

Secretion of *Plasmodium falciparum* Rhoptry Protein into the Plasma Membrane of Host Erythrocytes

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Abstract. The rhoptry is an organelle of the malarial merozoite which has been suggested to play a role in parasite invasion of its host cell, the erythrocyte. A monoclonal antibody selected for reactivity with this organelle identifies a parasite synthesized protein of 110 kD. From biosynthetic labeling experiments it was demonstrated that the protein is synthesized midway through the erythrocytic cycle (the trophozoite stage) but immunofluorescence indicates the protein is not localized in the organelle until the final stage (segmenter stage) of intraerythrocytic development. Immunoelectron microscopy shows that the protein is localized in the matrix of the rhoptry organelle and on

membranous whorls secreted from the merozoite. mAb recognition of the protein is dithiothreitol (DTT) labile, indicating that the conformation of the epitope is dependent on a disulfide linkage. During erythrocyte reinvasion by the extracellular merozoite, immunofluorescence shows the rhoptry protein discharging from the merozoite and spreading around the surface of the erythrocyte. The protein is located in the plasma membrane of the newly invaded erythrocyte. These studies suggest that the 110-kD rhoptry protein is inserted into the membrane of the host erythrocyte during merozoite invasion.

ERYTHROCYTE invasion by the malarial merozoite is a multi-step process, initiated by receptor-mediated binding of the parasite to its host cell (9). Electron microscopic studies show that penetration of the erythrocyte by merozoites involves invagination of the erythrocyte membrane where the apical end of the merozoite contacts the host cell (1). A moving membrane junction is formed and the contact maintained while the merozoite is internalized into a vacuole, which eventually forms the parasitophorous vacuole. Intracellular development of the parasite occurs inside this vacuole. Although it is well documented that invasion occurs by erythrocyte membrane invagination, the biochemical mechanisms whereby the parasite induces such a profound alteration in the rigid membrane-cytoskeletal organization of the erythrocyte are not understood.

Endocytosis is not observed in erythrocytes except in drug-induced instances (25). It has been proposed that the malarial parasite must initiate the membrane changes by some heretofore unknown process. Implicated in this unusual process are the rhoptries, a pair of electron dense organelles found in plasmodia and in other closely related members of the apicomplexa which are obligate intracellular parasites. Rhoptries of *Toxoplasma*, *Sarcocystis* and *Besnoitia* species have all been implicated in the invasion process. In *Toxoplasma*, a penetration enhancement factor possessing lytic activity has been identified, and is believed to be secreted by the rhoptries during invasion (15). In plasmodia the rhoptries are club-shaped organelles located randomly in the cytoplasm in the preinvasive stages of the parasite, that appear to subtend ducts to the exterior of the apical portion of the

merozoite at the time of invasion. Electron microscopic studies of *Plasmodium knowlesi* merozoites suggest that the contents of rhoptries are lost from the organelle during or shortly after invasion (1, 2, 12). However, no direct involvement of rhoptry components in invasion has been revealed.

Intraerythrocytic development of the *P. falciparum* parasite proceeds through several well defined stages during its 48-h cycle: the ring stage (0–16 h), trophozoite stage (16–24 h), schizont stage (24–44 h), and segmenter stage (44–48 h). Each schizont produces 16 merozoites, each containing 2 rhoptries. Ring stage parasites do not contain rhoptries and thus the assembly at the schizont stage must be de novo. Recently, monoclonal antibodies against merozoite antigens of various *Plasmodium* species have been found to identify rhoptry proteins. In *P. falciparum*, two different families of rhoptry proteins have been reported, one containing proteins of 155, 145, 132 and 110 kD (10, 26) and another containing proteins of 82, 65, and 40 kD (5, 7, 11, 16, 20, 23). In the present study, we have characterized a 110-kD protein located in *P. falciparum* merozoite rhoptries. With the availability of a monospecific antibody directed against the 110-kD protein it was possible to show directly that a rhoptry protein is secreted into the erythrocyte membrane at the time of merozoite invasion.

