RHOPTRY PROTEINS OF PLASMODIUM SPECIES

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RHOPTRY PROTEINS OF PLASMODIUM SPECIES

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ABSTRACT

Rhoptry proteins of *Plasmodium* sp. participate in host cell invasion and intracellular parasite development. In this review, the major rhoptry proteins of *P. falciparum* are discussed with respect to their importance in the biology of *Plasmodium* species and as malaria vaccine candidates. The morphology and organization of the rhoptries in *Plasmodium* species are compared with those of other apicomplexans, and the contributions of apical complex proteins to invasion in ookinetes and sporozoites are discussed. Furthermore, the significance of host cell binding by apical complex proteins, and their role in host cell invasion among the different invasive stages is also reviewed.

INTRODUCTION

Apical organelles of the Apicomplexa are unique among protozoans. The organelles include among other structures, the rhoptries, micronemes, dense granules, spherical bodies, polar rings, and conoid. The organelles are electron dense, contain mostly proteins and participate in the process of host cell invasion or transmembrane migration (Sinden, Hartley and Winger 1985). Lipids and carbohydrates have also

been described within the organelles (Zinecker et al. 1998; Foussard, Leriche and Dubremetz 1991). Proposed functions of apical complex proteins range from host cell attachment during invasion, parasitophorous vacuole formation and modification, escape from the vacuolar membrane and nutrient acquisition through enzymatic activity (Dyson, Grahame and Evennett 1994; Carruthers and Sibley 1997; Dubremetz et al. 1998; Sam-Yellowe 1996). Much effort has centered on understanding the biological role of the apical organelles in Apicomplexan parasites of medical and veterinary importance and in determining if the apical proteins have diagnostic or vaccine potential. Morphologically the apical organelles are similar across the phylum (Dubremetz, Ferreira and Dissous 1989; Etzion, Murray and Perkins 1991; Leriche and Dubremetz 1991; Machado et al. 1993; Sam-Yellowe et al. 1998; Petry and Harris 1999). It has become apparent that the organelles secrete their contents sequentially during the process of invasion and that secretion might be triggered by specific stimuli (Carruthers, Moreno and Sibley 1999). Organelles such as the dense granules may exist as different subpopulations with subsets functioning during parasite contact and others functioning following parasite entry (Carruthers and Sibley 1997). Secondly, both humoral and cellular immune responses are generated to apical complex proteins in infected hosts, suggesting their exposure during parasite infection. In this review, the rhoptries of *Plasmodium* species will be discussed and compared with the rhoptries of other apicomplexans. The role of the rhoptries in invasion and parasite development will be considered along with the role of micronemes and dense granules in the invasion process. The review will also consider the implications for structural conservation of organelle proteins among the invasive stages of *Plasmodium*.

MEROZOITE INVASION

Erythrocyte invasion by *Plasmodium* species is mediated in part by the specific recognition of receptors on the erythrocyte surface by apical complex proteins following initial contact of the merozoite with the host cell. Invasion proceeds through a mechanism resembling induced endocytosis. Specific recognition of host cell receptors by apical complex proteins occurs in all invasive stages of the Apicomplexa (collectively called zoites) (Aikawa and Seed 1980; Bannister and Mitchell 1989; Sinden, Hartley and Winger 1985). The zoite is a highly specialized stage designed to maintain transmission between host cells in vertebrates and

between vector and vertebrate hosts in hemoparasites. In *Plasmodium* species, these include the ookinetes which penetrate the mosquito mid-gut, non-infective sporozoites which penetrate the salivary glands in the mosquito, infective sporozoites which invade hepatocytes in the liver and merozoites which invade erythrocytes for the development of the blood stages. All these invasive stages possess an apical complex consisting of rhoptries and micronemes, and in some cases dense granules, conoid, polar rings and subpellicular microtubules (Bannister and Mitchell 1988). In the three invasive stages contents of the apical complex organelles are required for effective host cell invasion. Motility of the invasive stages appears to be a necessary condition that enhances invasion into the host cell. Within the mosquito midgut ookinetes actively escape the blood meal and have to traverse the peritrophic matrix chitin network, and midgut epithelia where they come in close contact with the plasma membrane of the host cell (Sinden 1998). In ookinetes chitinases are present within micronemes and appear concentrated within an electron dense collar of the subpellicular complex during the secretion process (Langer et al. 2000). Gliding motility has been described for sporozoites with the involvement of the CS and TRAP proteins (Menard et al.1997; Sultan et al.1997). Merozoites exhibit reduced motility. However, during reorientation of the merozoite to initiate host cell entry and for entering the PV, merozoite motility appears to be necessary (Pinder et al. 2000). The presence of *P. falciparum* (Pf) myosin A (PfmyoA) whose expression is limited only to the mature merozoites, predominantly in the apex argues for its involvement in merozoite motility (Pinder et al. 2000). Figure 1 shows a schematic illustration of a *Plasmodium* merozoite showing the apical complex, with the prominent paired flask-shaped rhoptries. Rhoptries and micronemes are thought to participate in host cell recognition and rhoptries are also thought to participate in host cell recognition, and parasitophorous vacuole (PV) formation (Sam-Yellowe 1996; Dubremetz et al. 1998). Dense granule components are also involved in host cell contact and in PV modification following parasite entry into the host cell. In *P. falciparum,* merozoite invasion into the host erythrocyte occurs in a rapid (<30 sec), complex, multi-step sequence involving the recognition of specific receptors on the erythrocyte surface. The sequence is thought to include the following: i) reversible attachment of merozoites to the erythrocyte surface by the major merozoite surface protein-1 (MSP-1), followed by binding of the microneme derived, 175 kDa erythrocyte binding antigen (EBA-175). The interaction of EBA-175 with the erythrocyte surface has

been well characterized and the binding domain for EBA-175 identified (Sim 1995). Both proteins bind to sialic acids on glycophorin. ii) irreversible attachment of the merozoite to the erythrocyte at the apical end leading to merozoite reorientation and discharge of rhoptry contents. iii) erythrocyte membrane deformation and moving junction formation; a 60 kDa merozoite cap protein-1 (MCP-1) with an oxidoreductase domain is localized to the junctions (Hudson-Taylor et al. 1995; Ladda, Aikawa and Sprintz 1969). No clear function has been established for the protein. iv) merozoite internalization accompanied by processing and sloughing of MSP-1 and parasitophorous vacuole membrane (PVM) formation; a 19 kDa fragment of MSP-1 is carried into the newly invaded cell. v) erythrocyte resealing and modification of the PVM. Additional merozoite surface proteins namely MSP-2, MSP-3, MSP-4 and MSP-5, may participate in the invasion process. However, their function remains unknown (Barnwell and Galinski 1998).

