MAHRP-1, a Novel Plasmodium falciparum Histidine-rich Protein, Binds Ferrroporphyrin IX and Localizes to the Maurer’s Clefts*

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Using a stage-specific cDNA library from Plasmodium falciparum we have identified a gene coding for a novel histidine-rich protein (MAHRP-1). The gene is exclusively transcribed during early erythrocyte stages and codes for a small transmembrane protein. The C-terminal region contains a polymorphic stretch of histidine-rich repeats. Fluorescence microscopy studies using polyclonal mouse sera revealed that MAHRP-1 is located at the Maurer’s clefts, which represent parasite-induced structures within the cytosol of infected erythrocytes. Biochemical studies showed that recombinant MAHRP-1 binds the toxic hemoglobin degradation product, ferrroporphyrin (FP) with a submicromolar dissociation constant and a stoichiometry determined by the number of DHGH motifs. The bound FP has increased peroxidase-like activity and is 10-fold more susceptible to H$_2$O$_2$-induced degradation compared with unbound FP. These properties of MAHRP-1 suggest it may play a protective role against oxidative stress, and its location at the Maurer’s clefts suggests a function in promoting the correct trafficking of exported proteins, such as P. falciparum erythrocyte membrane protein-1.

Plasmodium falciparum causes one of the most life-threatening infectious diseases of humans. Malaria is estimated to be responsible for up to 2 million deaths per year. The pathogenesis of the disease is associated with the intracellular erythrocytic cycle of the parasite involving repeated rounds of invasion, growth, and schizogony. The parasite appears to dispose of FP largely by sequestration in the parasite’s food vacuole as a granular pigment known as hemozoin (β-hematin). HRP2 is one of the best characterized. This soluble protein is found in the food vacuole but also in the erythrocyte cytosol (8, 11). Earlier studies suggested that HRP2 is ingested by the parasite’s cytostome from the red cell together with hemoglobin and thereby delivered to the food vacuole by bulk endocytosis. However, recent studies show that although ~75% of the hemoglobin is ingested by the parasite, more than 90% of HRP2 remains in the erythrocyte cytosol. Cytosolic HRP2 seems to concentrate mainly near the periphery of the erythrocyte, suggesting that it may not diffuse freely through the erythrocyte cytoplasm and could thereby avoid ingestion. These recent findings suggest the possibility that HRP2 could play a role in the detoxification of the by-products of hemoglobin degradation in the host cytoplasm. Recently, it has been shown that HRP2 modulates the redox activity of FP and that the HRP2-FP complex may have antioxidant properties. Hence, the localization of HRP2 near the erythrocyte membrane may function to protect the host membrane from oxidative stress and, thus, prevent premature host cell lysis.

The P. falciparum knob-associated histidine-rich protein (KAHRP) is another well characterized HRP. KAHRP is essential for the formation of knob-like protruberances on the surface of infected erythrocytes. These knobs act as platforms for the presentation of P. falciparum erythrocyte membrane protein-1

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MAHRP-1, a Novel P. falciparum Histidine-rich Protein

(PfEMP1), which is responsible for adherence of parasitized erythrocytes to vascular endothelial cells (14). This adhesion and subsequent accumulation of infected erythrocytes in the microvasculature are pivotal events in the pathogenesis of P. falciparum malaria, and these processes are considered to represent major virulence factors.

On route to the erythrocyte membrane, KAHRP is transiently located at the Maurer’s clefts, a parasite-derived vesicle-like structure in the erythrocyte cytosol (15). Other proteins have also been shown to be associated with Maurer’s clefts, including PfEMP3 (15, 16), Pf32 (17), and P. falciparum skeleton-binding protein 1 (PSBP1) (18). It has been suggested that these cleft structures may act as an intermediate trafficking compartment and may play a critical role in the organization and sorting of parasite proteins. In a host cell such as the erythrocyte, which is devoid of any organelles or trafficking machinery, such parasite-derived structures represent an interesting target for possible intervention.

Here we describe a novel P. falciparum membrane-associated histidine-rich protein, MAHRP-1, which localizes to the Maurer’s clefts. We identified the sequence encoding MAHRP-1 in a ring stage-specific cDNA library (19) and show that MAHRP-1 is exclusively expressed in the early erythrocytic stages but is present throughout the cycle. MAHRP-1 contains a predicted transmembrane domain and a polymorphic cluster of histidine-rich repeats. MAHRP-1 binds FP in vitro in a specific manner, promoting the peroxidase-like activity of FP and enhancing the degradation of FP by H2O2. These data suggest that MAHRP-1 might play an important role in generating the Maurer’s clefts or in protecting proteins within these structures against the noxious effects of FP and reactive oxygen species.