Materials and Methods

In Vitro Cultivation of *Plasmodium falciparum*

The FCR-3 (Gambia) strain of *P. falciparum* was cultured in vitro according to the method of Trager and Jensen (29). *P. falciparum* was grown in human

type A⁺ erythrocytes at 5% hematocrit in RPMI 1640-Hepes medium supplemented with 10% human serum and 20 mM glucose. To achieve parasite synchrony, schizont-infected cultures were fractionated by gelatin flotation (17). Parasites from the same synchronous culture were used in the experiments on the stage-dependent synthesis of the rhoptry protein. Schizont-infected erythrocytes concentrated by gelatin flotation, were resuspended with fresh erythrocytes at a parasitemia of 6% and reinvasion allowed to take place. The time of erythrocyte reinvasion when parasites were 0–6 h was defined as T-3. At each time point, T-3 (rings), 21 h (trophozoites), 33 h (mid-schizont), 41 h (segmenters, free merozoites) and 48 h (reinvaded rings), parasites were prepared for immunoblotting and IFA as described below.

Production of Monoclonal Antibody IB9

Hybridomas secreting mAb directed against *P. falciparum* antigens were produced as described (21). Hybridoma supernatant reacting with rhoptries were selected by IFA. One hybridoma, IB9, reacting with the rhoptry was selected and cloned by limiting dilution. Spent medium from in vitro-grown cloned hybridoma IB9 cultures were used as the source of mAb. Culture supernatants were concentrated 100 times by ultrafiltration (XM50; Amicon Diaflo, Danvers, MA).

Immunoprecipitation

100 μ l of mAb IB9 was incubated with 50 μ l of goat anti-mouse IgG-sepharose 4B (Cappel Laboratories, Malvern, IL) for 1 h at room temperature, and washed three times in Buffer A (1% BSA, 1% NP40, 1 mM EDTA in PBS). Extracts of schizont-infected erythrocyte labeled with [³⁵S]methionine were prepared (21). The beads were incubated with 100 μ l of [³⁵S]methionine-labeled parasite extracts for 1 h at room temperature, and then washed, twice in buffer A, once in buffer B (1% BSA, 1% NP40, 1 mM EDTA, 0.5 M NaCl in PBS), and once in buffer C (1% NP40, 1 mM EDTA in PBS). The beads were boiled in 100 μ l of electrophoresis sample buffer (0.1 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS and 0.001% bromophenol blue) with or without 100 mM dithiothreitol (DTT). The samples were subjected to electrophoresis on a 5–15% SDS-polyacrylamide gel. The gels were treated with Enhance (New England Nuclear, Boston, MA) before drying for exposure to X-Omat AR5 film.

Immunoblotting

Extracts of parasites collected at T-3 h, 21 h, 33 h, 41 h, and 48 h, were separated on 5–15% gradient SDS-PAGE gels under nonreducing conditions and transferred to nitrocellulose paper. The transfer was carried out in 20 mM Tris, 0.15 M glycine and 20% methanol at a constant current of 150 mA for 12 h at 4°C. Nitrocellulose paper was blocked in 0.1% Tween 20 in Tris-saline buffer (10 mM Tris, 0.9% NaCl pH 7.4) for 1 h at room temperature and then incubated with mAb IB9 in 20% fetal bovine serum in Tris-saline buffer. After washing, the antibody bound to the protein was detected by [¹²⁵I]rabbit anti-mouse IgG (New England Nuclear) (1 \times 10⁶ cpm/ml).

Indirect Immunofluorescence Assay (IFA)¹

Thin smears of *P. falciparum* cultures collected at T-3, 21, 33, 41, and 48 h were acetone-fixed for 10 min at 4°C and incubated with mAb IB9 for 1 h at room temperature in a humidified atmosphere. After three washes in PBS the slides were incubated with FITC-goat anti-mouse Ig (Boehringer-Mannheim) diluted 1:20 in PBS for 45 min at room temperature, washed in PBS and then in distilled water. Some slides were counterstained with ethidium bromide (10 mg/ml) for 30 s and then rinsed in distilled water. The slides were mounted with 50% glycerol in PBS, and examined by a Nikon Labophot microscope.