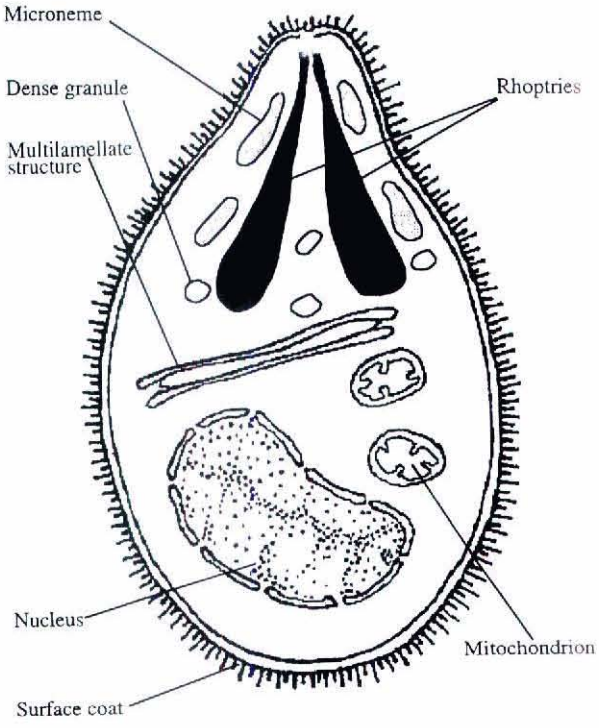

Figure 1. Schematic illustration of the apical complex in a *Plasmodium* merozoite.

Plasmodium falciparum invades the host erythrocyte using multiple erythrocyte receptors and parasite ligands thereby arguing for the presence of alternate invasion pathways by the merozoite (Barnwell and Galinski 1998). The most characterized pathway is that dependent on sialic acids on glycophorins (Perkins

and Rocco 1988). Parasite proteins binding to the human erythrocyte independent of this pathway may serve as ligands for a sialic acid independent pathway. The sialic acid independent pathway may represent an important route for merozoites invading the host erythrocyte in nature (Okoyeh, Pillai and Chitinis 1999). Parasite molecules involved in these alternate pathways together with those involved in glycophorin and sialic acid binding would constitute components of effective vaccines against malaria. In other *Plasmodium* species; such as in *P. vivax* and *P. knowlesi*, the Duffy glycoprotein, a chèmokine receptor, serves as the essential receptor for merozoite attachment. In these species subsequent steps of invasion may be mediated by reticulocyte binding proteins 1 and 2 (PvRBP-1 and PvRBP-2), located at the apical ends of *P. vivax* merozoites (Barnwell and Galinski 1998). A proposed location of *P. falciparum* apical complex proteins participating during erythrocyte invasion is shown in Figure 2.

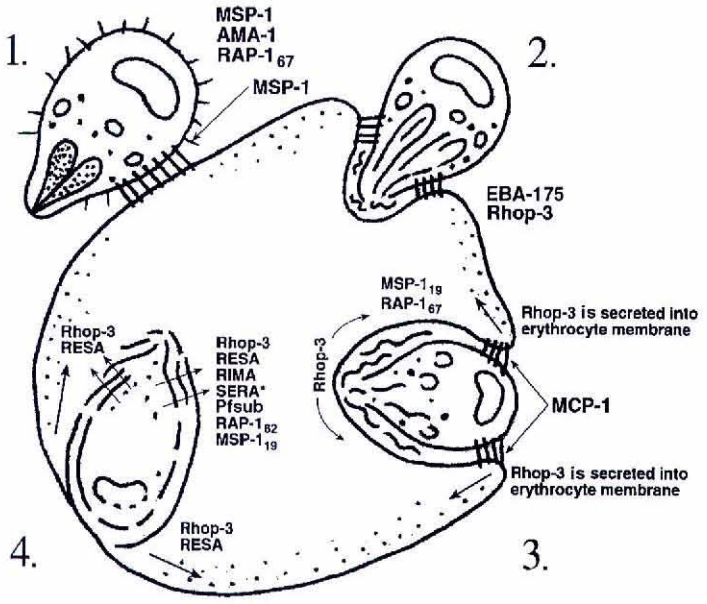

Figure 2. Proteins involved in merozoite invasion.
1. Merozoite attachiment to the erythrocyte with MSP-1
2. Merozoite reorientation with microneme and rhoptry discharge; beginning of junction formation
3. Merozoite internalization and PVM formation
4. Resealing of the erythrocyte membrane; dense granule discharge and PVM modification in the young ring stage parasite

* SERA is not an apical complex protein protein but is localized to the PVM.

