is composed of
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cytoskeleton like matrix (Bannister and Mitchell, 1995; Mitchell and Bannister, 1988). Two electron dense rhoptries and several micronemes are present in the apical complex. The ducts from these organelles converge onto the apical prominence. Several round, dense granules lie free in the apical cytoplasm. The nucleus lies within the posterior end of the merozoite. A mitochondrion is observed at the central portion of the merozoite (Fig. 1.5).

Figure 1.5. Electron micrograph of a merozoite. The merozoite shows an apical end Rhoptries (R), Micronemes (M) and organelles like Nucleus (N), Mitochondria (MT). The surface is covered with a surface coat (SC). (Dr. Peter David United Immunlogie Moleculaire des Parasites I.P.)

The plasma membrane of merozoites is much like that of eukaryotic cells, including the presence of numerous intramembraneous particles (IMP) (Mitchell and Bannister, 1988). Overlying the merozoite plasma membrane is a structured surface coat. In P. knowlesi, the entire surface of merozoite consists of clusters of fibrils that are closely spaced in a
reticulate pattern (Bannister et al., 1986). Each cluster of fibrils contains 5-10 parallel filaments that are 2-3nm thick and 18-22nm long. Thinner filaments are fewer in number and are approximately 40nm long. Thinner filaments either extend upward or bend parallel to the surface. In addition, between the tufts of filamentous bundles amorphous proteinaceous covering is present. Similar merozoite surface coat features have been observed for *P. falciparum* (Langreth et al., 1978), *P. vivax* (Galinski and Barnwell, 1996).

Invasion of erythrocytes by *Plasmodium* merozoites is a complex multistep process. The initial contact between the merozoite and erythrocyte occurs at any point on the merozoite surface (Dvorak et al., 1975). At this stage, merozoite can detach and can readhere to another erythrocyte. After initial attachment, reorientation of merozoite takes place to bring its apical end in contact with the erythrocyte surface. The rhoptries and micronemes present at the apical end release their contents to make an indentation on the erythrocyte surface, which leads to the formation of a tight, irreversible junction between apical end of merozoite and erythrocyte surface (Aikawa et al., 1978; Bannister et al., 1986; Bannister and Mitchell, 1989; Stewart et al., 1986). As the merozoite enters the invagination, the junction, which is in form of a circumferential zone of attachment between the erythrocyte and merozoite, moves around the merozoite. When entry is completed, the junction closes behind the parasite and merozoite finds itself inside a vacuolar membrane, known as parasitophorous vacuolar membrane (PVM) (Aikawa et al., 1978) (Fig 1.6).
Figure 1.6. Schematic diagram showing different events associated with merozoite invasion. A. Attachment B. apical reorientation C. junction formation and beginning of rhoptry discharge D and E. penetration of merozoite past the tight junction into a forming parasitophorous vacuole F and G pinching off the junction and resealing of the red blood cell membrane. The membrane surrounding merozoite is termed as parasitophorous vacuolar membrane (Cowman et al., 2000).

Thus, the process of invasion begins after merozoite recognizes receptors on the erythrocyte surface and forms an irreversible junction. It has been observed that the susceptibility of human erythrocytes to invasion by *P. falciparum* could be decreased by neuraminidase or trypsin treatment. Neuraminidase removes sialic acid residues while trypsin cleaves glycophorin A and C from the erythrocyte surface. This observation supports the role of erythrocyte sialoglycoproteins in invasion. Also, monosaccharides, N-acetylglucosamine, N-acetyl galactosamine and N-acetyleneuraminic acid could specifically block parasite invasion *in vitro*, indicating that the parasites bind to red cells in a lectin-like fashion.
(Jungery et al., 1985, Bharara et al., 2004). This suggests that *P. falciparum* merozoites recognize and interact with the sugar domains of glycophorin molecules for successful invasion into erythrocytes. It has been observed that not all strains of *P. falciparum* invade neuraminidase and trypsin treated erythrocytes. This indicates that *P. falciparum* merozoites invade via a limited number of receptors present on erythrocyte surface. However, when *P. falciparum* is continuously cultured in erythrocytes with limited receptors, the parasite switches over its invasion phenotype. For example, Dd2, which is dependent on sialic acid for invasion, maintained on neuraminidase treated erythrocytes, switches to invade neuraminidase treated erythrocytes. Furthermore, the change in phenotype was maintained even after the removal of selection pressure (Dolan et al., 1990).

Merozoite entry into the erythrocyte is not a passive process and requires motility and energy. Since the mature erythrocyte does not have any phagocytic or receptor mediated endocytic activities, the driving force for invasion comes from the parasite. Cytochalasin treatment of merozoites prevents invasion, whereas prior treatment of erythrocytes with cytochalasin does not affect invasion (Miller et al., 1979). Cytochalasins are fungal products that bind actin and prevent actin polymerization. Cytochalasin treated merozoites attach to red cells and form a junction, but movement of the junction over the merozoite surface is arrested. This suggests that an actin based parasite molecular motor functions to drive the movement of the junction. Similarly in *Toxoplasma gondii*, another Apicomplexan parasite like *P. falciparum*, host cell invasion and junctional movement has been shown to be dependent on parasite actin and not host cell actin (Dobrowolski and Sibley, 1996). Actin in the merozoites exists as one-third in the soluble form (G-actin) and the remaining in the filamentous form (F-actin) (Field et al., 1993).
Immunofluorescence studies using actin-specific antibodies showed that F-actin distribution was confined to the poles and periphery of merozoites (Webb et al., 1996). Myosin, which is also known to participate in gliding motility and invasion of *T. gondii* tachyzoites (Dobrowolski and Sibley, 1997) possess a similar localization in *P. falciparum* merozoites (Webb et al., 1996). Tardieux et al., using F-actin affinity chromatography isolated five major actin-binding proteins of molecular masses 75, 70, 48, 40, 34kDa from *P. knowlesi* merozoites. The 70kDa actin binding protein was identified as heat shock protein (HSC70) by using peptide microsequencing and further confirmed by Western blotting and binding of the protein to ATP-Agarose. A doublet of 32/34kDa protein coeluted with HSC70. HSC70/32/34 complex inhibited polymerization of rabbit skeletal muscle actin in vitro. The average length of the actin filaments polymerized in presence of this complex was significantly shorter than in the absence of this complex, consistent with capping activity. The capping or uncapping of actin filaments ends by HSC70/32/34kDa complex during invasion could provide a mechanism for localized actin filament growth and movement of the parasite into the host cell (Tardieux et al., 1998). Furthermore, pull down assays with antibodies against C-terminal domain of MIC2 of *T. gondii* and TRAP of *P. falciparum* coimmunoprecipitated aldolase and actin from respective parasite cell lysates (Jewett and Sibley, 2003). Aldolase is tetrameric and each monomer has the capacity to bind to F-actin, thus enabling it to crosslink and form filament bundles (Wang et al., 1996). Ultrastructural studies in salivary gland sporozoites localized aldolase to the periphery of secretory micronemes containing TRAP. The release of microneme contents in the anterior simultaneously brings aldolase, TRAP and F-actin to the parasite membrane, where motor is engaged (Buscaglia et al., 2003). Thus, binding of aldolase to the C domain of TRAP family of proteins might provide a bridge between the
extracellular domains engaged with host cell receptors and parasite cytoskeleton. However, whether this holds true for blood stage merozoites still needs to be confirmed.

These studies suggest that all molecular events involved in host cell invasion are well coordinated and regulated. A signal induces release of binding proteins from micronemes at the appropriate time point and a trigger or switch may activate an actin myosin motor to move the tight junction. Ward et al., studied the effect of staurosporine, an inhibitor of Protein kinase C, which is a serine/threonine kinase, on invasion of *P. knowlesi* merozoites. Similar to cytochalasin treatment, staurosporine did not prevent initial attachment and junction formation but inhibited invasion. Based on the fact that staurosporine affects the phosphorylation of actin and actin motor organization and effect is similar to cytochalasin treatment, role of Protein kinase C in junctional movement was proposed (Ward et al., 1994). In other organisms, calcium plays a central role in mediating some signal transduction pathways. Ca\(^{2+}\) has been shown to be indispensable for the normal growth of *P. falciparum*. EGTA, a calcium chelator when added after 20-26hrs of invasion leads to morphologically abnormal parasites, arrested in the mature trophozoite stage of the cycle (Wasserman et al., 1982). Addition of EGTA after 24-30hrs of invasion did not affect the development of schizonts and release of merozoites. However, reinvasion of fresh erythrocytes by these merozoites was impeded. This effect was overcome by addition of excess Ca\(^{2+}\) and not Mg\(^{2+}\), suggesting that Ca\(^{2+}\) plays a crucial role in mediating invasion (Wasserman et al., 1982). Similarly, chelation of Ca\(^{2+}\) leads to inhibition of invasion of *P. knowlesi* free merozoites (Johnson et al., 1981). Some studies have suggested intracellular Ca\(^{2+}\) and modulators such as calmodulin are involved in *P. falciparum* merozoite invasion (Matsumoto et al., 1987; Wasserman and
Chaparro, 1996; Wasserman et al., 1990). Finally, studies from McCallum Deighton and Holder concluded that requirement of Ca\(^{2+}\) for *P. falciparum* merozoite invasion is extracellular and not intracellular (McCallum-Deighton and Holder, 1992). However, more specific inhibitors of intracellular Ca\(^{2+}\) flux such as BAPTA/AM and mediators of signal transduction might reveal a role for intracellular calcium mediated signal transduction.