Immunoelectron Microscopy with IB9

Parasite pellets containing mature schizonts and reinvasion merozoites were fixed in 0.05% glutaraldehyde in 0.1 M cacodylate pH 7.4 for 15 min at 4°C. The fixed cells were centrifuged at 10,000 g for 5 min, dehydrated in graded alcohol, and then embedded in LR White resin (Ernest F. Fullam, Inc., Latham, NY). The sections were collected on Formvar carbon-coated

1. Abbreviations used in this paper: DOC, deoxycholate; IFA, immunofluorescence assay; NP-40, nonidet P40.

nickel grids. Grids with sections were blocked in PBS containing 0.5% BSA for 10 min followed by incubation with concentrated mAb IB9. The grids were incubated for 3.5 h at room temperature, washed in PBS, and then incubated with protein A bound to 5-nm gold particles for 30–60 min at room temperature. Some sections were stained with uranyl acetate.

Samples reacted with mAb IB9 before embedding were prepared by washing parasite pellet in 0.1 M cacodylate buffer after fixation, followed by PBS-0.5% BSA, then reacting pellet with 1:10 dilution of mAb IB9. The samples were incubated for 2 h at room temperature with manual agitation, washed in PBS and incubated with protein A-gold (20 nm) for 30 min. The pellets were washed in PBS, dehydrated in alcohol, and embedded in LR white resin.

Results

Rhoptry Proteins Identified by Reactivity with mAb IB9

Immunoblots of total parasite extracts demonstrate that mAb IB9 recognized two proteins of 110 and 100 kD respectively (Fig. 1, lane a). Under reducing conditions of SDS-PAGE, neither protein was detected, indicating that the epitope recognized by mAb IB9 is reduction labile (Fig. 1, lane b). Upon immunoprecipitation of [³⁵S]methionine labeled *P. falciparum* extracts with mAb IB9, a 110-kD protein was detected, and in addition, minor proteins of 155, 140, and 130 kD, respectively, were also immunoprecipitated (Fig. 1, lane c). When the immunocomplexes were separated by SDS-PAGE under non-reducing conditions, the same [³⁵S]methionine-labeled proteins were identified although they all migrated slightly faster (Fig. 1, lane d), further indication that they contain intradisulphide bonds. Different methods of immunoprecipitation, were used to clarify the relation of the additional proteins immunoprecipitated with mAb IB9. Under all conditions, the 155-, 140-, and 130-kD protein were immunoprecipitated (data not shown). We assume that detection of these proteins is due to coprecipitation.

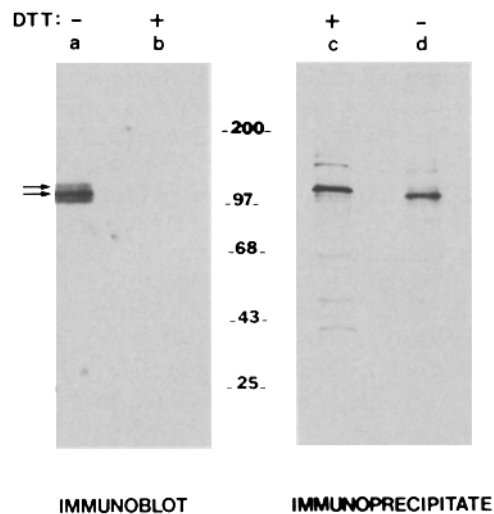


Figure 1. Immunoblotting and immunoprecipitation with mAb IB9. (a and b) Immunoblot. Extracts of mature schizont-infected cells were solubilized (a) without or (b) with DTT (100 mM) and processed for immunoblotting with mAb IB9 as described. (c and d) Immunoprecipitation. Cultures labeled with [³⁵S]methionine were extracted with 1% NP-40/0.1% DOC in PBS and immunoprecipitated with mAb IB9 as described. Immunocomplexes were boiled with electrophoresis sample buffer with DTT (c) or without DTT (d). Arrows indicate 110- and 100-kD proteins.

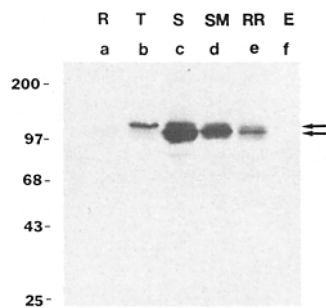


Figure 2. Stage-dependent synthesis and processing of the 110-kD rhoptry protein. Synchronized *P. falciparum*-infected erythrocytes were collected at different points of the intraerythrocytic development and processed for electrophoresis and immunoblotting with mAb 1B9 as described in Materials and Methods. (a) Rings (3 h); (b) Trophozoites (21 h);

(c), Schizonts (33 h); (d), Segmenters (41 h); (e), Reinvaded rings (48 h); (f), Uninfected erythrocytes. Arrows indicate 110- and 100-kD proteins.