Similar protein localization is thought to occur in other *Plasmodium* species. Following merozoite entry, the forming PVM is modified by rhoptry and dense granule proteins to accommodate the growing parasite and to interact with the intracellular network for macromolecular transport that soon develops (Haldar 1994). Dense granule proteins undergo exocytosis and become associated with the PVM. Proteins such as the ring-infected erythocyte surface antigen (RESA) shown to associate with spectrin in the cytoskeleton (Foley, Murray and Anders 1990), and ring infected membrane antigen (RIMA) are translocated to the forming PVM (Trager et al. 1992) following merozoite entry. Recently subtilisin- like proteases, PfSUB1 and PfSUB2 from a subset of the dense granules were described (Blackman et al. 1998; Barale et al. 1999). The proteases may function in the initial steps of invasion during processing of the MSP-1 fragment, MSP-1_{42} to MSP-1_{19} and leading to subsequent formation of the PV (Barale et al. 1999). The apical complex proteins are thought to function in a regulated sequence. In *P. falciparum*, invasion may be influenced by an apically located calcium-calmodulin complex (Matsumoto et al. 1987). In *Toxoplasma gondii*, the sequence of invasion is regulated by secretory pathways involving the micronemes, rhoptries and dense granules (Carruthers and Sibley 1997). Dense granule and microneme secretion is regulated by intracellular calcium levels following parasite attachment and parasite entry (Carruthers, Moreno and Sibley 1999). In *Theilaria parva* discharge of the rhoptry-microneme complex, upon entry of the sporozoite into a lymphocyte occurs independently of calcium (Shaw 1997). However, it appears that the initial sporozoite contact with the host cells leads to intrasporozoite calcium mobilization (Shaw 1997). These data suggest that signals associated with a specific type of host cell may influence the invasion process. Furthermore, the data suggest that the apical complex proteins may be used in different ways during host cell invasion by the different parasites to achieve intracellular parasitism.

Several proteins have been identified and localized to the apical complex in *Plasmodium* species, using monoclonal and polyclonal antibodies. However, the organelles are thought to contain many more proteins of as yet unknown function. This has been clearly demonstrated in the rhoptries, where SDS-PAGE analysis of isolated rhoptries from *Plasmodium* species shows the presence of a complex mixture of proteins exceeding the number characterized in the literature (Etzion, Murray and Perkins 1991; Sam-Yellowe et al. 1995; Sam-Yellowe et al. 1998; Sam-Yellowe,

Fujioka and Aikawa 1999). For a few of the apical complex proteins identified, such as the ring-infected surface antigen (RESA), Rhop-3, RAP-1/2. apical membrane antigen-1 (AMA-1) and MAEBL, genes encoding the proteins have been isolated and characterized, and homologues identified in other *Plasmodium* species suggesting that the proteins probably have a similar function in the different species (Table 1).

Table 1. Summary of rhoptry proteins of *Plasmodium* species

Rhoptry proteins	*Plasmodium sp.*	References
Py 235	rodent, human	Borre et al.1995; Preiser 1999
MAEBL	rodent, human	Kappe et al. 1998
Rhop-1/2/3 (Rhop-H)	rodent, human	Sam-Yellowe, et al. 1988; Lustigman et al. 1988
RAP-1/2/3	human, rodent	Howard et al. 1998
AMA-1	rodent, human, Simian	Thomas et al. 1990; Kappe & Adams 1996; Dutta et al. 1995
Pf60	human	Grellier et al. 1994
ROPE	rodent	Werner et al. 1998
Py 160	rodent	Murakami & Tanabe 1985
Py 68, 80,105, 130, 140 kDa	rodent	Heinne et al. 1998
PyAG1	rodent	Heinne et al. 2000
Pf 225	human	Roger et al. 1987
Pf 55	human	Smythe et al. 1988
Pf 52	human	Storey et al. 1992
PvRBP-1	human	Barnwell & Galinski 1998
PvRBP-2	human	Barnwell & Galinski 1998
PfRBP1-Ha	human	Rayner et al. 2000
PfRBP2-Hb	human	Rayner et al. 2000

Plasmodium falciparum rhoptry proteins such as the high molecular weight rhoptry proteins (Rhop-H) bind to the erythrocyte membrane and may participate in merozoite entry (Sam-Yellowe and Perkins 1990). Furthermore, AMA-1 with epitopes expressed on the merozoite surface may also have a role in invasion

(Thomas, Waters and Carr 1990). In recent studies, a newly described chimeric rhoptry protein, MAEBL, possesses shared domains from the Duffy-binding like (DBL) erythrocyte binding protein family, derived from micronemes and the AMA-1 rhoptry protein family (Thomas, Waters and Carr 1990; Kappe et al. 1998). The DBL family plays an important role in erythrocyte binding (Sim 1995).

MORPHOLOGY AND ORGANIZATION OF PLASMODIUM RHOPTRIES

In *Plasmodium falciparum* six rhoptry proteins have been molecularly characterized (see Table 1). The genes encoding the proteins do not share any obvious overall structural features. However, AMA-1 and MAEBL share cysteine rich domains of Duffy-like binding proteins probably containing the erythrocyte binding domain and the extracellular domain of AMA-1 (Kappe et al. 1998; Noe and Adams 1998). A consensus motif KNV found in all rhoptry proteins was previously reported (Suarez et al. 1994). No typical repetitive sequences are found in rhoptry proteins. The number of rhoptries found in invasive stages of the different apicomplexans vary, as do the number of proteins located in them. Only a single rhoptry was identified in sporozoites of *Cryptosporidium parvum*, and as many as four or more rhoptries have been identified in sporozoites and tachyzoites of *T. gondii*. This unique morphogenesis is intriguing and raises important questions concerning the regulation of organelle biogenesis. For example, a) how is organelle distribution regulated in the different zoites? and b) do the rhoptries and micronemes secrete their contents through a common duct? How is secretion regulated? Apical organelles have been isolated and characterized from several apicomplexans. Morphologically rhoptries from different species appear similar; they are electron dense and surrounded by a limiting membrane (Leriche and Dubremetz 1991; Etzion, Murray and Perkins 1991; Machado et al. 1993; Sam-Yellowe, Fujioka and Aikawa 1999). When sporozoites of *C. parvum* were fractionated, the rhoptries were not separated into a distinct fraction but remained associated with the apical tip of the zoite in fractions of 1.2-1.3 M sucrose concentration (Petry and Harris 1999). No proteins have been identified in the limiting single membrane of the rhoptry organelle. If any proteins are inserted into the organelle postranslationally, it will be important to identify proteins on the surface of the organelle that may be involved in protein import and sequences responsible for targeting the organelle (Johnson, Lahti and Bradley 1993). Rhoptry proteins identified so far are distributed to the body or neck of the rhoptries.