**1.3 Development of the parasite inside the erythrocytes:**

**1.3.1 Ring Stage:**

When extracellular merozoites invade erythrocytes, merozoite becomes round in shape due to rapid degradation of the inner membrane complex. Apical organelles of the merozoite such as polar rings, micronemes and rhoptries also break down at this stage. Giemsa stained smears viewed under light microscopy show the parasite as a signet ring structure (Fig.1.7). This thin discoidal flat ring form with a thick rim of cytoplasm houses the major organelles such as nucleus, mitochondria, plastid, most of the ribosomes and endoplasmic reticulum. The ring enters the trophozoite stage 18-26 hrs post invasion (Bannister et al., 2000).

![Ring](image)

**Figure 1.7. Ring stage of *P. falciparum.*** Giemsa stained smears made at ring stage of the parasite shows it as a thin discoidal flat ring with a thick rim of cytoplasm.
1.3.2 Trophozoite stage:
As the trophozoite grows, it becomes irregular in shape because of the absence of the rigid pellicular complex. The nucleus of the trophozoite contains finely granular and filamentous chromatin material. The presence of abundant euchromatin in the nucleus of the trophozoite suggests that the nucleus is transcriptionally active.

The trophozoite stage is marked by the digestion of host cell cytoplasm and digestion of hemoglobin. The trophozoite ingests host cell cytoplasm through a circular structure, called cytostome, which is located along the plasma membrane (Aikawa et al., 1966). The host cell cytoplasm enters into the cytosomal cavity, and a bulge of host cell is pinched off at the cytosomal orifice together with the cytosomal wall, forming a food vacuole within the parasite cytoplasm. The digestion of hemoglobin by Plasmodium takes place within the food vacuoles. During hemoglobin degradation, most of the liberated heme is detoxified by polymerization into hemozoin, which is stored within the food vacuoles (Dorn et al., 1995; Egan et al., 1994) (Fig.1.8).

![Figure 1.8. Trophozoite stage of P. falciparum.](image)

Figure 1.8. Trophozoite stage of P. falciparum. The trophozoite appears as a compact rounded structure with a cytoplasm (blue) and hemozoin pigment.
1.3.3 Schizont stage:
During schizony, nuclear divisions and cytoplasmic organelle development occur. As nuclear divisions progress, the nucleus becomes dumbbell shaped. During nuclear division, the nuclear membrane remains intact, except at the centriolar plaque (Aikawa and Beaudoin, 1968). Also, various merozoite organelles, which disappeared during ring stage, reappear at this stage. Repetitive nuclear division leads to the formation 10-30 daughter merozoites (Fig. 1.9). This stage prevails for 8-10 hrs.

![Figure 1.9. Schizont stage of *P. falciparum*.](image)

Schizonts have 6-12 merozoites usually arranged in a rosette and hemozoin pigment, which is seen at early and late schizont stage.

Daughter merozoites are enclosed within the parasitophorous vacuolar membrane and erythrocyte membrane. Rupture of parasitophorous vacuole membrane and erythrocyte membrane releases daughter cells. Studies with *P. falciparum* lines expressing green fluorescent protein targeted to the parasitophorous vacuole and host erythrocyte cytosol showed that merozoite release involves a primary rupture of the parasitophorous vacuole membrane followed by secondary rupture of the erythrocyte plasma membrane (Wickham et al., 2003). A role of cysteine protease falcipain-1 in erythrocyte invasion was recently suggested.
(Greenbaum et al., 2002). However, disruption of falcipain-1 did not alter parasite development (Sijwali et al., 2004), but surprisingly led to reduction in oocyst production in mosquito (Eksi et al., 2004). Serine repeat antigen (SERA-5), a serine protease localizes to the parasitophorous vacuole in mature parasites. Furthermore, inhibition of processing of SERA-5 using a protease inhibitor leupeptin, affects rupture of mature schizonts infected erythrocyte, suggesting that SERA-5 might be involved in merozoite release (Delplace et al., 1988).

1.4 Parasite molecules involved in erythrocyte invasion:
Invasion of erythrocytes by *Plasmodium* is a complex multistep process. It includes attachment of merozoite, reorientation of merozoite in such a way that its apical end is in contact with erythrocyte surface, junction formation and then entry into parasitophorous vacuole. Recognition of host cell surface receptors by parasite ligands is a crucial step in invasion process. The initial adhesive contact between the merozoite and erythrocyte membrane can take place at any point at the merozoite surface and may involve the merozoite coat filaments (Dvorak et al., 1975). Dvorak et al., also showed that merozoite-erythrocyte contact leads to heavy perturbations in the red cell membrane. This observation suggests that these interactions are quite dynamic in nature. At this stage, merozoite can detach and then can re-adhere to another erythrocyte. Merozoite then reorientes itself such that its apical portion is in contact with the erythrocyte surface. Transmission electron micrographs showing erythrocyte loosely wrapped around merozoites with their fibrillar extensions connected to the erythrocyte surface (Bannister et al., 1986; Mitchell, 1987) suggests that these adhesive interactions might enable merozoites to point its apical pole toward the red cell surface. Once, the apical end is in contact with the erythrocyte surface, a much closer membrane-to-membrane adhesion occurs and an
irreversible tight junction is formed between the two cells (Aikawa et al., 1978; Bannister et al., 1975). Thus, with the formation of tight junction, the merozoite commits itself to invasion into host cell.

The interactions, which take place between merozoites and erythrocytes during the initial contact phase of invasion, are mediated by specific receptors on the erythrocyte membrane and ligands on the merozoite. A number of proteins that are located in the micronemes and rhoptries have been identified and are thought to play a role during the process of red cell invasion (Fig. 1.10).

![Figure 1.10. Schematic representation of a *Plasmodium* merozoite showing localization of different molecules involved in erythrocyte invasion. Parasite molecules involved in erythrocyte invasion localizes either in the apical organelles like rhoptries (Rh), micronemes (Mn) or on the merozoite surface coat. (Cowman et al., 2000).](image_url)
1.4.1 Merozoite Surface proteins:

Merozoite surface proteins include proteins, which are expressed on surface of merozoite, and till date approximately 10 proteins have been identified as merozoite surface proteins, referred to as MSP1-10. Out of these four major proteins have been studied in detail: Merozoite surface protein 1 (MSP-1) (Holder and Freeman, 1984; Miller et al., 1993), MSP-2 (Clark et al., 1989; Smythe et al., 1988), MSP-3 (Mccoll et al., 1994; Oeuvary et al., 1994) and MSP-4 (Marshall et al., 1997). MSP-1 is a large merozoite surface protein with a relative mobility in SDS-PAGE of 185-215kDa. The expression of MSP-1 starts around early schizont stage (2-4 nuclei). MSP-1 then moves to the surface of merozoite at around 6-8 nuclei stage (Margos et al., 2004). MSP-1 undergoes extensive proteolytic processing very late in schizogony (David et al., 1984; Holder and Freeman, 1984). MSP-1 gets cleaved into fragments of sizes 83kDa (N-terminus), 30kDa and 38kDa (central portion) and 42kDa (C-terminus). 42kDa remains bound to the plasma membrane by a GPI anchor (Holder et al., 1987; Stafford et al., 1994). This 42kDa fragment is processed further into 33kDa and 19kDa fragments during invasion (Blackman et al., 1991). The 19kDa portion of MSP-1 that includes two-cysteine rich epidermal growth factor (EGF) like regions (Blackman et al., 1991; Haldar et al., 1985) remain attached to the merozoite surface throughout invasion and is seen within the infected erythrocytes as the internalized merozoites develop into rings (Blackman et al., 1990, 1996; Mcbride and Heidrich, 1987).

