Characterization of a Membrane-associated Rhopty Protein of Plasmodium falciparum*

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Invasive forms of apicomplexan parasites contain secretory organelles called rhoptries that are essential for entry into host cells. We present a detailed characterization of an unusual rhoptry protein of the human malaria parasite Plasmodium falciparum, the rhoptry-associated membrane antigen (RAMA) that appears to have roles in both rhoptry biogenesis and host cell invasion. RAMA is synthesized as a 170-kDa protein in early trophozoites, several hours before rhoptry formation and is transiently localized within the endoplasmic reticulum and Golgi within lipid-rich microdomains. Regions of the Golgi membrane containing RAMA bud to form vesicles that later mature into rhoptries in a process that is inhibitable by brefeldin A. Other rhoptry proteins such as RhopH3 and RAP1 are found in close apposition with RAMA suggesting direct protein-protein interactions. We suggest that RAMA is involved in trafficking of these proteins into rhoptries. In rhoptries, RAMA is proteolytically processed to give a 60-kDa form that is anchored in the inner face of the rhoptry membrane by means of the glycosylphosphatidylinositol anchor. The p60 RAMA form is discharged from the rhoptries of free merozoites and binds to the red blood cell membrane by its most C-terminal region. In early ring stages of the parasite RAMA is found in association with the parasitophorous vacuole.

Plasmodium falciparum malaria is one of the most important infectious diseases of humans, accounting for ~2 million deaths each year. The stages of the parasite that grow and multiply in red blood cells (RBCs) cause all the pathological effects associated with the disease, and accordingly invasion of red blood cells is one of the most important steps in the parasite life cycle. Three sets of secretory organelles, the rhoptries, micronemes, and dense granules are involved in and are essential for the invasion process. The understanding of the role of these organelles provides important knowledge about the basic biology of malaria and potential therapeutical targets.

Rhoptries of Plasmodium parasites are paired club-shaped organelles located at the apical end of merozoites, the form of the parasite that invades RBCs. Following the attachment of merozoites to the RBC surface, rhoptries discharge their contents onto the RBC membrane. Rhoptry organelles disappear after internalization of merozoites and thus are formed de novo with each erythrocytic cycle. Rhoptry formation occurs late in the erythrocytic stages of the parasite, and elucidation of rhoptry biogenesis of malaria parasites has been hindered by the lack of early organelle markers. Most of our knowledge is based on microscopic examinations, which suggest that rhoptry biogenesis follows the secretory pathway route, with rhoptry organelles being formed by sequential fusion of post-Golgi vesicles (2, 3), although why particular vesicles are selected is unclear.

Rhoptry contents include both protein and lipid components, which assemble to form membrane-like structures. Protein constituents of the rhoptry contents are still being defined. To date several rhoptry proteins have been identified and studied. Some have been characterized at the molecular level, whereas others are defined by immunological reagents (reviewed in Refs. 4 and 5). Most rhoptry proteins are expressed relatively late in the maturation of the parasite, are either soluble or transmembrane, undergo limited proteolytic modifications, and are located in the body of schizont rhoptries. Rhoptry proteins have been implicated as having a role in the invasion process. For example, RhopH3 has been shown to be discharged and to bind to RBC membrane components (6, 7), and antibodies to RAP1 and RAP2 inhibit RBC invasion by Plasmodium merozoites in vitro (8–10). Furthermore, immunization of monkeys with RAP1 conferred partial immunity against P. falciparum infection (11). Limited functional data is available for rhoptry proteins. For example, RAP1 is involved in transport of RAP2 to the rhoptry, but neither RAP1 nor RAP2 are required for invasion (12), whereas the gp76 rhoptry protein is a serine protease that cleaves RBC membrane components band 3 and glycoporphin A after being discharged from rhoptries (13).

Previous work identified an 833-bp cDNA clone, named Ag512, that encoded a fragment of a 55-kDa protein localized to rhoptries by indirect immunofluorescence (14). Labeling studies with 3Hmyristate and 3Hglucosamine were consistent with the presence of a GPI moiety, although an attachment motif for such an element was not encoded within the then known cDNA sequence. We present a characterization of the protein encoded by this gene, now designated the rhoptry-associated membrane antigen (RAMA), and show that it encodes an unusual rhoptry protein that is made early in parasite development.
maturation and appears to have a role both during rhoptry biogenesis and the red cell invasion process.