Immunological methods have been used to identify sucrose gradient fractions containing rhoptries in different apicomplexa. However, with the identification of proteases and other enzymes within different organelles, it will become possible to distinguish more carefully fractions containing a specific organelle. Among the most commonly studied organelle, the rhoptries possess a density in the range 1.12-1.18g/ml. Immunological cross reactivity was observed among merozoite rhoptry proteins of rodent *Plasmodium* species and *P. falciparum* (Sam-Yellowe et al. 1998) suggesting the conservation of rhoptry proteins across *Plasmodium* species. Recent evidence suggests that this cross reactivity may be shared by the ookinete and sporozoite (T. Y. Sam-Yellowe and C. Perry, unpublished observations). In *Eimeria tenella*, limited cross reactivity was observed among the different zoite stages (Tomley 1994). The *P. falciparum* rhoptry protein Pf60.1 encoded by a multigene family has homology to *Babesia* sp. RAP-1 (Grellier et al. 1994). Unlike other rhoptry proteins identified, Pf 60.1 possesses a high level of genetic polymorphism that has been found useful in typing *P. falciparum* strains (Carcy et al. 1995).

RHOPTRY PROTEINS OF *P. FALCIPARUM*

Table 1 summarizes rhoptry proteins of *Plasmodium* species. Rhoptry proteins of *Plasmodium* species are generally non-polymorphic and do not typically possess the multiple amino acid repeats associated with other asexual stage proteins. Some of the proteins are lost during merozoite release, some persist on the merozoite and others are carried into the newly formed ring stage indicating distinct roles in merozoite release and host cell invasion. Protection afforded by purified rhoptry proteins of *P. falciparum* have since been described for several proteins, with T and B cell epitopes identified in proteins such as AMA-1, Rhop-3 and RAP-1/2 /3 (Howard, Jensen and Franklin 1993; Jakobsen et al. 1993a; Jakobsen et al 1993b; Jacobson et al. 1998; Lal et al. 1996; Yang et al. 1996; Perrin et al. 1985; Ridley et al. 1990a; Ridley et al. 1990b; Brown and Coppel 1991; Siddiqui et al. 1987; Stowers et al. 1996; Thomas et al. 1994). Relative to AMA-1 and RAP-1/2/3, very little is known about the immunological properties of the individual Rhop-H proteins. Both B and T cell epitopes of *P. falciparum* antigens are essential for the induction and development of sustained protective immunity to *P. falciparum*. The major rhoptry proteins of *P. falciparum* are discussed below.

RHOPTRY-ASSOCIATED PROTEINS -1, -2 and -3 (RAP-1, RAP-2 RAP-3)

The RAP-1 protein, synthesized in the trophozoite stage as an 84 kDa protein is converted to an 86 kDa precursor protein (Pr86) that is processed sequentially from the N-terminus to proteins of 82 kDa (p82) and 67 kDa (p67) in the schizont stage. The RAP-1/2/3 proteins are located in the body of the rhoptries by IEM. Both p82 and p67 are localized to the merozoite surface and may be involved in merozoite invasion (Howard et al. 1998). The N-terminus of RAP-1 contains an octapeptide repeat of the structure KSSSPSXT/V- QTSGS/L and is conserved among different geographic strains of *P. falciparum* (Howard and Peterson 1996). RAP-1 associates non-covalently with proteins of 39 kDa (RAP-2) and 37 kDa (RAP-3) to form a stable complex. The p67 fragment is thought to function following merozoite entry, before sealing of the PVM (Howard et al. 1998). Gene-targeted disruption of the RAP-1 gene had no effect on merozoite invasion into the host erythrocyte. Similarly targeted disruption of the rhoptry protein ROP1 (Kim, Soldati and Boothroyd 1993) in *T. gondii* had no effect on parasite invasion. However, the mutant *P. falciparum* parasites produced truncated RAP-1 which failed to translocate into the rhoptries. The RAP-2 protein was also not translocated to the rhoptries suggesting that RAP-1 is essential for targeting of RAP-2 to the rhoptries (Baldi et al. 2000). No morphological changes were observed in the rhoptry organelle in the absence of RAP-1.

The lack of an effect on merozoite invasion resulting from RAP-1 disruption is intriguing in light of the immunogenicity of RAP-1 in infected individuals and in animal studies (Fonjungo, Stuber and McBride 1998; Fonjungo et al. 1999; Jacobsen et al. 1998; Alifrangis et al. 1999). RAP-1 may have a role not in host cell entry but in the biological function of the parasite. Human antibody responses to RAP-1 and RAP-2 appear to be age-dependent and are influenced by specific HLA (HLA DR and DQ) alleles suggesting that HLA allele products expressed at different ages govern the antibody levels achieved in adults and the rate at which RAP-1 immunity is acquired by an individual (Johnson et al. 2000).

APICAL MEMBRANE ANTIGEN-1 (AMA-1)

Apical membrane antigen -1, synthesized during the last four hours of schizogony, is an approximately 83 kDa protein, that is processed to a 66 kDa molecule with epitopes expressed on the surface of the merozoite. AMA-1 is localized to the neck of the rhoptries by IEM and is typical of a type 1 integral

membrane protein, with a hydrophobic signal sequence in the C-terminus and a cytoplasmic tail. Major structural features of AMA-1 are conserved in all AMA-1 genes characterized from different *Plasmodium* species, making this one of the most promising vaccine candidates of the blood stage. However, antigenic polymorphism among parasite strains has been described in AMA-1 and strain specific immune responses have been described in studies with *P. chabaudi* (Marshall et al. 1996; Good, Kaslow and Miller 1998).

Naturally acquired immunity to AMA-1 occurs in infected individuals in different geographic areas and age-related changes in antibody levels to AMA-1 were observed (Thomas et al. 1994). Immunodominant T-cell epitopes in AMA-1 have been mapped (Lal et al. 1996; Amante et al. 1997), and nine of seventeen putative T-cell determinants induced PBMC proliferation in a Kenyan study. Cells from individuals with life-long exposure to malaria responded the most, compared to cells from donors who were not previously exposed. Some of the most potent proliferative T cell responses were from peptides located in the highly conserved regions of AMA-1 (Lal et al. 1996).