Rhoptry Proteins at Different Stages of Parasite Development: Relationship of 110- and 100-kD Proteins

To determine the relationship of the 110- and 100-kD proteins, extracts of parasites were collected at different stages of intraerythrocytic growth and immunoblotted with mAb 1B9 (Fig. 2). At the ring stage *T*-3 h a 100-kD protein was barely detectable (Fig. 2 a). At the trophozoite stage *T*-21 h, (Fig. 2 b) an intensely labeled protein band of 110 kD was detected. At the schizont stage, *T*-33 h (Fig. 2 c) a 100-kD protein was seen along with the 110-kD species. The 100-kD antigen was the predominant band at the segmented schizont stage *T*-41 h (Fig. 2 d) at which point, free merozoites and newly reinvaded rings could be seen on Giemsa-stained

smears. The 100-kD antigen persisted into the next cycle of rings *T*-48 h (Fig. 2 e) indicating that this protein was present in erythrocytes newly reinvaded by the merozoites. Uninfected human erythrocytes (Fig. 2 f) did not show any of the antigens associated with the rhoptry. Biosynthetic labeling studies not shown here confirmed that the 110-kD protein is synthesized at the trophozoite stage and processed to the 100-kD species at the schizont stage. Thus it would appear that the 100-kD species present in ring forms (Fig. 2 a and 2 e) is the processed form persisting from the previous cycle.

Stage-dependent Localization of Rhoptry Proteins

To localize the rhoptry protein throughout the parasite's developmental cycle, thin smears were prepared, of cultures at the different developmental stages, from the same samples used for immunoblotting and metabolic labeling. Interesting differences in the overall distribution of the proteins between the parasite stages was apparent (Fig. 3). Immediately after reinvasion, a faint ring of fluorescence was detected around the membrane of the erythrocyte and also around the newly formed ring (Fig. 3 a, arrows). In trophozoites ~21 h after reinvasion a diffuse fluorescence was detected in the parasite cytosol with a concomitant decrease in the staining of the erythrocyte membrane (Fig. 3 b). At the schizont stage, fluorescence was more intense, indicating higher amounts of the protein but its distribution appeared to be uniform throughout the cytosol of the parasite (Fig. 3 c). In the segmented schizonts, containing fully differentiated merozoites