EXPERIMENTAL PROCEDURES

P. falciparum Material—P. falciparum 3D7 parasites were cultured in vitro using routine methods (15), and parasite extracts were prepared as previously described (16). Time-course parasite extracts from a synchronized culture expression were kindly provided by Dr. T. Wu and prepared as described (17). The P. falciparum cDNA library in XZAP phage (Stratagene) was a gift from Dr. D. Fidock.

DNA Amplification and Sequencing—The sequence of the RAMA locus was obtained from sequencing Ag512-positive cDNA clones and by gene walking by performing vectorette PCR reactions (18) on libraries prepared from P. falciparum genomic DNA, and directly sequencing the products. For intron mapping, corresponding RAMA fragments from genomic and cDNA were PCR-amplified and sequenced. For production of recombinant proteins, RAMA fragments were PCR amplified from cDNA (see Table I for primer sequences). DNA sequencing was performed as previously described (16). The protein sequence was analyzed using proteomic tools at www.expasy.org.

Recombinant Protein Expression and Raising Polyclonal Antibodies—Recombinant RAMA fragments were expressed and purified as GST fusion proteins as described previously (16) using pGEX-3X and pGEX-4T vectors. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The C-terminal fragment of RhopH3 protein, Ag44 (19), expressed as a GST fusion, was kindly provided by F. Motornia.

Polyclonal rabbit antisera to recombinant proteins were raised and tested as described previously (16). Polyclonal mice antisera were raised in 6- to 8-week-old BALBc female mice in a similar fashion. Four mice were used for each protein, and injections were carried out intraperitoneally using 25 μg of recombinant protein. SDS-PAGE and Immunoblotting—Recombinant proteins and parasite lysates were fractionated by SDS-PAGE on 12% polyacrylamide gels and blotted as described (18). Primary antibodies were detected with anti-rabbit or anti-mouse immunoglobulin conjugated to horseradish peroxidase and developed as described (16).

Immunocytochemical Methods—Indirect immunofluorescence assays (IFAs) by confocal microscopy were performed as described (16). Anti-RAMA and anti-Ag44 (RhopH3) raised rabbit and mouse sera, anti-FGRP and anti-PEER2 rabbit antibodies (Malaria Research and Reference Reagent Resource Center catalogue numbers MRA-29 and MRA-72, respectively), anti-merozoite surface protein 4 (MSP4) mouse antibodies (kindly provided by Dr. L. Wang), monoclonal anti-RAP1 or anti-RAP2 antibodies (kindly provided by Dr. L. Martin), and rabbit anti-AMA1 antibodies were used as primary antibodies. Alexa 488- and Alexa 568-conjugated anti-mouse and anti-rabbit antibodies (Molecular Probes, Eugene, OR) were used as secondary antibodies. Fluorescence resonance energy transfer (FRET) was performed using the acceptor photobleaching method (20), with Alexa 488- and Alexa 564-conjugated secondary antibodies. Image analysis was done using National Institutes of Health Image computer software. FRET was quantified as a function of increased fluorescence intensity of the donor fluorophore from the formula, \( E = 1 - (F_{\text{FRET}}/F_{\text{p}}) \), where \( E \) is the FRET efficiency and \( F_{\text{FRET}} \) and \( F_{\text{p}} \) are fluorescence intensities of the donor fluorophore before and after photobleaching, respectively. Immunoelectron microscopy was performed as described before (21) using raised rabbit anti-RAMA-D serum.

Brieffield and Low Temperature Treatment—The parasite culture was synchronized by the sorbitol lysis method twice (17), then split five ways, and parasites from one part were harvested as T0 sample. The four remaining parts were then cultured as follows. BFA (Sigma) was added to one culture flask to a final concentration of 5 μg/ml, from a 5 mg/ml stock in 99.7% (v/v) ethanol. An equal volume of 99.7% (v/v) ethanol to that delivered with BFA was added to one flask serving as a control. Both flasks were incubated at 37 °C. Two other flasks with standard culture media were incubated either at 13 °C or at 17 °C. After 8-h incubation parasites were harvested, and along with the T0 sample analyzed by immunoblots with anti-RAMA sera.

Triton Solubility—Parasites were harvested and incubated for 30 min at 4 °C in the ice-cold prepared as previously described (22). After 2 min centrifugation at 10,000 × g, at 4 °C, the supernatant was collected, the pellet was resuspended in an equal volume of Triton extraction buffer and incubated for 30 min at 37 °C. After centrifugation, the supernatant and pellet were collected. Equal volumes of each fraction were analyzed by SDS-PAGE and immunoblotting with anti-RAMA sera.