THE HIGH MOLECULAR WEIGHT RHOPTRY PROTEINS OF *P. FALCIPARUM* (Rhop-H)

The Rhop-H proteins of *P. falciparum* exist in a noncovalent complex consisting of genetically unrelated proteins of 140 kDa Rhop-1, 130 kDa Rhop-2 and 110 kDa Rhop-3. (Sam-Yellowe, Shio and Perkins 1988; Lustigman et al. 1988). The Rhop-H proteins are secreted into the erythrocyte membrane during merozoite invasion suggesting a role for the proteins in invasion (Sam-Yellowe, Shio and Perkins 1988). Similar secretion and association of the ring infected surface antigen (RESA) with human and mouse erythrocytes has also been shown (Klotz et al.1987). The three Rhop-H proteins are erythrocyte binding proteins. They bind the erythrocyte membrane in association with the serine rich antigen (SERA) (Sam-Yellowe et al. 1991, Perkins and Zeifer 1994; Sam-Yellowe et al. in press, b). Gene sequence information is unavailable for Rhop-1 and –2. Like the Rhop-1 and Rhop-2 proteins, the Rhop-3 is synthesized in the trophozoite stage as an approximately 110 kDa polypeptide that is processed to 100 kDa in the segmented schizont stage. Processing of the mature Rhop-3 protein to the 100 kDa fragment occurs by C-terminal processing (Doury et al. 1997). The Rhop-3 protein is highly hydrophobic,

lacks typical membrane spanning regions, and possesses an unusual molecular structure for *P. falciparum*. Seven exons (two mini-exons) and six introns make-up the Rhop-3 gene. Human sera from different endemic areas reacted strongly with the C-terminus of Rhop-3 (Yang et al. 1996; Brown and Coppel 1991; Wang, T. and Sam-Yellowe, T. in preparation). Rhop-3 specific antibodies inhibit invasion in vitro and in vivo (Sam-Yellowe and Perkins 1990; Siddiqui et al. 1987), suggesting that Rhop-3 may play an important role during invasion. We recently identified the Rhop-3 gene homologue in rodent *Plasmodium* species (Anthony et al. 2000; Sam-Yellowe et al. in press, c), suggesting a similarity in the biological role of Rhop-3 among *Plasmodium* species.

OTHER *P. FALCIPARUM* RHOPTRY PROTEINS

A GPI (glycosyl phospatidyl inositol) anchored rhoptry protein of 55 kDa was described in *P. falciparum* (Smythe et al. 1988). The properties of this protein in merozoite invasion are unknown. A 52 kDa rhoptry protein was described in *P. falciparum* (Storey 1992). A rabbit polyclonal antiserum but not a monoclonal antibody specific for the 52 kDa protein inhibited merozoite invasion in vitro (Storey 1992). A 225 kDa rhoptry protein synthesized as a 240 kDa precursor protein and localized to the neck of *P. falciparum* rhoptries was also described (Roger et al. 1987). Antisera against a rhoptry protein of *Babesia divergens* identified a 60 kDa (Pf 60.1) *P. falciparum* protein encoded by a multi-gene family (containing approximately 140 members) (Grellier et al. 1994). The Pf60 gene family is highly polymorphic, and gene products may have a role in merozoite invasion (Carcy et al. 1995). Restriction fragment length polymorphisms (RFLPs) of Pf60 have been used to identify isolates. Recently two rhoptry proteins; *Plasmodium falciparum* reticulocyte binding protein 2 homologues (PfRBP2-Ha and -Hb), encoding proteins of 370 kDa and 383 kDa respectively, were described (Rayner et al. 2000). Both proteins possess homology to the PvRBP2 and to the *P. yoelii* 235 kDa rhoptry protein (Galinski, Xu and Barnwell 2000).

RHOPTRY PROTEINS OF OTHER PLASMODIUM SPECIES

The role of *Plasmodium* rhoptry proteins in merozoite invasion and in conferring immunity was demonstrated in passive immunization studies by Freeman, Trejdosiewicz and Cross (1980). Merozoite specific antibodies, subsequently shown

to be specific for a 235 kDa (Py235) *P. yoelii* rhoptry protein inhibitied *P. yoelii* parasitemia. Immunization with the purified 235 kDa rhoptry protein modified host cell specificity from erythrocytes to reticulocytes and led to parasite clearance (Holder and Freeman 1981). The Py235 is encoded by a large multigene family that may be responsible for maintaining clonal variation of *P. yoelii* merozoites (Borre et al. 1995; Preiser et al. 1999). Py 235 possesses a domain with similarity to a partial sequence of the *P. vivax* rhoptry protein PvRBP-2 (Keen et al. 1994) and binds preferentially to mouse erythrocytes (Ogun and Holder 1996). The repetitive organellar protein (ROPE) is a 229 kDa protein identified in *P. chabaudi* and shown to resemble a cytoskeletal protein (Werner, Taylor and Holder 1998) due to the presence of dimeric alpha helical coiled-coils reminiscent of spectrin. A potential leucine histidine (LH)-zipper was identified in the primary sequence. ROPE recombinants were recognized by postinfection antiserum arguing in favor of a possible involvement of the protein in merozoite invasion and PV formation. The chimeric protein MAEBL described in *P. yoelii* strain YM, is also thought to function during merozoite invasion. Both ROPE and MAEBL were localized to the apical end of merozoites following IFA with recombinant specific antibodies. The rhoptry protein AMA-1 has been identified in all *Plasmodium* species (human, rodent, simian) examined so far (Kappe and Adams 1996; Thomas et al. 1994; Dutta, Marshall and Chauhan 1995). In addition, Rhop-3, RAP-1/2 and Py 235 genes have been identified in *P. falciparum*, *P. vivax* and different rodent *Plasmodium* species based on EST sequences available in the public data bases. A *P. yoelii* rhoptry protein of 160 kDa was identified by monoclonal antibodies (Murakami and Tanabe 1985). Its function is unknown. Heinne et al. (2000) identified a 68 kDa *P. yoelii* rhoptry protein, PyAG1 possessing Arf-1-GTPase activity. The gene encoding PyAG1 was identified using a rhoptry specific monoclonal antibody (Heinne et al. 1999).