The merozoite surface is covered with a 15-20nm thick coat consisting of clusters of filaments. Filaments are 2nm thick and are sensitive to trypsin and papain digestion, indicating a proteinaceous nature of these filaments. Further, they appear at the early schizont stage and could mediate intercellular adhesions at distances of 15-150nm. Based on
facts that MSP-1 and filaments appear at the same time and filaments are proteinaceous in nature, MSP-1 could correspond to the long fibrils that tether merozoite to the erythrocyte or comprise 20nm fibril bundles. Thus, MSP-1 might participate in the initial surface interaction between the merozoite and erythrocyte (Banister et al., 1986). MSP-1 contains conserved sequence blocks with unknown functions. Herrera S et al., demonstrated that recombinant MSP-1 protein 190L, which represents such a block of native MSP-1, binds to inner red blood cell membrane skeleton protein spectrin (Herrera et al., 1993). Also, association of 190L with naturally occurring spectrin deficient red blood cells is drastically reduced. In contrast, Perkins and Rocco showed that the binding capacity of MSP-1 is dependent on terminal sialic acid residues present on erythrocyte membrane glycoproteins (Percins and Rocco, 1988).

Recently, two nonglycosylated exofacial regions of human band 3 in the erythrocyte were identified as a crucial host receptor binding the C-terminal processing product of MSP-1. A major segment of Band 3 receptor (5ABC) bound to MSP142. 5ABC blocked the interaction of MSP42 with erythrocytes in vitro. Recombinant MSP119 also bound to 5ABC as well as red cells. This binding was not affected by neuraminidase treatment but was sensitive to chymotrypsin treatment (Goel et al., 2003). Moreover, two regions of MSP-9 (also known as acidic basic repeat antigen, ABRA) interacted directly with 5ABC during invasion by *P. falciparum* (Li et al., 2004). Two recombinant MSP-9 peptides MSP9/Delta1, MSP9/Delta2 blocked erythrocyte invasion in *in vitro* invasion assays. Li et al., also showed that MSP-9 and MSP142 existed as a stable complex. Thus, the merozoite exploits a specific complex of coligands on its surface to target a single erythrocyte receptor during invasion.

Role of MSP-2, MSP-3, and MSP-4 in erythrocyte invasion is not well characterized. A glycosylphosphatidylinositol (GPI) anchor has been
demonstrated for *P. falciparum* MSP-2 (Gerold et al., 1996; Smythe et al., 1988) and MSP-4 (Marshall et al., 1997). MSP-3 neither possesses a classical hydrophobic transmembrane domain nor a GPI modification to anchor it to the plasma membrane (McColl et al., 1994). Based on the fact that merozoite surface is relatively easy to strip, MSP-3 could be noncovalently harnessed to the surface by electrostatic forces. Coiled coil helices of MSP-3 could entwine and contribute to the plush appearance of the organized fibrillar bundled clusters observed on the merozoite surface. These coiled coil helices could also form homotypic or heterotypic multimeric protein bundles (Cohen and Parry, 1990, 1994; Lupas, 1997), which would be consistent with the 8-20nm bundles of fibrils extending from the surface (Bannister et al., 1986). Whether one or more of these proteins has erythrocyte adhesive properties and contributes to the 18-20nm fibril adhesions between the merozoite and erythrocyte remains to be investigated.

1.4.2 Role of apical organelle proteins in erythrocyte invasion:

Binding of parasite ligands to specific receptors on the erythrocyte surface is an integral part of the invasion process. The molecules, which are involved in invasion, are localized in the apical organelles. Rhoptries, micronemes and dense granules, which form the apical portion of the merozoite, store proteins responsible for red cell invasion.

1.4.2.1 Dense Granules:

These organelles are spheroidal membranous vesicles with densely granular lumen and electron-lucent perimeter. Dense granules are mostly distributed between rhoptries and the nucleus, lying free within the cytoplasm of the apical half of the merozoite. When merozoites enter host cells, dense granules move to the surface of the merozoite, fuse with
the pellicle, and release their contents into the parasitophorous vacuolar space (Bannister et al., 1975). Dense granules are also associated with the formation of channels from the parasitophorous vacuole (Torii et al., 1989). Immunoelectron microscopy performed with antibodies specific for proteins in dense granule labeled specifically dense granules of *P. knowlesi* merozoites. No labeling was observed for rhoptries and micronemes. This suggests that the content of dense granules is different from that of rhoptries and micronemes. Molecules that have been isolated from dense granules include Ring-infected Erythrocyte surface Antigen (RESA) (Aikawa et al., 1990), Ring membrane Antigen (RIMA) and two subtilisin-like proteases (SUB1 and SUB2).

### 1.4.2.1a Ring Membrane Antigen (RIMA):

Immunoelectron microscopic studies localized this protein to the dense granules. RIMA is expressed at late schizont stage and in free merozoites. It is also localized on the membrane of the newly invaded ring just after invasion. Exact function of this protein is unknown (Trager et al., 1992).

### 1.4.2.2b Ring-infected Erythrocyte Surface Antigen (RESA):

RESA is a 155kDa protein, which is released from merozoites and becomes associated with the erythrocyte membrane at the time of invasion. RESA has been shown to be associated with spectrin present on red cell membrane (Foley et al., 1991). Similarly the interaction of RESA with spectrin was observed using steady-state fluorescence polarization, where the thermal denaturation of fluorescein-labeled spectrin was studied in presence of recombinant RESA. The studies showed that RESA partially protected spectrin against heat-induced conformational changes. Furthermore, erythrocytes infected with RESA- laboratory strain (FCR3) were more susceptible to heat induced fragmentation than erythrocytes infected with a RESA+ strain of the parasite (Da Silva et al., 1994). In order to study the function of this
protein in detail, RESA was deleted in *P. falciparum*. Absence of RESA did not influence erythrocyte invasion or membrane rigidity. However, the parasite became susceptible to heat shock. Exposure of parasite for 6hrs at 41 degrees during ring stage resulted in significant inhibition of growth. Thus, RESA might be involved in protection of the infected erythrocyte cytoskeleton during febrile episodes (Silva et al., 2005).

### 1.4.2.2c Proteases:

Two subtilisin-like proteases have been characterized in dense granules that play an important role in erythrocyte invasion. PfSUB1 gets post-translationally processed during secretory transport to dense granules and by its autoproteolytic processing activity converts itself to mature active form. Upon merozoite release, the enzymatically active form (p47) is secreted from the parasite in a soluble form. Immunoelectron microscopic studies with PfSUB1, localizes p47 to dense granules in free merozoites and mature schizonts of *P. falciparum*. The subcellular localization and timing of expression of p47 suggests its role in erythrocyte invasion (Blackman et al., 1998).

Another subtilisin like protease PfSUB2 is expressed at late schizont stage and free merozoites (Barale et al., 1999) as a large putative type-I integral membrane protein and undergoes extensive post-translational modification similar to PfSUB-1. PfSUB2 has been shown to take part in the final processing of MSP-1 during invasion (Hackett et al., 1999; Barale et al., 1999). Gene targeting studies performed with SUB2 in *P. berghei* confirmed that SUB2 is essential for the parasite's survival, as PbSUB2 knockout parasites did not survive.

### 1.4.2.2 Rhoptries:

Differentiation of rhoptries initiates from spheroidal 80-120nm structures during early schizont stage (8 nuclei) and continues till 16
nuclei stage where it is in mature form with tapered apex (Margos et al., 2004). The staining of rhoptries with ethanolic phosphotungstate indicates a proteinaceous composition (Banister et al., 1986). The rhoptries consist of two distinct parts: an electron dense rounded basal bulb and a less dense rhoptry duct that ends just beneath plasma membrane covering the apical prominence (Bannister et al., 2000). In mature rhoptries of *P. falciparum*, different sets of rhoptry proteins are contained in the basal bulb and duct. Crewther et al., showed that rhoptry associated protein (RAP1/2) complex is situated in the bulb (Crewther et al., 1990), while p225 protein and RHOP1-3 complex is located in the duct (Roger et al., 1988; Sam yellowe et al., 1995).

Besides playing a role in host cell invasion, rhoptries help in the formation of parasitophorous vacuole during invasion (Bannister et al., 1989).