Organelle Isolation and Triton X-100 and Trypsin Treatment—Intact rhoptries were isolated as described (23) with saponin lysis used instead of nitrogen cavitation. The organelle pellet was resuspended in phosphate-buffered saline. For trypsin treatment, 0.07% (w/v) phosphatidyl-chloromethyl-ketone-treated trypsin (Sigma) was added to a final concentration of 1 mg/ml. The preparation was then incubated at room temperature for 30 min, after which soybean trypsin inhibitor (Sigma) was added to a final concentration of 2 mg/ml. When required, organelle preparation was treated with Triton X-100 (1% v/v final concentration) by incubating on ice for 30 min. Samples of equal volumes were run on SDS-PAGE and examined by immunoblots with anti-RAMA sera.

Cytochalasin B Treatment, Preparation of Rhoptry Discharge Fraction, and RBC Binding Assays—Externally attached merozoites were obtained by cytochalasin B (Sigma) treatment performed as described (24). After treatment and incubation, thin blood films were prepared and analyzed by IFA.

Isolated free merozoites in incomplete RPMI media were incubated for 30 min with trypsin-treated RBCs prepared as described (25). Spent media supernatant was collected by spinning down the cell pellet at 10,000 × g for 2 min. RBC binding assays were performed as described (26) with incubation carried out for 4 h at 37 °C, using either the spent media supernatant, or recombinant proteins: RAMA-B, -C, -D, -E, Ag44, or GST (control) at equal molar amounts corresponding to 5 μg of RAMA-E. Fresh culture media was used as a negative control. Equal volumes of eluates were examined by SDS-PAGE, and immunoblots were probed by either anti-RAMA or anti-GST sera. For competition RBC binding assays, fresh RBCs were first incubated for 2 h at 37 °C with 0, 0.01, 0.1, 1, or 10 μg of recombinant RAMA-E, and then for 2 h at 37 °C with spent media supernatant. Equal volumes of eluates were examined by SDS-PAGE and immunoblotting. In parallel blots, RAMA-E and p60 were detected with either anti-GST or GST-prebiorbed anti-RAMA-D sera, respectively. Protein bands were quantified using NIH Image and the results, in arbitrary optical density (OD) units, were plotted on a graph.

RESULTS

RAMA Sequence and Primary Structure Analysis—Screening of a number of cDNA libraries with an Ag512 probe detected two hybridizing clones that provided 1470 bp of RAMA sequence, including the stop codon. A combination of gene walking by vectorette PCR and ZAP cDNA library screenings provided clones spanning the RAMA ORF and 200 bp of the 5′-untranslated region. The 20-bp-long sequence immediately upstream from the putative start site shows a base bias similar to that observed in other malarial genes, and the −4 to −1 positions are in an agreement with the consensus sequence for malarial translation initiation sites (27).

To examine the RAMA gene structure, a series of PCR amplification and sequencing reactions on genomic and cDNA templates were performed. Four introns of conventional structure were identified.
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with the RAMA gene. Southern hybridization analysis with digoxigenin-labeled RAMA fragments as probes indicated that the RAMA ORF exists in a single copy within the P. falciparum genome (data not shown). The RAMA locus was mapped to chromosome 7 by pulse field gradient electrophoresis in agreement with a previous report assigning Ag512 to this chromosome (28).

The RAMA ORF is a structure of 3215 bp and 5 exons, a relatively unusual arrangement for P. falciparum genes and one similar to that of the high molecular weight rhoptry antigen, RhopH3 (29). The introns are located at the periphery of the RAMA gene, and the second exon comprises most of the 2583-bp-long coding sequence (Fig. 1A). BLAST searching of the P. falciparum data base at www.plasmodb.org located RAMA ORF within bases 2451–5649 of the chrBLOB_004173 contig, and the confirmed RAMA sequence was 98% identical with the chrBLOB_004173.gen_1 product predicted by Genefinder. However, the first draft of the P. falciparum genome does not contain RAMA and BLAST searches against the currently available sequence no longer detect the RAMA gene, because a number of sequence contigs await final chromosomal assignment. The predicted RAMA protein is 861 residues long and is predominantly hydrophilic. At the N terminus there is a 15-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 21-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 15-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 21-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 15-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 21-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 15-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 21-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 15-residue hydrophobic region consistent with the presence of a signal peptide. The C-terminal region has a 21-residue hydrophobic region consistent with the presence of a signal peptide.