RHOPTRY PROTEIN FUNCTION

The rhoptries are secretory organelles. Their biogenesis through the endoplasmic reticulum and Golgi support this view. Futhermore, as demonstrated in *T. gondii*, the rhoptries are acidic, further evidence for their secretory property (Shaw, Roos and Tilney 1999). Rhoptries of other apicomplexans will need to be investigated to confirm the similarity in organelle pH. Rhoptry proteins are important vaccine candidates for malaria due to their highly conserved structure. Three leading

candidates for an asexual stage malaria vaccine are AMA-1, RAP-1 and RAP-2 (Facer and Tanner 1997). The collective data so far indicates that rhoptry proteins have diverse roles. Rhoptry proteins are expressed on the merozoite surface, bind to the host erythrocyte, are secreted into the host cell membrane during invasion (Thomas et al.1994; Howard et al.1998; Sam-Yellowe, Shio and Perkins1988; Sam-Yellowe and Perkins 1991) and rhoptry proteins with proteolytic activity have also been described (Braun-Breton, Rosenberry and Pareira da Silva 1988).

Rhoptry specific antibodies inhibit invasion in vitro and infected individuals make antibodies that recognize rhoptry proteins, a strong indication that rhoptry proteins are accessible to the host immune system.

Rhoptry proteins of *T. gondii* participate in host cell entry, PVM modification and pore formation in the vacuole for solute transport (Dubremetz et al. 1998), and as such present a good target for immunological responses. *Toxoplasma gondii* proteins of 36 kDa to 38 kDa recognized by a monoclonal antibody and localized to rhoptries and dense granules possess acid phosphatase activity (Metsis, Pettsersen and Petersen 1995). In addition, reactivity of *T. gondii* apical organelle specific antibodies was obtained in *Hammondia hammondi* and *Neospora caninum*, suggesting a conservation of many organelle proteins across the apicomplexa (Riahi et al. 1999). Plasmid DNA vaccines encoding GRA1, GRA7 and ROP-2 provided partial protection against challenge with *T. gondii* (Vercammen et al. 2000), suggesting the potential uses of other apical complex genes such as those from *Plasmodium* species in DNA immunization studies. A 22 kDa merozoite specific rhoptry protein of *Eimeria neischulzi* with an unspecified role, is released during invasion and localized to the PV (Rick, Dubremetz and Entzeroth 1998). In addition, no function has been described for RAP proteins of *Babesia*. In *Babesia* multiple copies of the RAP-1 rhoptry gene family were reported in all species of *Babesia* examined (Dalrymple et al. 1993). The immunogenicity of RAP-1 and the inhibition of *Babesia* infection in vitro using RAP-1 specific antibodies suggests that RAP-1 may play a significant role during bovine erythrocyte invasion. In addition, a 48 kDa *B. caballi* rhoptry protein was investigated as a marker protein for serologic ELISA to identify infected horses (Ikadai et al. 2000). When whole rhoptries of *Babesia* were used for immunizing cattle, partial protection was observed, suggesting that other rhoptry proteins in addition to RAP-1 may induce inhibitory antibodies (Machado et al. 1999) in infected animals. In *Theilaria parva* which parasitizes bovine lymphocytes the apical complex

organelles do not function directly in invasion. The organelles discharge during the escape of the parasite from the enclosing vacuole following cell entry. Three apical complex proteins of *T. parva* have been described and they include a 104 kDa rhoptry-microneme protein and two microsphere proteins (Iams et al. 1990; Skilton et al. 1998). Their role in invasion is not known.

BINDING OF RHOPTRY PROTEINS TO ERYTHROCYTES

The erythrocyte binding proteins (EBPs) of *Plasmodium* species can be classified into four groups; 1) The variable merozoite surface protein-1 (MSP-1) which binds to sialic acids on glycophorin (Perkins and Rocco 1988); 2) The microneme derived EBPs, such as EBA-175 of *P. falciparum* which also binds to sialic acids on glycophorin (Sim 1995), the Duffy antigen binding proteins of *P. vivax* and *P. knowlesi* which are also of microneme origin; 3) The reticulocyte binding proteins, PvRBP-1 and PvRBP-2 also apically derived, participate in *P. vivax* merozoite invasion into reticulocytes (Barnwell and Galinski 1998). Py 235 of *P. yoelii* which shares an area of limited homology with PvRBP-2 binds preferentially to mature erythrocytes and not reticulocytes. However, the specific binding domain for Py 235 has not been identified. 4) The high molecular weight rhoptry proteins of *P. falciparum*, Rhop-H that binds human and mouse erythrocytes, mouse reticulocytes, membrane ghosts and inside-out-vesicles (IOVs) (Sam-Yellowe and Perkins 1991). The Rhop-H proteins bind the erythrocyte membrane in association with the serine rich antigen (SERA). The *P. falciparum* Rhop-H complex and SERA also bind to liposomes, showing a preference for phosphatidylserine (PS) containing liposomes substituted with short-chain fatty acids (Perkins and Ziefer 1994). Native MSP-1 and peptide fragments from MSP-1 induce the binding of Rhop-H and SERA to intact human erythrocytes (Sam-Yellowe and Perkins 1991). The Rhop-3 gene homologue was recently identified in the rodent *Plasmodium* species, *P. yoelii*, *P. berghei* and *P. chabaudi* (Anthony et al. 2000; Sam-Yellowe et al. in press). The rodent *Plasmodium* Rhop-3 gene product shares erythrocyte binding properties with the *P. falciparum* protein suggesting a similarity in the function of the proteins among *Plasmodium* species

The receptor binding domain of the Rhop-3 protein has not been defined. No typical adhesion motifs were identified in the protein sequence (Brown and Coppel 1991). *P. falciparum* invades mouse erythrocytes and the Rhop-3 protein is also

secreted into the mouse erythrocyte membrane as seen in human erythrocytes (Sam-Yellowe, Shio and Perkins 1988; Sam-Yellowe and Perkins 1990).