**1.4.2.2a Low molecular weight complex (RAP1, 2, 3):**

The low-molecular-weight RAP1, 2 and 3 complex consists of at least two gene products RAP-1 and RAP-2. RAP-1 is synthesized as an 80kDa protein (p80) during early schizont stage, which gets proteolytically processed to 65kDa protein (p65) during late schizont stage (Harnyuttanakorn et al., 1992). Studies performed by *in vitro* invasion assays and monkey immunization trials showed that RAP-1 plays a role in erythrocyte invasion (Howard et al., 1998). In order to study the function of RAP-1, RAP-1 gene was disrupted using gene-targeting technology, producing parasites that express truncated forms of RAP-1. Immunoprecipitaton studies showed that truncated RAP-1 did not form a complex with RAP-2 and RAP-3. Baldi et al., also showed that in RAP-1 knockout parasites, RAP-2 did not traffic to the rhoptries. Instead, RAP-2 was found to be located in the compartments that appear related to the lumen of the endoplasmic reticulum. These results suggest that RAP-1 is
involved in targeting RAP-2 to the rhoptries but is not essential for mediating erythrocyte invasion (Baldi et al., 2000). Similarly, disruption of RAP-3 is not essential for merozoite invasion. This could be because RAP-3 has homology with RAP-2 and thus can complement the loss of function of RAP-3 (Baldi et al., 2002).

1.4.2.2b High molecular weight complex:

*P. falciparum* rhoptry proteins of 140, 130 and 110 kDa were identified by co-immunoprecipitation with monoclonal antibody Mab1B9 (Cooper et al., 1988). Erythrocyte binding studies have demonstrated that this high molecular weight complex binds to inside-out vesicles (IOVs) prepared from human erythrocytes. 140/130/110 kDa complex is also involved in binding of erythrocyte membranes prepared by hypotonic lysis. However, these proteins did not bind to intact human erythrocytes. So, a binding site on the cytoplasmic face of the erythrocyte membrane implies that rhoptry proteins might be translocated across the bilayer membrane during merozoite invasion. The rhoptry protein complex bound equally well to normal erythrocytes as well as erythrocytes treated with neuraminidase, trypsin and chymotrypsin indicating that the binding site is independent of glycophorin and major surface proteins. The rhoptry complex also bound to liposomes containing neutrally, positively, and negatively charged phospholipids. However, liposomes containing phosphatidylethanolamine compete effectively for rhoptry proteins binding to mouse erythrocytes. These studies suggest that the 140/130/110 kDa protein complex might interact directly with sites on the lipid bilayer of the erythrocyte membrane (Sam-Yellowe et al., 1991, 1992).

1.4.2.2c Reticulocyte-binding protein homologues:

*P. vivax* merozoites primarily invade reticulocytes. Two proteins from *P. vivax*, namely *P. vivax* Reticulocyte binding protein 1 and 2 (PvRBP-1 and
PvRBP-2) colocalize at the apical portion of the merozoite and specifically adhere to reticulocytes. PvRBP-1 is a transmembrane anchored disulfide linked protein, and along with PvRBP-2 forms an adhesive protein complex (Galinski M et al., 1992). Members of the Py235 protein family in *P. yoelii* are homologous to PvRBP-1 and PvRBP-2 and have been thought to be involved in defining specificity of red cells for invasion by *P. yoelii* merozoites. Preiser P et al., using micromanipulation, studied transcription of Py235 in single parasites of *P. yoelii*. A single Py235 transcript was detected in uni-nucleated trophozoite, but following nuclear division, additional Py235 transcripts were observed within the maturing schizont. However, each merozoite released by a single schizont expresses a single but distinct transcript. Thus, each of the progeny merozoite from a single schizont could express different ligands of the Py235 family. This type of clonal phenotypic variation provides the parasite a survival advantage in the mammalian host (Preiser et al., 1999). This phenomenon is genetically and functionally distinct from antigenic variation, which is mediated by the *var* multigene family of *P. falciparum*.

Two homologs from *P. falciparum* (PfR2Ha and PfR2Hb) share similar gene structure and aminoacid homology with Py235 rhoptry protein family and PvRBP-2 (Rayner et al., 2000). PfR2Ha and PfR2Hb protein released into the supernatant failed to show binding with erythrocytes and reticulocytes. However, antibodies against pfR2Ha/Hb inhibited merozoite invasion. This observation indicates that these proteins play some role in the process of erythrocyte invasion (Triglia et al., 2001). Targeted gene disruption of PfRH2a and PfRH2b shows that PfRH2b mediates an invasion pathway through a chymotrypsin sensitive, trypsin/neuraminidase-resistant receptor. Also, PfRH2b functions independently of other related proteins (Duraisingh et al., 2003). PfRh3,
another homologue of PvRBP-2 is a pseudogene, which is transcribed but not translated (Taylor et al., 2001). However, proteomic analysis has shown that PfRh3 might be expressed in sporozoites (Florens et al., 2002).

PfNBP1 (*P. falciparum* normocyte binding protein 1) also referred to as PfRh1, homologue of PvRBP-1, mediates invasion through a novel receptor on erythrocyte surface. This receptor is sialic acid dependent and trypsin resistant. Antibodies against PfNBP1 could inhibit invasion of trypsinized erythrocytes. Also, *P. falciparum* strains that express truncated PfNBP1 are unable to invade trypsinized erythrocytes (Rayner et al., 2001). Taylor et al., studied variations in the sequence, transcription, and protein expression of PfRH family of proteins. The apparent redundancy observed in the PfRH family at the level of gene number and variations in transcription and protein level may endow different invasion profiles to *P. falciparum* isolates (Taylor et al., 2002).

**1.4.2.3 Micronemes:**

Micronemes are much smaller than rhoptries. There are up to 40 micronemes per merozoite. Micronemes look like long-necked bottles, about 160nm long and 65nm at their widest diameter. Electron micrograph of a merozoite shows that micronemes bear bristle like filaments, each 3-4nm thick and 25nm long on their external surfaces. Micronemes originate from a single Golgi like cisterna near the nucleus along a band of two or three subpellicular microtubules to the merozoite apex, where they dock to the rhoptry tips (Bannister et al., 2003). Proteins like apical membrane antigen–1 (AMA-1), erythrocyte binding protein family (EBPs), which are involved in interaction with red cell, are located in the micronemes.
1.4.2.3a Apical Membrane Antigen 1:
AMA-1 is a type I integral membrane protein. Expression and post-translational N-terminal proteolytic cleavage of AMA-1 is restricted to the final stages of schizogony, during which the protein is localized within the neck of the rhoptry (Crewther et al., 1990). However Healer et al., using immunoelectronmicroscopy showed that mouse monoclonal antibody against 83kDa labels micronemes rather than rhoptries (Healer et al., 2002). Also, *Toxoplasma gondii* homologue (TgAMA-1) localizes in the micronemes (Donahue et al., 2000; Hehl et al., 2000). In *P.falciparum*, Bannister et al., while studying the structure and development of micronemes confirmed that AMA-1 is present in the micronemes (Bannister et al., 2003).

AMA-1 ectodomain comprises of three-cysteine rich domains, domain I, II and III. Domain I is preceded by a N-terminal prosequence. PfAMA-1 is initially routed to secretory organelles at the apical end of the merozoite where the 83kDa precursor (PfAMA183) is converted to a 66kDa form (PfAMA166). After translocation of PfAMA166 onto the merozoite surface, it is further cleaved between domain II and III or at a membrane proximal site following domain III giving rise to PfAMA144 or PfAMA148 respectively. The cleaved fragments are shed from the parasite surface, leaving the transmembrane and cytoplasmic domain with or without domain III attached (Howell et al., 2001). The crystal structure of three domains of *P. vivax* solved at 1.8 Å shows that domain I and II belong to PAN motif. PAN motif defines a super family of proteins implicated in receptor binding. The presence of PAN motif and invasion inhibitory epitope on domain II, suggests its role in receptor binding during red cell invasion (Pizzaro et al., 2005).

In order to study function of AMA-1 in erythrocyte invasion, domain1/2 of *P. yoelii* were expressed on the surface of COS-7 cells. *P. yoelii*
domains 1/2 mediated adhesion to mouse and rat erythrocytes but not to human erythrocytes, indicating its role in erythrocyte invasion. This binding was sensitive to trypsin and chymotrypsin but not to neuraminidase treatment of erythrocytes (Fraser et al., 2001). Recently, Kato et al., expressed domains I/II/III of *P. falciparum* AMA-1 on the surface of CHO-K1 cells and studied their ability to bind erythrocytes. These domains failed to bind normal human erythrocytes. In contrast, domain III bound to trypsin treated erythrocytes. Furthermore, domain III failed to bind trypsin treated K$x$ (null) (Mcleod erythrocytes) erythrocytes, which lack K$x$ protein. Thus, AMA-1 might mediate invasion through a process that involves exposure or modification on the erythrocyte surface protein, K$x$, by a trypsin like enzyme (Kato et al., 2005). In order to study the role of AMA-1 in erythrocyte invasion, Triglia et al., disrupted AMA-1 in *P. falciparum*. Although, PfAMA-1 could be targeted by homologous recombination, no knock out parasite was obtained (Triglia et al., 2000), implying that AMA-1 is essential for the survival of the parasite. Electron microscopic analysis of *P. knowlesi* merozoites incubated with red cells in the presence of antiAMA-1 antibodies showed that random, initial, long-range (12nm) attachment between red cells and merozoites occurs normally. However, bound merozoites failed to reorient, so that there was no close junctional contact. This suggests that either AMA-1 is directly responsible for reorientation of merozoite or the molecule may initiate the junctional contact, which is then dependent on Duffy binding protein for the completion of invasion process (Mitchell et al., 2004).