Reactivity of Anti-RAMA Antisera—The predicted 861-residue RAMA protein was too large to encode the 55-kDa protein previously identified, and we were concerned whether the RAMA gene truly encoded a rhoptry protein and what relationship it had to Ag512. To examine this we raised antisera to four fragments of RAMA spanning most of the protein sequence (Fig. 1B). All four RAMA fragments were produced as abundant soluble GST fusion proteins and the protein preparations were relatively free of contaminating Escherichia coli proteins (data not shown). All expressed proteins were recognized by anti-GST serum, with detected bands corresponding to those observed on Coomassie-stained gels (data not shown).

Expressed recombinant RAMA fragments were used to produce polyclonal antibodies in rabbits and mice. All raised antisera reacted with the corresponding fusion proteins as assessed by immunoblots (data not shown), and showed characteristic patterns of rhoptry labeling in indirect immunofluorescence assays (IFAs) confirming that RAMA was indeed a rhoptry protein (Fig. 3A). The rabbit antisera were then used to identify one or more parasite proteins in extracts of parasites from an asynchronous in vitro culture, by performing immunoblots under non-reducing conditions (Fig. 2A). Antisera raised against fragments RAMA-B and RAMA-C recognized the same protein of apparent molecular mass of 170 kDa (p170), whereas antisera against fragments RAMA-D and RAMA-E recognized predominantly a protein of apparent mass of 60 kDa (p60). Both anti-RAMA-D and anti-RAMA-E sera also reacted with p170, however to a lesser extent. No bands were observed in non-parasitized RBCs or when using pre-bleed sera (data not shown).

The molecular mass of RAMA predicted from its amino acid sequence is 103.7 kDa. We suggest that p170 is the full-length RAMA running higher than its predicted size, a feature quite common for Plasmodium proteins (30). The p60 is the same protein as that of 55 kDa observed by Smythe and colleagues (14) using affinity-purified human sera against the Ag512 clone (fragment D of RAMA). We hypothesized that p60 is a product of proteolytic cleavage of the full-length protein (p170) comprising the C-terminal part of RAMA and confirmed this in experiments described below.

Processing of RAMA in P. falciparum Parasites—To establish the relationship between observed RAMA forms we used brefeldin A (BFA), a fungal toxin known to block protein trafficking and processing within the secretory pathway by disrupting the Golgi apparatus and accumulating proteins in the endoplasmic reticulum (ER) (31). We also used low temperature blocks of protein trafficking to and within the Golgi appa-

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Footnotes:

2 F. Glenister, personal communication.

Characterization of RAMA

Expression of RAMA in Erythrocytic Stages of P. falciparum—To study the expression pattern of RAMA protein, we performed immunoblots on parasite lysates extracted from various stages of a synchronized culture using rabbit antisera against fragments B and D (Fig. 2C). The results indicate that RAMA is first produced between 15 and 20 h after invasion, in late ring/early trophozoite stages, and is present until schizont rupture. Both p170 and p60 were observed to appear at the same stage, indicating that proteolytic processing occurs shortly after RAMA expression. Although full-length and processed RAMA forms are present throughout the late erythrocytic stages, the amount of p60 increases with time relative to p170, suggesting accumulation of a final product. Immunoblotting of the same set of parasite samples, using mouse anti-RhopH3 sera showed that this rhoptry protein appears later, between 28 and 40 h (data not shown). This is in agreement with previous studies showing that RhopH3 is first detected in malaria parasites in trophozoite stages, at 30 h post-infection (3).

Localization and Trafficking of RAMA within P. falciparum—Specific anti-RAMA sera were used in confocal microscopy IFA to determine the localization of different RAMA forms in malaria parasite cells (Fig. 3). The results show a punctate pattern in schizont stages, characteristic of a rhoptry location. Furthermore, RAMA co-localizes with known rhoptry proteins RAP1 and RhopH3 (data not shown). The antisera recognizing p170 (anti-RAMA-B and anti-RAMA-C) as well as the sera predominantly immunoreactive with p60 (anti-RAMA-D and anti-RAMA-E) showed the same pattern in IFA, indicating that both protein forms localize in the same intracellular compartment and that processing of RAMA most likely takes place in rhoptries (Fig. 3A). In early parasite stages RAMA staining was observed in a diffuse pattern and some concentrated foci, which presumably represent secretory pathway compartments and some form of pre-rhoptry organelles (Fig. 3B). The diffuse staining in younger parasites is in agreement with the report of dispersed, unstacked Golgi in Plasmodium parasites (33). To ascertain RAMA trafficking through the compartments of the secretory pathway, we performed double staining IFA using mouse anti-RAMA-E sera and rabbit antibodies to either ER marker (PigP) or Golgi marker (PFPERD2) proteins. In both cases RAMA showed partial co-localization with these proteins, indicating transient localization within the ER and Golgi compartments (Fig. 3C). Anti-RAMA-D rabbit serum was used in electron microscopy to localize the protein within the rhoptry organelle, and a characteristic appearance is shown in Fig. 4A. Immunogold staining was observed at the rhoptry periphery, indicating association of RAMA with the rhoptry membrane. Gold particles seemed to concentrate in discrete islands instead of uniform peripheral distribution.