In additional studies the Rhop-H and SERA proteins bound to intact erythrocytes following treatment of the erythrocyte with membrane modulating agents such as 2-(2-methoxyethoxy) ethyl-8-(cis-2-n-octylcyclopropyl) octanoate (A2C) and myristoleyl alcohol (MA) (Ndengele et al. 1995). These results suggest that membrane pertubation might be a requirement for Rhop-H protein binding to erythrocytes during merozoite invasion. The EBPs in group 2 are characterized by cysteine rich motifs referred to as Duffy binding-like (DBL) domains which mediate binding to the erythrocyte membrane. Additional EBP homologues with the conserved cysteine-rich motifs have been described in the rodent *Plasmodium* species, *P. berghei*, *P. chabaudi*, *P. vinckei* and *P. y. yoelii* (Kappe et al. 1996), and in *P. cyanomolgi* (Okenu et al. 1997). Furthermore, regions containing the adhesion motifs have also been mapped in *P. knowlesi* and *P. vivax* (Ranjan and Chitinis 1999).

The EBPs of the rodent *Plasmodium* species investigated in *P. berghei* and *P. yoelii*, possess sequence homology to the Duffy binding domain of the DBL family of EBA-175. A new chimeric protein designated MAEBL possesses sequence homology to the ectodomain fragment of AMA-1 (duplicated in the chimera) and the Duffy binding domain of the DBL family of EBA-175. MAEBL bound to human erythrocytes in a rosetting assay, following expression of the protein in COS-7 cells (Kappe et al. 1998). Major questions still unresolved include the following: a) is apical complex protein binding to the erythrocyte by itself necessary for merozoite invasion?; b) are EBPs of apical origin necessary for parasite survival? Further investigations are required to address these important questions.

RHOPTRY BIOGENESIS

Studies have shown that rhoptries are formed and distributed concurrently with other subcelullar organelles, with proteins destined for the organelle synthesized and routed through the rough endoplasmic reticulum (RER) and Golgi in a regulated secretory pathway. Treatment of parasites with the fungal metabolite Brefeldin A (BFA) which blocks anterograde transport from the ER to the Golgi, and parasite incubation at 15°C block protein transport in the cis-Golgi, further evidence for the Golgi and functional secretory pathway (Haldar 1994). In *P. falciparum*, using transcript analysis of different developmental stages in the schizont and subcellular

fractionation of rhoptries at different stages of "maturity", assembly of the rhoptries was found to be staggered, with the detection of transcripts encoding the high and low molecular weight rhoptry proteins at different times (Jaikara et al. 1993). Pre-rhoptry compartments were also identified suggesting that the rhoptries are formed from vesicular compartments that may contain one or more proteins at the onset of schizogony (Jaikara et al. 1993).

For RAP-1, Py 235 and MAEBL post-translational processing and import of the proteins are inhibited by BFA, while for Rhop-3, post-translational import into the organelles, but not processing is inhibited by BFA (Howard and Peterson 1995; Ogun and Holder 1994; Sam-Yellowe 1996). The Rhop-H and RAP-1/2/3 proteins like MAEBL are synthesized before the formation of the organelles. MAEBL and Rhop-3 are observed as part of the intracellular network prior to their presence in the rhoptries (Sam-Yellowe et al. in press, a; Noe, Fishkind and Adams 2000). MAEBL accumulates in a short-lived tubular reticular network of the ER (TuRNER) at the onset of schizogony (Noe, Fishkind and Adams 2000). An ER chaperone protein, BiP, and Pf39 (an ER resident calcium binding protein) colocalized with MAEBL. In addition the cis-Golgi marker ERD2 also colocalized with MAEBL (Noe, Fishkind and Adams 2000). Disruption of the RAP-1 gene resulted in the retention of RAP-2 in the ER lumen (Baldi et al. 2000). A rhoptry gene encoding a protein with ADP-ribosylation Factor-1 GTPase-activating protein (Arf1-GAP) was recently identified in *P. yoelii*. Gene homologues were identified in other *Plasmodium* species (Heinne et al. 2000). The presence of Arf1-GAP and the data from RAP-1 gene disruption studies provides further evidence for the function of a classical secretory pathway involving the Golgi, ER and coated vesicles in the biogenesis of the rhoptries. To fully understand the mechanisms involved in rhoptry protein secretion and sorting, subcellular fractionation of the rhoptries, electron microscopy and molecular techniques such as gene disruption will be required. Processing of rhoptry proteins occurs within the pre-rhoptry compartments or within the mature organelle to "activate" the rhoptry proteins. Evidence from *T. gondii* in association with the ROP1 rhoptry protein supports the former hypothesis (Soldati et al. 1998).

APICAL ORGANELLES OF OOKINETES AND SPOROZOITES

The ookinete and sporozoite make up the other invasive stages of *Plasmodium* species. The ookinete traverses the mid-gut epithelium of the mosquito,

while the sporozoites develop within the oocyst and penetrate the salivary glands where they attain infectivity. Following a blood meal the sporozoites invade hepatocytes. Despite the similarity in mode of host cell entry and the implication of apical complex organelles in the invasion process of the mosquito invasive stages, apical complex organelle morphology, structure and organization has been poorly studied. Earlier studies indicated that ookinetes lacked rhoptries (Aikawa 1988). Additional studies indicated the presence of rhoptries in *P. yoelii* ookinetes (Sinden, Hartley and Winger 1985). Ookinetes can be transformed easily in vitro (Sinden, Hartley and Winger 1985) and methods are available to isolate the apical complex organelles for further study. Future morphological investigations are required to clarify the organization of the apical complex in ookinetes. There are indications that rhoptries and micronemes are more numerous in the ookinete (Sinden 1999) than in merozoites or sporozoites. Following contact of ookinetes with cells of the mid-gut epithelia, rhoptry and microneme contents are secreted, a PV vacuole is formed but soon after degraded (Sinden 1999). The ookinete transforms into an oocyst upon reaching the basal lamina. A transmission electron micrograph showing the apical complex organelles of *P. gallinaceum* ookinetes is shown in Figures 3A and B. Unlike the merozoite and similar to the sporozoite the ookinete has a well developed conoidal ring (R) and polar ring (P) structure associated with a network of microtubules (MT) that make extrusion of the apical tip possible (Figs. 3A and B). This would certainly facilitate "burrowing" of the ookinete through the mid-gut wall.