MATERIALS AND METHODS

FP (porcine), bovine serum albumin (essentially fatty acid free (BSA)), saponin, and ortho-phenylenediamine (OPD) were obtained from Sigma. Albumax was provided by Invitrogen. All primers were manufactured by Invitrogen. Escherichia coli BL21 strain and plasmid vector pGEX-6P-2 were obtained from Amersham Biosciences. E. coli TunerDE3pLacI was obtained from Novagen. The sequenator vector pGEM-EF-T was provided by Promega, Wallisellen, CH. Glutathione S-transferase (GST) was expressed from the pGEX-6P-vector and purified as described for the recombinant MAHRP-1c. Secondary antibodies for immunoblot analyses were goat anti-mouse IgG (chain specific) alkaline phosphatase-labeled (Jackson ImmunoResearch Laboratories, West Grove, PA). Secondary antibodies for immunofluorescence labeling were goat anti-mouse IgG conjugated to Alexa 568 (Molecular Probes) and goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma or Dako). Rabbit antisera recognizing KAHRP, PfEMP3, P. falciparum exported protein-1 (EXP-1), and PfEMP1 were kindly donated by Kathy Davern and Dr. Jacqui Waterkeyn, Walter and Eliza Hall Institute of Medical Research, and Prof. Klaus Lingelbach, Philippus-University, Marburg, Germany, and Professor Chris Newbold, John Radcliffe Hospital, Oxford, UK.

Methods

In Silico Analyses—One of the ring stage-specific gene fragments reported in Spielmann and Beck (19) coded for a histidine-rich protein (AJ290925). Query of the malaria sequence data base (www.plasmodb.org) returned the complete gene, now referred to as mahrp1. The predicted intron sequence was removed from the complete gene sequence to obtain the open reading frame. This was translated into amino acid sequence using ExpASy Molecular Biology Server (www.expasy.ch). Protein primary structure analysis was performed using www.expasy.ch.

Amplification and Sequencing of Full-length MAHRP-1—The sequencing coding for MAHRP-1 was amplified from a collection of DNA derived from the following P. falciparum strains: 3D7, K1, SN250, ITG2F6, IFA18, Mad20. Primers used for amplification by PCR were: forward primer, 5’-GGTTCCACTCTGGTGAACTGTCCTC-3’, and reverse primer, 5’-CTAATCTGGCTTTTGTGGACATCAGTG-3’. The PCR products were cloned into the sequencing vector pGEM-T. The commercially available SP6 and T7 primers were used for sequencing reactions.

Expression of Recombinant MAHRP-1c—The sequencing coding for the C-terminal domain of MAHRP-1 was PCR-amplified from both 3D7 and ITG2F6 genomic DNA using primers 5’-CAGATCAAGATGAAGCTG-3’ and 5’-CTAATTGTTTGGTATCAGTG-3’. The PCR product was cloned into the Smal restriction site of GST in pGEX-42B by cycle restriction ligition to yield the plasmid pMAHRP-1c. E. coli BL21 strain (for the 3D7 fusion protein) and E. coli Tuner DE3pLacI (for the ITG2F6 fusion protein) were transformed with plasmid pMAHRP-1c and used for expression of recombinant MAHRP-1c. Luria broth was inoculated with E. coli harboring pMAHRP-1c and cultured overnight at 37 °C containing one (for 3D7) or both (ITG2F6) of the recombinant antibodies: mouse anti-FP (100 μg/ml) and goat anti-FP (34 μg/ml). Fresh Luria broth containing antibodies was inoculated with the overnight culture at a dilution of 1:10 and cultured at 37 °C to an A600 of 1.0. Expression of MAHRP-1c was induced by adding isopropyl-1-thio-β-galactosidase to a final concentration of 1 mM. Induced cultures were grown for 3–6 h at 37 °C.

Purification of Recombinant MAHRP-1c—Induced cultures were harvested by centrifugation and resuspended in lysis buffer (2 mg/ml lysozyme, 2 mM phenylmethylsulfonyl fluoride, 2 mg/ml DNase) in 137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4, 1.8 mM KH2PO4, pH 7.4 (phosphate-buffered saline (PBS)) and lysed by sonication. The supernatant containing the soluble GST fusion protein was purified using the Glutathione Affinity Gel Purification Module (Amersham Biosciences) recommended by the manufacturer. Purity and integrity of the protein was checked on a 12.5% SDS-PAGE. 500 ml of culture yielded ~2–4 mg of recombinant protein. To test whether the histidine residues could also be used for further purification, an aliquot of the protein preparation was run over a Ni2+ chelate column (Qiagen) according to the supplier’s protocol (data not shown).