Association with Lipid Rafts and Membrane Topology—We demonstrated that RAMA is released from a membrane-bound fraction after Triton X-114 phase separation and treatment with phospholipase D, an enzyme known to specifically cleave within GPI molecules (data not shown), thus confirming the GPI-anchoring aspect of RAMA. Interestingly, we also observed RAMA in the detergent-insoluble pellet. To further address this issue, we examined RAMA solubility in the non-ionic detergent Triton X-100 at different temperatures. In contrast to extraction with Triton X-100 at 4 °C, where some RAMA was found to be insoluble, extraction at 37 °C solubilized RAMA completely (Fig. 4B). Thus a pool of RAMA appeared to be associated with detergent-resistant microdomains or lipid rafts (34). This was later confirmed by detection of RAMA in lipid rafts isolated on sucrose gradient (35).

To determine the membrane topology of RAMA, we used trypsin treatment of isolated intact rhoptries with or without Triton X-100 preincubation. Any protein on the inner side of the rhoptry membrane, including proteins present within the rhoptry body, would not be affected by trypsin treatment. However, permeabilization of the rhoptry membrane by Triton X-100 would render any such protein sensitive to trypsin di-
gestion. As shown in Fig. 4C, RAMA was susceptible to trypsin treatment only after the rhoptry membrane was disrupted by Triton X-100, as RAMA forms p60 and p170 were detected after incubation of rhoptry preparation with trypsin alone (lane 2). A similar degradation pattern was observed for RhopH3, used as a control for internal rhoptry proteins (data not shown). Therefore, we conclude that RAMA is located on the inner surface of the rhoptry membrane. In addition, this experiment confirms that the processing of RAMA takes place in rhoptry organelles, because the p170 was detected in the isolated organelles, and no ER and/or Golgi contamination was detected in the rhoptry fraction (data not shown).

**Interaction of RAMA with Other Rhoptry Proteins**—The early appearance of RAMA, its transient localization within the Golgi apparatus, and association with lipid rafts suggested that RAMA might play a role during rhoptry formation, perhaps trafficking other rhoptry constituents into the nascent rhoptries. To test that, we used fluorescence resonance energy transfer (FRET), an immunofluorescence technique that detects protein-protein interactions based on photon transfer between two fluorophores that are in close proximity. The range over which FRET exhibits measurable efficiencies is 1–10 nm, with a sixth order dependence on separation, which is within the molecular range of interacting proteins (20). We tested for FRET by double staining parasites with anti-RAMA-E and either anti-RhopH3, anti-AMA1, anti-RAP1, or anti-RAP2 antibodies. We double stained with anti-RAMA-E and anti-RAMA-D or anti-MSP4 sera as positive and negative controls, respectively. FRET was performed using the acceptor photobleach method (20), and ten cells were analyzed for each pair of labeled proteins and FRET efficiency for each was quantified. The median values (±S.D.) were as follows: 0.11 (±0.08) for RAMA-RAMA, −0.01 (±0.02) for RAMA-MSP4, −0.04 (±0.02) for RAMA-AMA1, −0.12 (±0.05) for RAMA-RAP1, and 0.07 (±0.06) for RAMA-RhopH3.
for RAMA-AMA1, 0.07 (±0.05) for RAMA-RhopH3, 0.07 (±0.03) for RAMA-RAP1, and 0.05 ± 0.03) for RAMA-RAP2. The negative values obtained are due to marginal photobleaching of the donor fluorophore. The FRET efficiencies for RAMA-RhopH3, RAMA-RAP1, and RAMA-RAP2 were statistically higher than the negative control (p values < 0.0001, Mann-Whitney test), whereas no statistical difference was observed between FRET efficiencies of RAMA-AMA1 and the negative control. Furthermore, the FRET efficiency of RAMA-RAMA, RAMA-RhopH3, RAMA-RAP1, or RAMA-RAP2 was not dependent on acceptor density. The results of the experiment indicate that RAMA is in close apposition with RhopH3, RAP1, and RAP2 but not with AMA1. We hypothesize that interaction with RAP2 may be indirect, via RAP1, because RAP1 has been shown to traffic RAP2 to rhoptries (12).