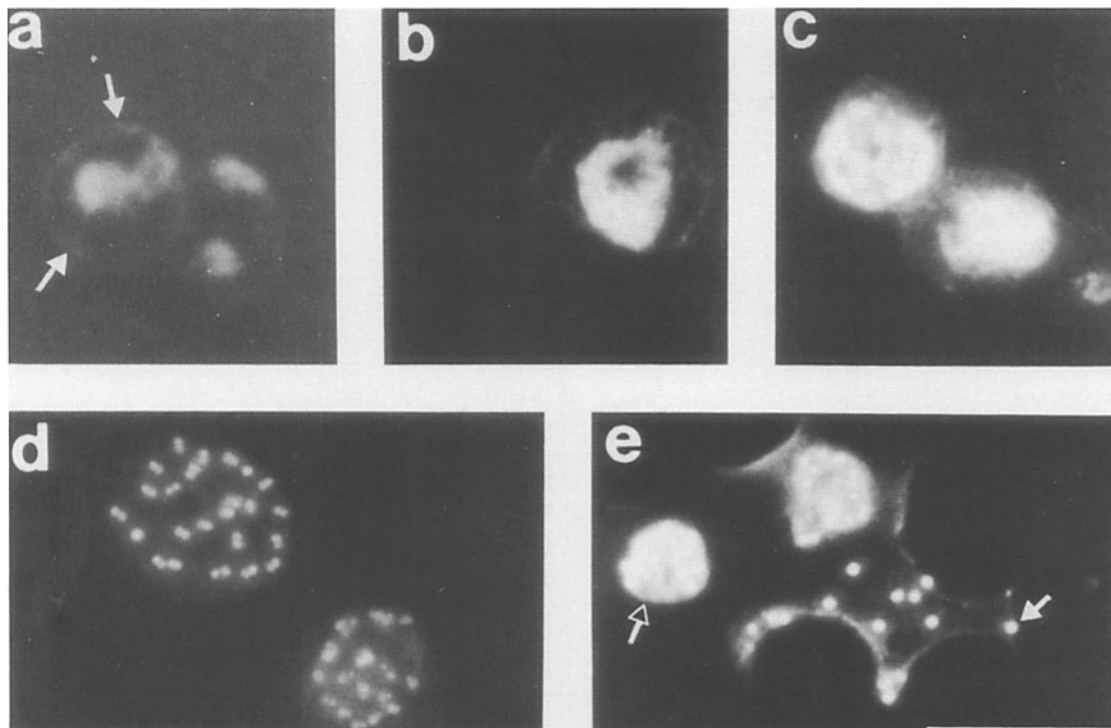


Figure 3. Immunofluorescence localization of the 110-kD protein in *P. falciparum* at different stages. Acetone-fixed smears of parasites from different developmental stages of the intraerythrocytic cycle were incubated with mAb 1B9 and FITC-goat anti-mouse IgG. A, early ring stage (3 h); fluorescence around parasite and around erythrocyte membrane is denoted by arrows. (B) trophozoite (21 h); (C) schizonts (33 h); (D) segmenter (41 h); punctate pattern of immunofluorescence shows the paired organelles in mature segmented schizonts. (E) Free merozoites in the process of reinvading erythrocytes (closed arrow) (48 h). Mature schizonts are also present in field (open arrow). Bar, 10 μ m.