Production of Specific Sera—C57BL6 mice were immunized subcutaneously with 10 μg of recombinant MAHRP-1c excised from polyclonal gels. Mice were boosted three times within 7 weeks and bled 1 week after the last immunization. Specificity of sera was tested on Western blots with recombinant MAHRP-1c, recombinant GST, and parasite protein extracts. Four different mice antisera were generated, each of which gave equivalent reactivity profiles.

Western Blot Analyses—Samples were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose (Hybond-C extra, Amersham Biosciences) for 1.5 h using a Trans-Blot semi-dry electrophoretic unit (Bio-Rad). The membrane was washed twice for 5 min in TNT (0.1 mM Tris/HCl, 1.5 mM NaCl, 0.05% Tween 20, pH 8.0) and blocked in TNT and 5% milk powder overnight at 4 °C. The primary antibody was diluted in 15 ml of TNT and 1% milk powder, and the secondary antibody was diluted in 15 ml of TNT and 0.1% milk powder. The membrane was washed 4 times in TNT and twice in Tris buffer (0.1 mM Tris/HCl, 0.5 mM MgCl2, pH 9.5). The membrane was stained in 20 ml of 0.05% Coomassie blue containing 5% glycerol and 0.02% sodium dodecyl sulfate (15 mg/ml) and 200 μl of nitro blue tetrazolium (30 mg/ml 70% N,N-

PARASITE CULTURE AND PREPARATION OF PARASITE PROTEIN EXTRACTS—P. falciparum 3D7 strains were cultured at 5% hemocrit as described (20) using RPMI medium supplemented with 5% human serum or 0.5% Albumax (21). Parasites were synchronized with 5% sorbitol (22).

For total parasite extracts infected erythrocytes from a 10 ml culture (5% hematocrit, 10% parasitemia) were lysed with 0.3% saponin for 20 min on ice, washed in PBS, and resuspended in Laemmli sample buffer. Triton-X-114 phase separation was performed as previously described (23). Briefly, 10 ml of parasite culture (15–20% parasitemia) was saponin-treated, and the pellet was washed with PBS and then resuspended in 500 μl of human tonicity phosphate-buffered saline (HTPBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM sodium phosphate, pH 7.2) containing 0.5% Triton-X-114 and 1 mM phenylmethylsulfonyl fluoride. The solution was incubated at 20 °C until use. After thawing, the extract was kept on ice for 30 min with intermittent mixing. The extract was spun at 15,000 x g for 20 min to pellet insoluble material. The supernatant, containing the detergent-soluble proteins, was layered over a 0.4 ml ice-cold sucrose cushion (6% sucrose, 0.06% Triton-X-114 in HTPBS), incubated at 37 °C for 40 min and centrifuged at 100,000 x g for 5 min at 4 °C. The resulting three phases were treated as follows. (i) The upper layer (detergent-depleted fraction) was collected; (ii) the sucrose cushion was discarded; (iii) the detergent pellet, containing the membrane proteins, was resuspended in 0.5 ml of HTPBS, the purification over a sucrose
MAHRP-1, a Novel P. falciparum Histidine-rich Protein

(A) mahrp-1 gene structure

MAHRP-1 protein structure

(B) MAHRP-1 aa sequence

(C) 3D7

FIG. 1. Primary structure of P. falciparum 3D7 MAHRP-1. A, schematic representation of the mahrp1 gene and deduced MAHRP-1 protein. The N-terminal domain is shown hatched, followed by the transmembrane domain (TM), shown in light gray. The C-terminal histidine-rich domain is shown in dark gray, with DHGH indicating the characteristic repeat motif. B, P. falciparum 3D7 MAHRP-1 amino acid (aa) sequence. The 18-amino acid long transmembrane domain is highlighted in gray, and the C-terminal repeats are indicated in bold. The C-terminal sequence cloned as GST fusion protein and referred to as recombinant MAHRP-1c is underlined.

FIG. 2. Length polymorphisms in the histidine-rich regions of mahrp1 among different strains of P. falciparum. A, schematic representation of SspI and NcoI restriction enzyme recognition sites in mahrp1. B, RFLP pattern of SspI- and NcoI-digested mahrp1 alleles of six different strains, visualized by agarose gel electrophoresis. C, amino acid sequence alignments of MAHRP-1 from 6 different P. falciparum strains. Repeats are indicated in bold. Amino acid numbering according to the 3D7-coding sequence.