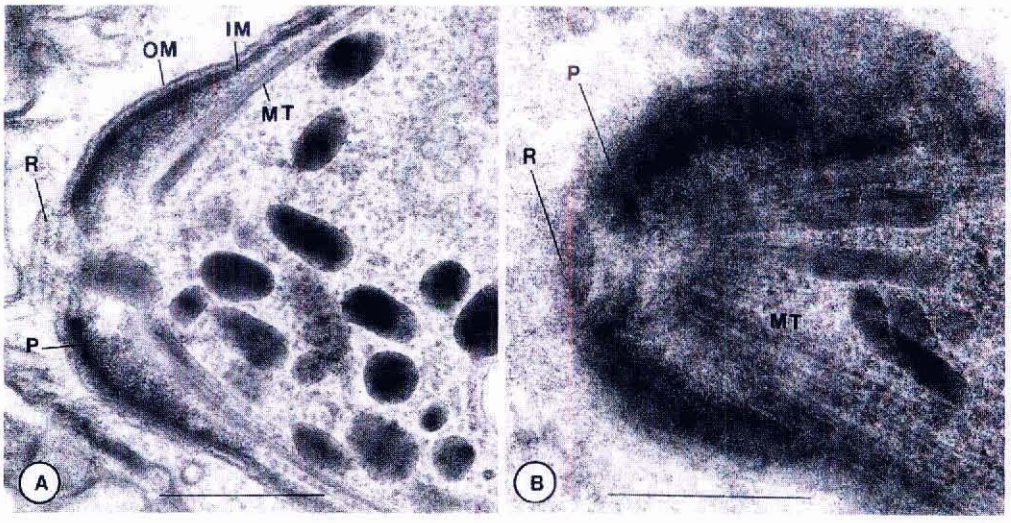

Figure 3. Transmission electron micrograph of *Plasmodium gallinaceum* ookinetes.
Panels A and B show the apical region containing apical complex organelles. OM = outer unit membrane; IM = inner membrane complex; MT = microtubules; P = polar ring; R = conoidal ring; Bar = 0.5 micron

In the sporozoite stage, the circumsporozoite (CS) protein and thrombospondin anonymous protein (TRAP) originating from the micronemes have significant functions in the development of the oocyst and sporozoites, motility of the sporozoites, and invasion into hepatocytes (Beier and Vanderberg 1998; Frevert and Crisanti 1998). A microneme protein circumsporozoite- and TRAP-related protein (CTRP) was identified in *P. berghei* ookinetes (Dessens et al. 1999). CTRP is a microneme protein and represents the first apical complex protein identified in the ookinetes (Dessens et al. 1999). Disruption of the *CTRP* gene resulted in reduction in ookinete motility and infectivity of the midgut epithelium leading to poor development of oocysts. The inclusion of CTRP in the large family of adhesion proteins due to the presence of specific structural motifs suggests that CTRP may be involved in cell adhesion (Dessens et al. 1999). Investigations using specific gene deletion of the *CS* (Menard 1997), *TRAP* (Sultan et al. 1997) and *CTRP* genes in *P. berghei* sporozoites and ookinetes confirm their role in the oocycst and sporozoite development and in the activity of the ookinete. However, invasion of host cells by ookinetes, sporozoites and merozoites results in the formation of a "parasitophorous vacuole" which in the case of the ookinete rapidly disappears since the ookinete transformation into oocysts occurs extracellularly (Sinden 1999). The micronemes in the ookinete also contain and secrete chitinases (Langer et al. 2000) which are used for penetrating the chitin-containing peritrophic matrix as the ookinete makes its way to the midgut. The chitinases unlike CSP, TRAP and CTRP lack adhesive domains. All three major apical complex organelles are derived from the ER and Golgi (Sinden 1999). Rhoptries and micronemes of ookinetes form aggregated electron dense particles that move anteriorly within the cytoplasm of the ookinete following their formation (Sinden 1999).

CONCLUSIONS AND FUTURE CHALLENGES

Apical complex proteins are unique among the protozoa. To fully understand the role of the organelles, isolation of the organelles, molecular characterization of organelle contents and identification of conserved motifs among the genes will be required. Furthermore, the basis for specific types of protein-protein associations i.e. protein complexes and protein-membrane interactions will be identified. Improvements in the techniques for organelle isolation, and establishment of in vitro targeting and import experiments are needed. Future research efforts should continue

to utilize the techniques of targeted gene deletion, gene disruption and parasite transformation to determine the function of specific rhoptry genes and gene products. With the availability of genomic data bases and expressed sequence tags (ESTs), newly identified genes can be rapidly analyzed. The use of bioinformatics, proteomics and microarray technology will undoubtedly enhance our understanding of organelle biology at the molecular level and further provide an explanation for the mechanism of organelle function during host cell invasion. It appears that many *P. falciparum* field isolates can invade the host erythrocyte using mechanisms independent of sialic acids (Okoyeh, Pillai and Chitinis 1999; Cowman et al. 2000). This observation underscores the need to determine what role the non-sialic acid binding proteins play during invasion. Furthermore, peptides in MSP-1 and EBA-175 have been reported to bind independently of glycophorin A and sialic acid (Jakobsen et al. 1998; Nikodem and Davidson 2000). These data suggest that proteins involved in major and alternate invasion pathways will be required in the development of effective vaccines against malaria. The new technologies will permit rationale vaccine design that takes into account important domains of apical complex molecules.

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