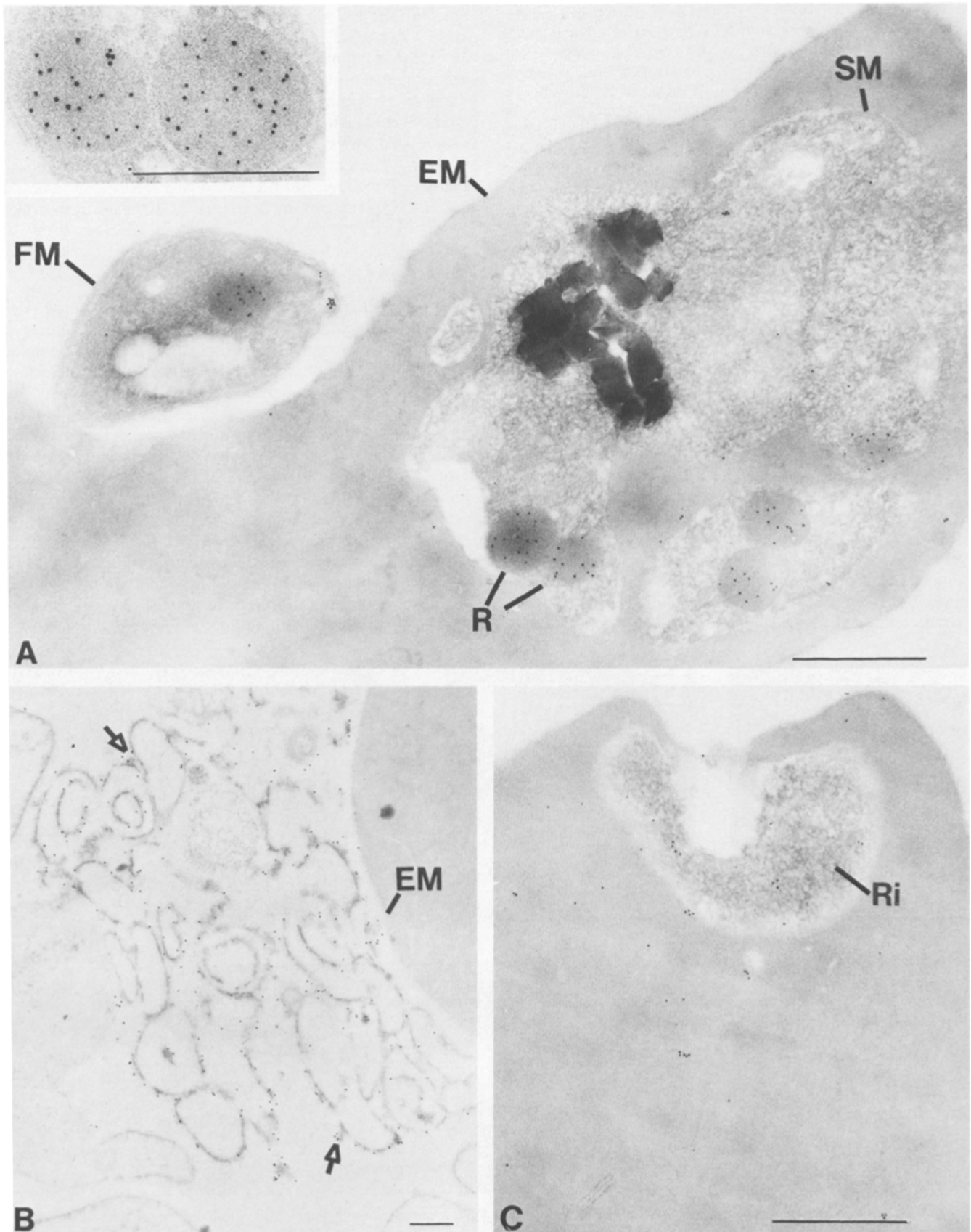


Figure 4. Immunoelectron microscopic localization of the rhoptry protein using mAb 1B9 and protein A-gold. (A) post-embedded immunolabeling of mature schizont-infected erythrocyte showing localization of 5-nm gold particles over rhoptries. *EM*, Erythrocyte membrane; *SM*, schizont membrane; *FM*, free merozoite; *R*, rhoptries. (Inset) High magnification of paired rhoptry organelles showing localiza-