1.4.2.3b Erythrocyte binding proteins family (EBPs):
The erythrocyte binding protein family is found in a wide range of *Plasmodium* species. EBPs are defined by the presence of cysteine rich Duffy binding like (DBL) domains (Adams et al., 1992). This family
includes *P. vivax* and *P. knowlesi* Duffy binding proteins. *P. vivax* 135-kDa protein bound to Duffy blood group antigen on erythrocytes. This binding was specifically inhibited by purified Duffy glycoprotein and anti-Fy6 monoclonal antibody against Duffy glycoprotein (Wertheimer et al., 1989; Barnwell et al., 1989). Similarly, *P. knowlesi* Duffy binding protein of 138kDa bound to Duffy glycoproteins of human as well as rhesus erythrocytes (Adams et al., 1990). Duffy blood group antigen is also the receptor for the chemokines interleukin-8 and melanoma growth stimulatory activity (MGSA). Anti-Fy6 monoclonal antibody blocked binding of IL8 and MGSA to Duffy antigen. Also, IL8 and MGSA inhibited invasion of human Duffy positive erythrocytes by *P. knowlesi* (Horuk et al., 1993). This suggests that IL8 and MGSA binds to Duffy antigen on erythrocytes and thus blocks invasion of *P. knowlesi* into Duffy positive erythrocytes. However, these proteins are not involved in the initial attachment and reorientation of merozoites during invasion process. Electron microscopic studies on cytochalasin-treated *P. knowlesi* merozoites showed that merozoite attaches normally to the erythrocytes. Merozoite then reorients itself and forms a junction between apical region and the erythrocytes. Further movement of junction is blocked due to cytochalasin treatment of the merozoite. When cytochalasin treated merozoites were incubated with Duffy negative erythrocytes, merozoites attach but junction formation was not observed. Therefore, the Duffy associated antigen appears to be involved in junction formation, and not initial attachment (Miller et al., 1979) (Fig.1.11). This was further proved by deletion of Duffy binding protein (DBP) from *P. knowlesi*. Deletion of DBP using homologous recombination resulted in complete loss of invasion of human erythrocytes by *P. knowlesi*. Electron microscopy studies with *P. knowlesi* merozoites reveal that DBP knockout parasites were unable to form junction with human erythrocytes (Singh et al., 2005).
Figure 1.11. Electron micrograph of a *P. knowlesi* merozoite showing the attachment between the apical end of a cytochalasin treated erythrocyte and a Duffy positive erythrocyte (A) or Duffy negative erythrocyte (B). C. represents the magnified image of the interaction between a cytochalasin treated merozoite and Duffy negative erythrocyte (Miller et al., 1979).

DBPs from *P. vivax* and *P. knowlesi* share similar structures and sequence. The ORF of DBP is encoded by five exons. Exon 1 encodes a signal sequence. Exon 2 consists of five regions. Region I is highly charged with negative charge predominating. The N-terminal cysteine rich region (region II) has twelve cysteine residues, all of which are conserved in position. The C-terminal cysteine rich region (region VI) includes the final 104 residues of exon 2 and immediately precedes the transmembrane domain. All cysteine residues and 11 of 13-15 aromatic aminoacids residues of region VI are conserved among *P. vivax* and *P. knowlesi*. Region II and region VI are separated by a nonhomologous and hydrophilic amino acid stretch that consists of regions III-V. Exon 3 encodes a transmembrane domain of around 20 aminoacids. Exons 4 and 5 encode cytoplasmic domain of 45 aminoacid residues (Adams et al., 1992) (Fig.1.12).
**Figure 1.12. Structure of genes encoding the erythrocyte binding proteins of *P. vivax*, *P. knowlesi* and *P. falciparum*.** The exon-intron boundaries are conserved for DBL genes. Duffy/EBA-175 protein has a signal sequence (ss) at the N-terminus and a transmembrane segment (tm) with a cytoplasmic domain at the C-terminus. The extracellular is divided into six regions based on sequence homology. Two conserved cysteine rich regions (region II and VI) separated by non-homologous region, region III-V, are found in each protein. *P. falciparum* erythrocyte binding proteins (EBA-175, EBA-181, EBA-140) have a duplication of DBL domains in region II.

In *P. falciparum*, the interactions between a merozoite and erythrocyte are mediated through sialic acid residues on erythrocyte membrane glycoporphins. In order to search for erythrocyte binding antigens that might mediate invasion, *P. falciparum* culture supernatants were incubated with erythrocytes. Four antigens with apparent molecular weight of 175k, 120k, 65k, and 46k from culture supernatants bound specifically to human and Aotus erythrocytes. Out of these four antigens, only 175k bound to human erythrocytes. The 175kDa antigen might be a receptor acting as bridge between erythrocytes and merozoites (Camus and Hadley, 1985). The 175kDa antigen is expressed during schizogony as a 190kDa precursor protein, which is recovered from the cell pellet in schizont-infected erythrocytes. Upon schizont rupture, this 190kDa is
released as 175kDa in the culture supernatants (Orlandi et al., 1990). These observations established the fact that *P. falciparum* invades red cells by means of a parasite receptor, the 175kDa Erythrocyte Binding Antigen (EBA-175). Red cell binding requires N-acetylneuraminic acid (Neu5Ac) on human erythrocytes with glycophorins and this binding was dose-dependent. Soluble Neu5Ac did not inhibit the binding of EBA-175 to erythrocytes, suggesting oligosaccharides are not the sole receptor for mediating this binding. Binding to glycophorin A was competitively inhibited by Neu5Ac (alpha 2-3)-gal and partially by Neu5Ac (alpha 2-6)-gal. Also, monoclonal antibodies against glycophorin A immunoprecipitated EBA-175. Selective cleavage of O-linked tetrasaccharides clustered at amino terminus of glycophorin A significantly reduced binding in inhibition assays. So, Neu5Ac (alpha 2,3) gal determinant on O-linked tetrasaccharides on glycophorin A appears to be required for EBA-175 binding (Orlandi et al., 1992; Klotz et al., 1992).

EBA-175 gene structure is similar to the DBP family of *P. vivax* and *P. knowlesi* except that it possesses two copies of N-terminal cysteine rich region (F1 and F2) (Sim et al., 1992). Regions II and VI contain cysteines and a number of hydrophobic amino acid residues such as tryptophans, phenyalanines, and tyrosines that are conserved in position. Twelve cysteine residues are present in the same position in F1 and F2 as in *P. vivax* and *P. knowlesi*. But, the F1 motif has one extra cysteine and F2 possess two extra cysteines when aligned with region II of *P. vivax* and *P. knowlesi* (Adams et al., 1992). The fact that region II is conserved suggests that this might be conserved for function, namely, binding to erythrocyte receptors. This was further confirmed when different regions of PvDBP and PkDBP were expressed on the surface on COS7 cells and tested for binding to red cells. Erythrocyte binding assays identified
Duffy-binding-like (DBL) domains are also found in the members of the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) family, encoded by *var* genes. The DBL domains of PfEMP-1 bind to a variety of endothelial receptors such as chondroitin sulphate A (CSA), heparin sulphate, ICAM-1, complement receptor-1 and CD31 to mediate cytoadherence. Thus, DBL domains mediate two essential pathogenic mechanisms in malaria, namely erythrocyte invasion and cytoadherence. The minimal binding region for the Duffy antigen lie in the central region of ~170 aminoacid stretch that includes cysteines 5-8 of DBL domains in PvRII and PkαRII (Singh et al., 2003). The receptor binding residues of parasite ligands that bind sialic acid residues on glycophorin A for
invasion as well as mediate adhesion to endothelial receptors like CR-1 and CSA also map to the central regions of DBL domains that are equivalent to the C5-C8 stretch of PvRII and PkaRII (Mayor et al., 2004). Thereafter, site-directed mutagenesis and quantitative binding assays were used to localize binding residues for DARC within this central region of PvRII. Thus binding of DARC, expressed as a recombinant protein in 293 cells, was studied with PvRII (wild type and mutants) expressed in E. coli. The studies showed that mutagenesis of some hydrophobic residues between cysteines 5-8 in PvRII reduced the binding to DARC by 70%, suggesting that hydrophobic interactions play a major role in the binding of PvDBP with DARC. Also, mutagenesis of some positively charged residues with in cysteines 5-8 leads to a decrease in binding of PvRII with DARC. These observations suggest that the binding site has dual characteristics with positively charged and hydrophobic regions. Moreover, Choe et al., showed that sulphated tyrosine 41 is critical for association of PvDBP with DARC (Choe et al., 2005). PvRII has a predicted pI of 8.9, with a number of positively charged residues such as arginine and lysine while N-terminal extracellular region of DARC is negatively charged with a predicted pI of 3.5. Thus based on these facts, it could be possible that positively charged residues in PvRII may play a role in initial recognition of DARC through sulphate of tyrosine 41 and further high affinity interactions and binding specificity is dependent on tyrosine which mediates hydrophobic interactions (Hans et al., 2005). Using similar strategy, an independent study from Vanbuskirk et al., showed that mutations that occurred in discontinuous clusters of conserved residues lead to complete loss of binding (Vanbuskirk et al., 2004).