RAMA Release and Binding to RBC Membrane—Double staining immunofluorescence, using anti-MSP4 sera and either anti-RAMA-B or anti-RAMA-E sera showed that p60/RAMA but not p170/RAMA is present in free extracellular merozoites (Fig. 3D). To examine RAMA fate in invading merozoites, we performed IFA on cytchalasin B (CytB)-treated parasites. Cytchalasins are fungal toxins and potent inhibitors of actin polymerization. When added to malaria culture, CytB and CytD arrest merozoites at the stage of irreversible attachment but do not block some of the early events of RBC invasion, such as rhoptry discharge (36, 37). As shown in Fig. 3E, diffusion of staining indicates that RAMA appears to be released from rhoptries of apically attached merozoites. To investigate whether RAMA is truly part of the rhoptry discharge and address the possibility that RAMA interacts with RBC membrane components we prepared spent media supernatant fraction containing material discharged from rhoptries by incubating free merozoites with trypsin-treated RBCs. We used this fraction in an RBC binding assay and analyzed the material eluted from RBC surface in immunoblot. As shown in Fig. 5A, RAMA was detected in isolated merozoites, in the spent media and also in the eluate from the RBC binding assay, but not in the control samples. The same pattern was observed for RhopH3 (data not shown) in agreement with reports that it is discharged from rhoptries during invasion and interacts with RBC membrane components (6). In contrast, MSP4 was detected only in the isolated merozoite sample (data not shown), thus confirming that the presence of RAMA in spent media is truly due to the rhoptry discharge and not merozoite death and degradation. These results confirm that RAMA is part of rhoptry discharge and show that after release RAMA interacts with one or more RBC membrane components. To map the binding region of RAMA we performed RBC binding assays using the expressed recombinant RAMA fragments. The elutes were analyzed in immunoblot with anti-GST sera. As shown in Fig. 5B, the only fragment of RAMA binding to RBCs and detected in eluted samples was fragment E. A recombinant fragment encoding the C terminus of RhopH3 was also found to bind RBC, thus mapping the binding region of RhopH3 to this part of the protein. No binding was observed for GST alone, indicating that the presence of fragment E and the C terminus of RhopH3 in eluates is not due to the GST portion of fusion proteins. Other faint bands observed on immunoblot were constant among the samples and represent co-eluted RBC proteins. The specificity of RAMA-E binding to RBCs was examined in a competition experiment, in which a fixed amount of culture supernatant was incubated with RBCs after preincubation with increasing amounts of RAMA-E. The amounts of p60 and RAMA-E binding to the RBC surface were quantitated using densitometry (Fig. 5C). RAMA-E completely inhibited the binding of p60 suggesting the RBC binding site of RAMA is contained completely within fragment E. As increasing levels of RAMA-E were added, binding to RBCs plateaued indicating saturable binding to a receptor.

To investigate whether RAMA contributes to the formation of the parasitophorous vacuole (PV), we performed IFA on isolated merozoites incubated in the presence of fresh RBCs. RAP1 was used as a second marker, because it has been demonstrated to be carried to ring stages and to associate with PV (12, 38). As shown in Fig. 3F, shortly after the red cell invasion RAMA co-localizes with RAP1 in a discrete compartment surrounding the parasite, which is the PV.
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Similar results were observed with RhopH3 as a second marker (data not shown).