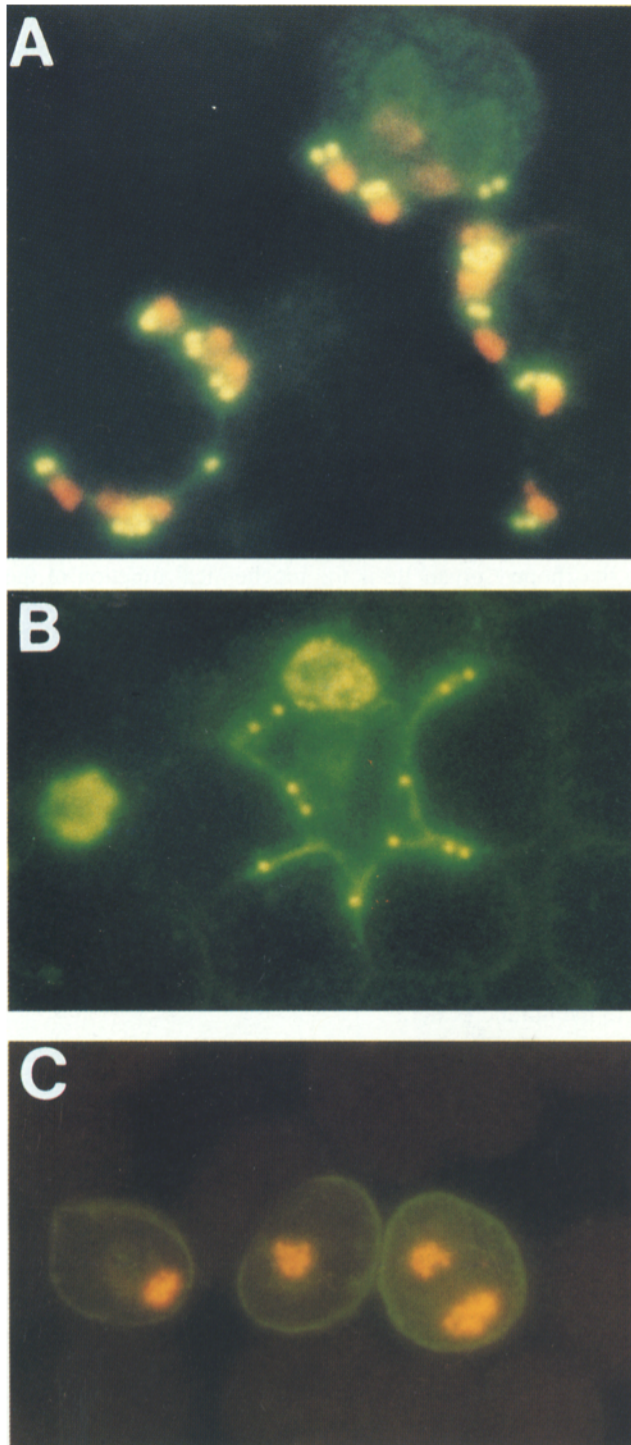


Figure 5. mAb 1B9 Immunofluorescence of merozoite invasion. Thin smears were incubated with mAb 1B9, FITC-goat anti-mouse IgG and counterstained with ethidium bromide. (A and C) to identify parasite nucleus. (A) Merozoites attached to erythrocytes. Erythrocyte at upper right has been invaded by two merozoites. (B) Merozoites in the process of invading; rhoptry protein is discharged around erythrocyte surface. (C) Erythrocytes invaded by merozoites. Rhoptry protein is localized in infected erythrocyte membrane.

and coinciding with the development of completely formed rhoptries, a bright punctate fluorescent pattern could be seen at the apical end of the merozoites (Fig. 3 d). In some cases a single fluorescent organelle could be seen but usually a pair of organelles was resolved. During merozoite release and reinvasion of erythrocytes, the fluorescence could be detected at the point of contact between the merozoite and erythrocyte and the areas immediately surrounding it (Fig. 3 e, arrows).

Electron Microscopic Localization of Rhoptry Protein

In mature schizonts and extracellular merozoites, the bound mAb 1B9 was localized by protein A-gold to electron-dense organelles located at the apical ends of fully differentiated merozoites (Fig. 4 A). In samples incubated with mAb 1B9 before embedding, the antibody reacted with concentric membranous whorls that appeared to be associated with merozoites from the rupturing schizont (Fig. 4 B). In immature parasites (ring stage) the antigen was distributed around the parasite in the parasitophorous vacuole membrane and in the membrane of the erythrocyte (Fig. 4 C).

Localization of Rhoptry Antigen During Reinvasion

In smears counterstained with ethidium bromide, the merozoite nucleus could be seen separately from the rhoptries (Fig. 5 A). During invasion, the rhoptry antigen was detected in the area immediately at the point of contact between the apical end and the erythrocyte membrane and appeared to spread out over the surface of the erythrocyte in a "halo-like effect" (Fig. 5 B). In newly formed rings, the rhoptry antigen was seen to persist in the erythrocyte membrane and weakly in the erythrocyte cytosol. The antigen was seen also around the ring form parasite (Fig. 5, A and C), and this may colocalize with the parasitophorous vacuole as shown in Fig. 5 C. Counterstaining with ethidium bromide showed the ring-stage nucleus to be distinct from the ring of fluorescence around the erythrocyte membrane (Fig. 5 C). The uninfected erythrocytes, stained faintly with ethidium bromide (Fig. 5 C) in the field did not show this membrane staining with the 1B9 antibody. The 110- and 100-kD protein is not synthesized at the ring stage and thus its presence in the erythrocyte membrane must originate from the merozoite of the previous cycle.

Discussion

Parasitic microorganisms often exhibit considerable specificity for the host cell selected for their intracellular development. This is also true in the case of the malarial parasite which has two distinct cycles in its vertebrate host localized in different host cells. The sporozoite invades and develops inside hepatocytes and the extracellular stage of the blood cycle, the merozoite, invades only the erythrocyte. Specificity of merozoite attachment is mediated by surface molecules of the erythrocyte and in the case of *P. falciparum* the receptor has been shown to be the sialic acid residues of glycophorins (18). Although variation between different strains of *P. fal-*

tion of rhoptry antigen. (B) concentric membranous whorls from preembedded sample. Protein A-gold particles of 20 nm are shown distributed around the concentric lamellae (arrows). (C) Post-embedded immunolabeling of ring-stage parasites (Ri). Gold particles of 5 nm are distributed around the parasite and over the erythrocyte cytoplasm. Bars, 500 nm.

ciparum in their requirement for sialic acid has been reported, all strains depend on glycophorin for optimal invasion into human erythrocytes (14, 17). The biochemical events involved in merozoite invasion after attachment to the cell surface are not understood, but are clearly intriguing in view of the highly ordered and rigid nature of the erythrocyte membrane-cytoskeletal network (3). It is assumed that the parasite induces a radical reorganization of the erythrocyte cytoskeleton, responsible for maintenance of cell shape and deformability. Based on electron microscopy it has been proposed that the contents of the rhoptries are involved in this process. However, the nature of the components of the rhoptry has been a point of debate and it has been proposed that they could be lipids. Membranous whorls being discharged from rhoptries have been described (27, 28). With the introduction of monoclonal antibodies several protein components of the organelle have been identified and it now appears that the major components are proteins. In this study, we were able to locate a 110-kD rhoptry specific protein on membranous whorls similar in morphology to those described by others (2, 27, 28).