Crystal structure of EBA-175RII domain shows that the molecule is composed of mostly α-helices with two antiparallel β sheets and several
bound sulfate molecules. The two subdomains of RII: F1 (residues 8-282) and F2 (297-603) share a very similar structure. All cysteines except cysteine 273 are involved in disulfide bridges. Also, all disulfide bonds formed are located within a given subdomain. Further analysis of crystal structure showed that EBA175RII exists in a dimeric form, with extensive interactions with the elongated monomers. The F1 domain of one monomer interacts with the F2 domain of the second monomer and vice versa. This dimeric organization leads to the formation of two prominent channels that contains four of the six observed glycan binding site. The F2 domain prominently lines the channels and makes majority of the glycan contacts. Based on these observations, Tolia et al., suggested two models for EBA-175RII binding to the red blood cell receptor glycophorin A. In the first model, RII dimer might assemble around the dimeric glycophorin A extracellularly and that dimerization occurs upon ligand-receptor binding at the erythrocyte surface during the invasion process. Alternatively, glycophorin A binds to RII in a straightforward manner and where monomers would assemble around glycophorin A. Thus, any preexisting dimers would dissociate to allow for receptor binding and mediate erythrocyte invasion (Tolia et al., 2005). The role of other domains of EBA-175 in red cell invasion is still not clear. Gilberger et al., studied the role of cytoplasmic domain in trafficking of EBA-175 to the micronemes. 3D7 (sialic acid independent) and W2mef (sialic acid independent) parasites disrupted for cytoplasmic domain still trafficked to the micronemes, suggesting that cytoplasmic domain of EBA-175 is not required for localization of this protein to micronemes in *P. falciparum*. However, cytoplasmic tail of EBA-175 encodes crucial information for its role in merozoite invasion as 3D7 and W2mef deleted for cytoplasmic tail invaded chymotrypsin treated erythrocytes significantly less than wild type parasites, consistent with the loss of function of EBA-175 protein as a result of truncation of the cytoplasmic tail. This suggests that the cytoplasmic domain of EBA-175 is essential
for the function of this protein in parasites that invade via sialic acid independent as well as sialic acid dependent pathways (Gilberger et al., 2003).

These studies have established the fact that EBA-175 plays a major role in erythrocyte invasion. However, when EBA-175 was deleted in *P. falciparum* laboratory strain 3D7 and W2mef, parasite switched to invasion pathway that is sialic acid independent. This implies that EBA-175 is not obligatory for the survival of the parasite (Reed et al., 2000). In contrast, antibodies against the receptor-binding domain, region II, blocked invasion of the parasites that are either sialic acid dependent or independent (Narum et al., 2000; Pandey et al., 2002). Thus, the role of EBA-175 as a crucial parasite ligand for erythrocyte invasion needs to be further elucidated.

*P. falciparum* laboratory as well as field isolates are able to invade erythrocytes treated with neuraminidase or trypsin, which cleaves sialic acid residues and glycophorin A respectively. Okoyeh et al., have shown that alternate invasion pathways commonly exist in Indian *P. falciparum* field isolates. Out of fifteen field isolates studied, 12 field isolates could use receptors other than sialic acid/glycophorin A for invasion into erythrocytes (Okayeh et al., 1999). Although, *P. falciparum* field isolates from Africa used glycophorin A as the major receptor but, there exists considerable variation among field isolates for invasion into neuraminidase and trypsin treated erythrocytes (Baum et al., 2003). This suggests that sialic acid/glycophorin A independent receptors can be used for erythrocyte invasion by *P. falciparum*. EBA-175 has been shown to interact only with sialic acid residues on glycophorin A, suggesting that molecules other than EBA-175 might be involved in mediating alternate invasion pathways. Reverse transcription of RNA transcripts
followed by amplification using oligonucleotide primers of DBL domains resulted in identification of DBL domains homologous to EBA-175. Products of these reverse-transcription-PCR amplifications included sequences of single copy genes (including EBA-175) and variably transcribed genes that cross hybridize to multiple regions of genome. Moreover, these variable transcribed genes showed diverse restriction patterns among different parasite lines. Thus, these transcripts had features similar to the var family of genes that modulate cytoadherence and antigenic variation of *P. falciparum* infected erythrocytes. Another class of DBL-encoding transcripts includes single copy genes, which are conserved. Characterization of the single gene identified ebl-1 gene that is related to EBA-175 and might be involved in erythrocyte invasion (Perterson et al., 1995). Analysis of *P. falciparum* genetic cross 3D7*HB3 linked ebl-1 to an inheritance from 3D7 parent of a more rapid proliferation type. This rapid proliferation could be a result of a relatively higher efficiency in invasion of red cells by progeny inheriting this linkage group (Peterson et al., 2000).

Analysis of *P. falciparum* genome database has identified four EBA-175 paralogues from *P. falciparum* lab strain 3D7. EBA-175 paralogues (EBA-181, EBA-165 and EBA-140) share similar exon intron structure as that of EBA-175. They share a similar signal sequence, 5' and 3' cysteine rich regions, transmembrane domain and a cytoplasmic tail. Therefore, EBA-175 paralogues might be responsible for mediating alternate invasion pathways.

**1.4.2.3c Erythrocyte binding antigen-140 (EBA-140):**

EBA-140/BAEBL shares same structural features and homology with EBA-175. EBA-140 localizes with EBA-175 in the micronemes. Although, receptor for EBA-140 is neuraminidase and trypsin sensitive, EBA-140
still binds to erythrocytes that lack glycophorin A. Deletion of exon 3 in the glycophorin C gene (GYPCDEX3) changes the serological phenotype of the Gerbich (Ge) blood group, resulting in Gerbich negativity. EBA-140 has reduced binding to erythrocytes with Gerbich mutation (Mayer et al., 2001). In another study, Maier et al., showed that EBA-140 binds to glycophorin C (Fig.1.13). Also, antibodies against EBA-140 inhibited the EBA140-glycophorin C pathway, resulting in reduction of merozoite invasion, suggesting that this interaction mediates an important invasion pathway into human erythrocytes. Furthermore, EBA-140 does not bind to glycophorin C in Ge-negative erythrocytes, and thus could not invade Ge negative erythrocytes using this invasion pathway (Maier et al., 2003). This indicates that Ge negativity has arisen in Melanesian populations through natural selection by severe malaria. In order to overcome this selection pressure, P. falciparum generates polymorphisms, which might provide versatility in invasion pathways, thus increasing P. falciparum fitness. Polymorphism studies with EBA-140 showed that even one amino acid change in F1 domain of EBA-140 changes the binding specificities of EBA-140 with erythrocytes. Out of four polymorphisms studied, three of them bound equally well to Ge negative erythrocytes. Also, these changes also allowed EBA-140 to bind to either neuraminidase treated or trypsin treated erythrocytes or both (Mayer et al., 2002). Thus, P. falciparum has evolved multiple invasion pathways dependent on polymorphisms in the EBA-140 ligand.