DISCUSSION

Smythe and colleagues used human antibodies affinity-purified on a particular clone designated Ag512 to identify a 55-kDa polypeptide that partitioned in the detergent phase in Triton X-114 phase-separated parasite extracts and was located in rhotries (14). The 55-kDa protein labeled with [3H]myristate and [3H]glucosamine indicating it was anchored to the rhotry membrane, probably via a GPI moiety. There has been a previous description of a GPI-anchored protease at the parasite surface possibly in rhotries, but no actual localization results have been presented, and the protein has not been further molecularly characterized since the initial report (39, 40). We used the unpublished sequence data of Smythe et al. to identify the remainder of the gene. To our surprise, we found that the 55-kDa polypeptide, which we now call RAMA for rhotry-associated membrane antigen, was in fact a processing product of a much larger protein with an apparent molecular mass of 170 kDa. The evidence that it is this gene that encodes the previously reported rhotry protein is strong. Four distinct regions of this gene were used to produce sera in two species of animal, which reacted with the protein fragment against which they were raised, but did not cross-react with any of the other recombinant proteins. All sera recognize the same 170-kDa polypeptide and react with rhotries by IFA. Two non-cross-reacting sera to non-overlapping regions of the C terminus recognize a 60-kDa polypeptide that is GPI-anchored, consistent with the presence of a GPI anchor motif encoded within the 3’-end of the RAMA coding sequence. Incubation of parasites with brefeldin A block the processing of p170 to p60 and lead to accumulation of the precursor form of the protein. The difference between the 60-kDa product and the 55-kDa polypeptide previously reported is due to the differing gel systems and molecular mass standards used in the two studies. The fact that Smythe and colleagues did not observe any full-length RAMA is probably due to several factors. First, the study used affinity-purified sera, which tend to be of low titer and thus less likely to react detectably with the more minor p170 product. Second, sera directed against the C-terminal portion of RAMA react much more weakly with p170. And finally, the affinity-purified antibodies were prepared against the Triton X-114 phase-separated material, and proteins of molecular mass greater than 80 kDa do not partition well in this phase (41, 42).

The onset of RAMA expression occurs quite early for a rhotry protein. Our experiment indicates that RAMA appears in malaria parasites between 15 and 20 h post-infection, several hours before the RhopH3 protein. RhopH3 is first detected at 30 h post-infection and appears before the RAP complex and before AMA1 (3). We have shown that, in immature parasite stages, RAMA has a diffuse pattern of staining in IFA, representing the trafficking of RAMA through the secretory pathway. In agreement with this and the model of rhotry biogenesis (2), we have shown that RAMA transiently localizes in the ER and Golgi compartments, before reaching nascent rhotries. Thus, RAMA is the earliest expressed rhotry protein currently known and is part of the rhotry precursor vesicles and the post-Golgi vesicles that subsequently fuse to form the newly developed rhotry organelles. Within the vesicles RAMA is located on the inner surface of the rhotry membrane with the body of the protein within the rhotry lumen, as determined by susceptibility to trypsin digestion. This finding is supported by data from immunoelectron microscopy showing gold particles at the periphery of the rhotry organelle, located along the membrane.

We have shown that p60 is a product of proteolytic cleavage of the full-length RAMA and comprises the C-terminal part of the protein. This processing event occurs in post-Golgi compartments, because it was blocked by the presence of BFA and incubation at low temperatures (32). This compartment is most likely nascent, or mature rhotries, because sera directed against N-terminal fragments of RAMA and recognizing only the p170 form, show the punctate pattern of rhotry staining in IFA, and by the fact that p170 is detected in the isolated rhotries in immunoblots. No polypeptide of intermediate size is seen by sera directed to the N-terminal regions of RAMA suggesting that the N terminus is rapidly and completely degraded during processing. The cleavage of the N-terminal part of RAMA resembles the removal of the pro region observed for rhotry proteins in Toxoplasma gondii, occurring in either the mature organelles or in the pre-rhotry vesicles (43, 44). The pro region of the T. gondii ROP1 protein has been shown to contain the targeting signals and be sufficient to localize recombinant fusions to rhotries (45). However, the pro region may have other roles as redundancy of targeting signals exists in rhotry proteins (46). It should be noted at this stage that the potential pro region of RAMA is quite large, comprising approximately half of the 861-residue protein. This is in contrast to other rhotry proteins, which are proteolytically processed by removal of much shorter pro-peptides. For example, RAP1 of P. falciparum is processed by removal of 190 residues (47), whereas in T. gondii, the pro regions of ROP1 and ROP2 comprise 83 and 97 residues, respectively (48, 49).