The biosynthetic studies on the 110-kD rhoptry protein highlight an interesting problem in organelle biogenesis. The proteins identified here begin to be synthesized at the trophozoite stage (*T*-21 h) and at the schizont stage, 12 h later they are still localized diffusely in the parasite cytosol. Rhoptries only begin to form late in schizogony (*T*-41 h). Why its proteins should be synthesized long before the formation of the organelle is an intriguing question. How the organelles are assembled de novo and sorted into the merozoites at the time of segmentation remains to be investigated. By immunofluorescence the rhoptry proteins can be seen to be discharged into the erythrocyte membrane at invasion and persist in the host membrane and cytosol for some time. Since the 110-kD protein is not synthesized in the ring stage, its presence in the erythrocyte membrane must originate from the invading merozoites. A *P. falciparum* protein of 155 kD, localized in micronemes and rhoptries has also been described as being secreted into the erythrocyte membrane during invasion (4, 19). Several other rhoptry proteins have been identified in *P. falciparum* and other malarial parasites (5, 7, 10, 11, 16, 20, 23, 26). In each case a mAb identified a family of proteins associated with the organelle. In two studies (10, 26), a monoclonal antibody was shown to immunoprecipitate proteins of 155, 140, 130, 110, and 100 kD, identical in size to those immunoprecipitated by mAb IB9. However, Holder, et al. (10) showed by immunoblot that only the 140-kD protein was detected indicating that the additional proteins were co-precipitated. In the present study, mAb IB9 only immunoblots the 110/100-kD proteins indicating additional proteins of 155, 140, and 130 kD are probably co-precipitated. We have shown here that all proteins of the 140/110 kD family contain intradisulphide bonds and other studies indicate that both the 80 and 40-kD proteins also contain intradisulphide bonds. It is interesting to note the biochemical similarity between the *P. falciparum* rhoptry proteins and many proteins destined for exocytosis in mammalian cells. Secretory proteins of mammalian cells often contain disulfide linkages e.g. insulin (6), antibodies (22), and pancreatic enzymes (13, 24) which are proteolytically processed from a precursor form to a functional form. The processing of the rhoptry proteins in mature schizonts and merozoites prior to reinvasion may

represent an activation step coinciding with formation of the mature rhoptry organelle. Recently, a cDNA clone encoding the carboxy terminus of a 105-kD rhoptry antigen has been obtained and found to react with affinity purified human antiserum (8). By immunoblot other proteins of 103 and 107-kD were identified and it is possible that these three proteins are the same as those identified in the present study, although details of the biosynthesis of the 105-kD protein were not reported (8). mAb IB9 gave positive immunofluorescence with different geographical strains of *P. falciparum*; 7G8 and It2 (Brazil), CDC-1 (Honduras), FC-27 (New Guinea), and FVO (Vietnam) indicating that the antigen is conserved (data not shown).

The present study demonstrates that a 110-kD rhoptry protein is secreted into the erythrocyte membrane and confirms for the first time that rhoptry contents are discharged from the merozoite during invasion. Although this had been postulated, no direct evidence for rhoptry protein secretion during invasion has been reported. The major question now relating to the rhoptry organelle is the biochemical function of the protein components. It will also be of interest to understand how a protein is inserted in a pre-existing plasma membrane and to identify the target erythrocyte proteins with which they interact. Secreted products of the *Toxoplasma* rhoptries have been reported to be lytic substances which may be involved in the penetration process during invasion (15) and preliminary studies suggest that the 110-kD protein is a protease. It is possible that some of the proteins of the *P. falciparum* rhoptries identified in this study and by others are proteases and the target proteins are components of the erythrocyte membrane cytoskeleton, spectrin, band 4.1, band 3, etc. Alternatively the proteins may have hydrophobic domains and by inserting into the lipid bilayer are capable of dissociating the highly regulated interactions of the erythrocyte membrane proteins.

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