1.4.2.3d Erythrocyte binding Antigen 181 (EBA-181):
EBA-181/JESEBL, parologue of EBA-175 is expressed at the same time as EBA-175 and colocalizes with this protein in micronemes of merozoites. EBA-181 binds to a novel sialoglycoprotein on the surface of human erythrocytes, which is trypsin resistant and chymotrypsin sensitive (Fig.1.13). The level of expression of EBA-181 differs among parasite isolates. Thus, role of this ligand for invasion appears to be
strain dependent as EBA-181 can be disrupted in W2mef parasites without affecting the invasion phenotype but cannot be targeted in 3D7
(Gilberger et al., 2003). Similarly as in case of EBA-140, polymorphisms in region II of EBA-181 altered its receptor specificity. However, in contrast with EBA-140 where all of the base substitutions were in F1 domain, EBA-181 had base substitutions in F1 as well as F2 domains of region II. Region II of these polymorphic EBA-181 when expressed on surface of Chinese Hamster Ovary (CHO) cells was able to bind erythrocytes treated with neuraminidase or trypsin or chymotrypsin. Moreover, EBA-140 showed binding to erythrocytes treated with neuraminidase, trypsin and chymotrypsin (Mayer et al., 2004).

All three ligands (EBA-175, EBA-181, and EBA-140) show some redundancy in function as the genes for all can be disrupted. However, this disruption is dependent on genetic background. For example, EBA-181 can be disrupted in W2mef but not in 3D7, whereas EBA-175 cannot be disrupted in D10. D10 parasite line already lacks EBA-140 gene, suggesting that a minimum complement of the parasite ligands are required for sialic acid dependent process.

**1.4.2.3e Erythrocyte binding Antigen-165 (EBA-165):**

EBA-165 possesses the same gene structure as of EBA-175, consisting of the F1/F2 domains, transmembrane region and a short cytoplasmic tail. However, genome database of Plasmodium (PlasmoDB) predicted an extra intron between F1 and F2 domain of EBA-165. RT-PCR analysis of EBA-165 shows that it is transcribed during late schizont stage. However, antibodies that recognize EBA-165 fusion protein could not detect this protein in the parasite (Triglia et al., 2001). Thus, EBA-165 is a pseudogene, which is transcribed but not translated (Fig.1.13). An extra intron between F1 and F2 domain might lead to frame shift and therefore the protein is not translated in the correct frame.
### Review of Literature

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Chromosome</th>
<th>Aminoacid</th>
<th>Receptor binding</th>
<th>Enzyme sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBA-175</td>
<td>7</td>
<td>1475</td>
<td>Sialicacid/glyA</td>
<td>-      -      +</td>
</tr>
<tr>
<td>EBA-140</td>
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<td>1210</td>
<td>Sialicacid/glyC</td>
<td>-      -      +</td>
</tr>
<tr>
<td>EBA-181</td>
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<td>1567</td>
<td>Not known</td>
<td>-      +      -</td>
</tr>
<tr>
<td>EBA-165</td>
<td>4</td>
<td>1431</td>
<td>Not expressed</td>
<td>-      -</td>
</tr>
</tbody>
</table>

Figure 1.13: Receptor binding specificities of EBA-175 and its paralogues to erythrocytes.

### 1.4.2.3 MAEBL:

MAEBL is a unique member of ebl family. MAEBL has similar structure as that of DBPs. However, MAEBL is an exceptional member of this family as its ligand-binding domain is homologous to that of domains I and II of AMA-1. MAEBL colocalizes with rhoptry proteins (RHOP H2, RAP-1) and not with micronemes proteins (EBA-175, EBA-140) (Blair et al., 2002). Besides being expressed during blood stage, the expression of MAEBL was also detected during sporozoite stage (Kappe et al., 2001). A targeted disruption of *P. berghei* MAEBL revealed that it is essential for sporozoite infection of salivary glands and was involved in the attachment to the salivary gland surface. In contrast, disruption of MAEBL gene neither affected sporozoite motility in vitro nor infectivity to the vertebrate host (Kariu et al., 2002).
1.5 Tools to study function of a gene:

Gene targeting studies are a powerful method for studying function of a gene in *P. falciparum*. *Plasmodium* is haploid and integrates only by homologous recombination. An important limitation of the current technology is that loss-of-function mutations cannot be selected in genes that play a critical role in parasite invasion or multiplication inside RBC. This indirectly indicates that a gene is important for the survival of parasite, as reported for MSP-1 (O'Donnell et al., 2000) and AMA-1 (Triglia et al., 2000). However, the actual function of the protein cannot be established. Sometimes, knockout of a parasite gene leads to change in the invasion phenotype of the parasite. This is frequently observed for genes, which are responsible for invasion of red cells. For example targeting EBA-175 in *P. falciparum* laboratory isolate, 3D7 using homologous recombination switches the invasion phenotype of the parasite to a sialic acid independent pathway (Duraisingh et al., 2003). Thus, sometimes gene knockout might not give accurate information about the function of a gene. Conditional mutagenesis could also be used to study function of many essential genes, including those involved in invasion. Conditional mutagenesis has been only successful applied in *P. berghei* using site-specific recombination and Flp/FRT system of yeast (Carvalho et al., 2004). Recently, Mital J et al., developed a tet regulatable promoter system to create a conditional knockout of *Toxoplasma gondii* AMA-1. The studies on TgAMA-1 depleted parasites showed that the parasites are severely impaired for attachment to host cells and are also defective in rhoptry secretion. Thus, TgAMA-1 mediated intimate association with the host cell is a critical step in the invasion pathway leading to discharge of contents from rhoptries, which are essential for the formation of junction during the invasion process (Mital et al., 2005).
Alternatively, the function of many genes can be determined using ribozyme and antisense technologies. Antisense RNA technology has been used for the inhibition of a wide array of genes in mammalian cells (Vanderkrol et al., 1988). However, further progress in antisense RNA was hampered, as antisense mediated gene inhibition was usually dependent on the presence of a considerable excess of antisense RNA to target RNA in the cell.

Ribozymes bind to substrate RNA through base-pairing interactions, cleave the bound target RNA and release the cleavage products. The cleavage products are then recycled so that they can repeat this process multiple times. The observation that such ribozymes can be repeatedly targeted to cleave virtually any pathogenic transcript in vitro (Uhlenbeck et al., 1987; Haseloff et al., 1988) led to much speculation about their potential therapeutic value in vivo (Cech et al., 1988; Usman et al., 2000). The utility of transcleaving ribozymes was however limited, as high level inhibition of target transcripts was difficult to achieve with ribozyme technology.

In 1998, Fire and colleagues discovered that the injection of double stranded (ds) RNA into Caenorhabditis elegans led to a sequence specific silencing (Fire et al., 1998). This phenomenon was referred to as RNA interference (RNAi) and is similar to previously described silencing phenomena such as posttranscriptional gene silencing (PTGS) (Jorgensen et al., 1990) in plants and quelling in fungi (Romano et al., 1992).

Further analysis of PTGS induced either by transgene or virus in plants identified RNA molecules, which were of uniform length of about 25 nucleotides (nt). Their accumulation required either transgene sense transcription or RNA virus replication. Thus, the 25nt antisense RNA is likely synthesized from an RNA template and represents the specificity
determinant of PTGS (Hamilton et al., 1999). The same phenomenon was recapitulated in *Drosophila melanogaster* embryo extracts, in which long dsRNA were cleaved into short interfering siRNAs of ~22nt (Zammore et al., 2000). Furthermore, the introduction of chemically synthesized 21-nt and 22-nt siRNAs to *Drosophila* embryo extracts facilitated the degradation of the target RNA (Elbashir et al., 2001). Short RNA products were subsequently found in fly embryos and worms that were injected with dsRNA as well as in *Drosophila* Schneider2 (S2) cells that were transfected with long dsRNA. These findings provided a new tool for studying gene function.

**1.5.1 Mechanism of RNA interference:**

dsRNA processing and target mRNA degradation have been shown to be biochemically separable activities. This led to the view that RNAi can be divided into distinct initiator and effector phases as described below. Number of proteins have been identified which carry out different functions to bring about the degradation of the target RNA.

**1.5.1.1 Initiation Phase:**

In the first step, long dsRNA is cleaved to produce siRNAs. siRNAs are 21-23nt dsRNA duplexes with symmetric 2-3-nt 3' overhangs and 5'-phosphate and 3'-hydroxyl groups (Elbashir et al., 2001). This structure is characteristic of an RNAselIII like enzymatic cleavage pattern, which led to the identification of the highly conserved Dicer family of RNAselIII enzymes as the mediators of dsRNA cleavage. Dicer has a distinctive structure, which includes a helicase domain and dual RNAselIII motifs. Dicer also contains a region of homology to the RDE1/QDE2/Argonaute family, which helps in shuttling of siRNAs to appropriate effector complexes (RISCs) (Bernstein et al., 2001).
However, sometimes cleavage of longer (~70nt) endogenous precursors with imperfect hairpin like structures by a Dicer like protein, Drosha, generates 22nt structures termed as microRNA (miRNA). miRNAs bind to sites that have partial sequence complementarity in the 3' untranslated region (UTR) of their target RNA, causing repression of translation and inhibition of protein synthesis (Pasquinelli et al., 2002). miRNAs have been cloned from various organisms and cell types (Pasquinelli et al., 2002) and play an important role during the developmental process. For example lin-4 RNA of *C. elegans* is a negative regulator of the developmentally important proteins lin-14 and lin-28 (Lee et al., 1993; Olsen et al., 1999; Moss et al., 1997; Wightman et al., 1993). lin-14 and lin-28 control the timing of developmental events in diverse cell types. The 22-nt lin-4 RNA contains homology to the target sequence in 3' UTR of lin-14 and lin-28. Thus, deletion of lin-4 target sequence causes an unregulated gain of function phenotype and controls the postembryonic development of *C. elegans* (Lee et al., 1993). In addition to cleavage by Drosha, other proteins involved in effector phase of RNAi are likely to function in both pathways (Hutvagner et al., 2002; Mourelatos et al., 2002).