We have determined that a pool of RAMA is associated with detergent-resistant microdomains or lipid rafts, sphingolipid- and cholesterol-rich sub-domains of liquid-ordered phase in the membrane bilayer. GPI-anchored and transmembrane proteins segregate into lipid rafts to form discrete functional clusters within biological membranes, implicated in the processes of signal transduction and vesicular trafficking and sorting within endocytic and secretory pathways (reviewed in Ref. 50). The previous biosynthetic labeling studies and the presence of a consensus GPI attachment site at the C terminus of the predicted protein are consistent with the presence of a GPI anchor in RAMA. We have confirmed the presence of the GPI moiety by Triton X-114 phase separation and treatment with phospholipase D, which cleaves to release RAMA into the aqueous phase of a phase-separated preparation (data not shown). In electron microscopy studies, gold particles were observed clustered in discrete islands rather than being uniformly peripherally distributed, an appearance consistent with a presence in discrete domains. The clustering and formation of lipid rafts is quite common within the Golgi apparatus and other compartments of the secretory pathway and facilitates proper protein trafficking (34). We suggest that, in the Golgi of malaria parasites, p170/RAMA aggregates into lipid rafts and marks these membrane stretches as the material that will form the proto-rhotries. As the parasite matures, we hypothesize that newly synthesized p170/RAMA binds rhotry-destined proteins such as RhopH3 and RAP1 and anchors them within rafts. The formation of complexes by RAP1 with RAP2 and RAP3 and by RhopH3 with RhopH1 and RhopH2 would result in concentration of all these proteins in the patches of membrane destined to become rhotries. These lipid rafts would then pinch out from the Golgi and form vesicles targeted to nascent and maturing rhotries. The machinery needed to traffic the resulting vesicles would likely require that vesicles also contain a transmembrane protein with signals within its cytoplasmic tail, which could interact with rhotry-targeting coat components of vesicles in the secretory pathway to ensure
proper trafficking (51). The identity of this protein is not known, but the recently described Rhop148 is a candidate (52). The mode of recruitment of such a transmembrane protein might be by means of interaction with RAMA. Within the rhoptry organelle, the proteolytic processing of RAMA and removal of the repeat regions would allow for disassociation of RAMA-bound proteins and distribution of rhoptry constituents in the growing organelle. Clearly, more work on RAMA is required, however, at this stage we can state that p170/RAMA has the characteristics of an organizing agent that marks membrane stretches and vesicles as nascent rhoptries and a possible escort protein for other rhoptry components.

FRET studies indicate that RAMA is found in very close association with the rhoptry proteins RAP1 and RhopH3. The distances over which FRET is detectable are so small that this finding suggests a direct protein-protein interaction. RAP1 is responsible for trafficking RAP2 to the rhoptry so a RAMA-RAP1 interaction could lead to both members of the RAP complex reaching the rhoptry. No binding domain within RAMA has yet been mapped, but the presence of prominent, highly acidic, repeat regions in the N terminus is notable. Repeats are quite common in Plasmodium proteins located on the merozoite surface or exported to the infected RBC membrane, but less so in rhoptry proteins. To date, pronounced tandem repeats have been observed in MAEBL, PfRhop148, and members of the PRHI family of rhoptry proteins (52–55). It is still not clear what is the role of repeat regions in malaria. In some cases they are involved in protein-protein interactions (56), as in many other non-malarial proteins, but in other cases they appear to be immunodominant epitopes of surface proteins and may be important in the immune response to malaria infection. RAMA repeats are conserved across the species barrier, 3 consistent with a possible functional role in binding to other rhoptry proteins. RAMA repeats are located within the pro-mature region, which suggests that any role they do play can only be during the trafficking and organelle maturation. The rapid degradation of this N terminus of RAMA within the rhoptry would result in release of bound proteins and allow them to then take part in the various RAP and RhopH complexes that are formed. Further binding studies are required to determine which regions of RAMA are closely associated with RhopH3 and RAP1.

Our experiments indicate that p60/RAMA is involved in the processes of invasion of merozoites into RBCs and formation and expansion of the PV. Following apical attachment of merozoites to RBCs p60/RAMA is discharged from rhoptry organelles allowing it to bind to the RBC membrane by virtue of sequences located within fragment E. Discharge of RAMA from rhoptries might occur either by insertion of the GPI anchor into the lamellated lipid present in the rhoptry (57) or by action of a rhoptry-dwelling phospholipase or protease. A recent report by Hiller and colleagues (58) indicated that parasite-derived lipid rafts play a role in invasion and formation of parasitophorous vacuole. The binding of RAMA-E is saturable suggesting a specific ligand-receptor interaction with a molecule on the RBC surface. Further examination is needed to determine which regions of the 82-residue E fragment are required for RBC binding and to identify its ligand molecule(s) on the RBC membrane. In early ring stages RAMA is found within the PV, most likely associated with the PV membrane, although its function at this stage is not known. Collectively, our results make a strong case that RAMA is an important protein of the malaria parasite, with roles in the processes of rhoptry biogenesis and the invasion of merozoites into RBCs.

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Characterization of RAMA

Characterization of a Membrane-associated Rhopty Protein of *Plasmodium falciparum*
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