**1.5.1.2 Effector Phase:**
siRNAs generated either by Dicer or Drosha cleavage are incorporated into a multiprotein complex referred to as RNA-induced silencing complex (RISC). siRNAs need to be 5' phosphorylated to enter into RISC (Nykanen et al., 2001). siRNAs lacking a 5' phosphate get phosphorylated by an endogenous kinase (Schwarz et al., 2002) and incorporation of siRNAs converts holo RISC to the active RISC form. The duplex siRNA is unwound, leaving the antisense strand to guide RISC to its homologous target mRNA for endonucleolytic cleavage. The target mRNA is then cleaved at a single site in the center of the duplex region between the
guide siRNA and the target mRNA, 10nt from the 5' end of siRNA (Elbashir et al., 2001) (Fig. 1.14).

**Figure 1.14. Mechanism of RNA interference.** RNAi is initiated by Dicer enzyme (two Dicer molecules with five domains each are shown), which process dsRNA into ~22nt siRNAs. The siRNAs are then incorporated into a multicomponent nuclease, RISC. RISC is then activated from a latent form, containing a double stranded siRNA to an active form, RIS, by unwinding of siRNAs. RISC\( ^* \) then uses the unwound siRNA as a guide to substrate selection. (Hannon, 2002)

In order to identify proteins in RISC complex, coimmunoprecipitation experiments were performed with Dicer. Coimmunoprecipitation of Dicer, which is associated with initiator phase, identified Arganoute protein, associated with RISC (Bernstein et al., 2001). Recently, structural studies with arganoute (Ago) proteins provided strong evidence
that the PIWI domain in the Ago protein contains the active site for the endonuclease activity of RISC, while PAZ domain has been implicated in RNA binding (Ma et al., 2004; Lingel et al., 2004). Two *Drosophila* proteins with RNA binding domains – the Vasa intronic gene (Vig) and the orthologue of human fragile X-mental retardation protein (FMRP) have also been found to associate with RISC. The functions of these two proteins in the process of target mRNA degradation is still not clear (Ishizuka et al., 2002; Caudy et al., 2002). Tudor SN (tsn), another protein with multiple repeats of the staphylococcal nuclease domain has been found to be associated with RISC (Caudy et al., 2003). Enzyme-inhibitor studies performed with Tsn seems to exclude a role for Tsn in siRNA directed target cleavage (Martinez et al., 2004; Schwarz et al., 2004), although its proposed activity could still be involved in the more extensive degradation of the initial cleavage products of RISC.

In *D. melanogaster*, affinity tagged Fmr1/Fxr, which is an RNA binding protein, copurifies with Ago2, components of large ribosomal subunit and the presumptive RNA helicase Dmp68 (Ishizuka et al., 2002). Knockdown of Dmp68 expression inhibited RNAi response in *Drosophila* S2 cells, suggesting its role in RISC. Although, proteins involved in RNAi efficiently act at different phases of target mRNA degradation but the important question that remained unanswered was how a very small amount of dsRNA leads to silencing. This raised the possibility that the silencing signal may be amplified. Genetic studies in plants and *C. elegans* led to the identification of RNA-dependent RNA polymerases (RdRPs) as key elements of such an amplification loop (Sijen et al., 2001; Vaistij et al., 2002,). However, human and *Drosophila* genome sequences lack an RdRP homolog, suggesting that amplification step might be restricted to only a subset of organisms that are competent for dsRNA mediated silencing. However, the sequence specific nature and a
relatively easy method of delivery still make it a better choice than antisense and ribozyme technology to study function of a gene in humans and *Drosophila*.

1.5.2 **Advantages of siRNA over dsRNA:**
Most common method used for inducing RNAi was either microinjection or electroporation or in *C. elegans* by soaking worms in dsRNA solution. However, exposure of cells to dsRNA such as poly(IC) induces the transcription of interferons that induces an antiviral state in cells. Furthermore, induction of interferons, in conjunction with other signals, could stimulate apoptosis (Gil et al., 2000). The antiviral state is characterized by the synthesis of number of proteins that recognize dsRNA. These proteins include a kinase, Protein Kinase R (PKR) which is activated by dsRNA, a 2'−5' oligoadenylated synthetase activates the endoribonuclease RNaseL, which degrades RNA. Once PKR gets activated, it inhibits protein translation by phosphorylating the alpha subunit of the translation initiation factor (EIF2α). EIF2α phosphorylation is followed by a general suppression of protein synthesis that directs the cells towards apoptosis. Thus, there occurs a nonspecific suppression of translation and degradation of mRNA (Paddison et al., 2002; Svoboda et al., 2001). siRNAs being less than 30bp fail to activate PKR and interferon pathways efficiently. Chemically synthesized siRNAs introduced into cells bypass the initial cleavage step and leads to target messenger RNA degradation (Elbashir et al., 2001). RNAi could also be induced in mammalian cells by transfection of long hairpin RNA expressed from an RNA polII promoter, which yields a population of siRNAs with several sequence specificities. siRNAs with single sequence specificity can be expressed either by tandem polIII promoter that express individual sense and antisense strands that associate in trans (Miyagishi et al., 2002) or a single polIII promoter that expresses short
hairpin RNA with sense and antisense strands of siRNA that associate in cis (Brummelkamp et al., 2002; Mcmanus et al., 2002; Paddison et al., 2002).

1.5.3 Applications of RNAi:

RNAi technology has become a method of choice to downregulate gene expression in a variety of systems. RNAi methods are now well established in *C. elegans* and *Drosophila*. RNAi can be used as a tool for studying functions of genes in those organisms, which are difficult to study by classical genetic approaches. Although, genetic crosses are feasible and have been performed for *T. gondii*, *P. falciparum* and *Trypanosoma brucei*, these parasites engage in sexual reproduction in experimentally difficult conditions, in cat, mosquito and tsetse respectively, thus complicating the performance of such experiments. RNAi although not a substitute for classical genetic approaches, can be used to improve the understanding of function a gene. In *T. brucei*, RNAi has been shown to be important for retroposon transcript abundance. RNAi has been further used to study numerous aspects of trypanosome biology including cytokinesis (Ngo H et al., 1998), flagellum ontogeny (Bastin et al., 2000), a mitochondrial RNA polymerase (Grams et al., 2002) and enzyme compartmentation in glycosomes (Guerra-giralez et al., 2002). Essential genes such as that encoding for topoisomerase II (Wang Z and Englund, 2001) and a gene required for flagellar attachment (Lacount et al., 2002) have been studied by observing progress to cell death after RNAi induction. In contrast to *T. brucei*, application of RNAi in *P. falciparum* is still at an early stage. Malhotra et al., using RNAi inhibited cysteine proteases of *P. falciparum*, important for hemoglobin degradation. Introduction of dsRNA to the parasite leads to reduction of the targeted mRNA, production of siRNAs from introduced dsRNA, and changes parasite morphology and hemoglobin degradation consistent
with cysteine protease inhibition (Malhotra et al., 2002). Similarly, down regulation of Pfmyb1 (transcription factor belonging to tryptophan cluster family) using RNAi exhibited 40% inhibition of growth when compared with control cultures and affected transition from trophozoite to schizont stage. Thus, Pfmyb1 is required for intraerythrocytic development and controls key genes for cell cycle regulation (Gisssot et al., 2005). However, other apicomplexan parasites T. gondii and L. major were found to be RNAi negative. It could be possible that the RNAi machinery does not exist in these parasites. In P. falciparum, studies have shown that addition of dsRNA to the culture reduces levels of mRNA and protein in the parasite, leading to gene specific phenotypes. However, this is not supported by the genetic makeup, as annotation of Plasmodium genome could not identify proteins involved during RNAi.