Cushion was repeated, and the pellet was resuspended in 0.5 ml of HTPBS. Both the detergent-depleted fraction (i) and the final detergent fraction (iii) were precipitated with tricarboxylic acid and analyzed by SDS-PAGE.

Localization Studies Using Indirect Immunofluorescence Microscopy—Infected erythrocytes were smeared onto glass slides and fixed in acetone:methanol (1:1 v/v) at –20 °C for 10 min. Slides were partitioned into wells using a hydrophobic pen and incubated for 1 h in a humid chamber with one or both of the following primary antibodies: mouse anti-MAHRP-1c (1:1000), rabbit anti-PFEMP3 (1:50), rabbit anti-PFEMP1 (1:200). The primary antibody was washed off with PBS or 1% Tween 20 in Tris-buffered saline, and the slides were then incubated with one or both secondary antibodies: fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:250) and Alexa 568-conjugated anti-mouse IgG (1:50). The slides were mounted in 90% glycerol containing 0.05% N-propyl gallate (Sigma) to reduce bleaching. Samples were viewed using an Olympus IX50 light microscope fitted with a fluorescence attachment and a SPOT RT digital camera or with a Leica TCS-NT laser-scanning confocal microscope at the Confocal Microscopy Facility, Monash University.

FP Binding Studies—The interaction between FP and the recombinant proteins was examined spectrophotometrically in PBS, pH 7.4. Concentrated stock solutions of FP (10 mM) were freshly prepared in 0.1 M NaOH and diluted in water as required. Absorption spectra were recorded using a Cary 1E spectrophotometer. The absorption spectrum of FP exhibits a time-dependent change due to gradual self-association events (24). Therefore, spectroscopic measurements of FP in the presence of protein were recorded 2 min after mixing the FP stock into the protein solution.

Redox Activity of FP—The H2O2-mediated decomposition of FP at pH


cushion was repeated, and the pellet was resuspended in 0.5 ml of HTPBS. Both the detergent-depleted fraction (i) and the final detergent fraction (iii) were precipitated with tricarboxylic acid and analyzed by SDS-PAGE.
7.4 was monitored by measuring the decrease in absorbance of FP (5 μM) in the presence or absence of either MAHRP-1c, BSA, or GST (all 2 μM). The reaction (in a final volume of 950 μl) was initiated by the addition of 50 μl of 2 mM H₂O₂ and monitored at 20 °C using a Carry 1E spectrophotometer. The initial measured value for the FP concentration was set as 100%.

To monitor the peroxidase-like activity of FP at pH 7.4, samples (200 μl) containing 5 mM OPD and 5 μM FP with or without protein (MAHRP-1c, BSA, or GST; 0.1–2.0 μM) were added to the wells of a 96-well plate. The reaction was initiated by the addition of 10 μl of 40 mM H₂O₂, and plates were incubated at 20 °C for 20 min. The oxidation of OPD was measured at 490 nm after the addition of 50 μl of 3 M HCl.

RESULTS

Identification and Primary Structure of MAHRP-1—Using suppression subtractive hybridization, we identified a set of genes that are exclusively transcribed in the ring stage of P. falciparum 3D7 strain, as reported in Spielmann and Beck (19). Among the 13 ring stage-specific gene fragments obtained,
The C-terminal domain of MAHRP-1 (3D7 strain, amino acids 131–242), referred to as MAHRP-1c, was deduced from the open reading frame. The translated sequence comprises 249 amino acids, with a predicted molecular weight of 28.9 kDa and a pI of 5.92. MAHRP-1c contains a predicted transmembrane domain (amino acids 106–124) but no predicted N-terminal signal sequence. The region N terminus to the transmembrane domain has a histidine content of nearly 30% and contains 6 tandem repeats of the amino acid sequence, DHGH, with additional preceding DH repeats.

We examined the degree of conservation of MAHRP-1 among different strains of P. falciparum. Digestion of six strains with restriction enzymes SspI and NcoI resulted in four distinguishable fragments (Fig. 2). The major length polymorphism was found in the 160-bp fragment in the histidine-rich region of the C-terminal domain, between amino acids 195 and 249. Sequence analysis revealed that alleles differed in the number of DHGH repeats, whereby 3D7 has 6 and ITG2F6 has 13 repeats.

Immunochromatograph Characterization of MAHRP-1 in Parasitized Erythrocytes—The C-terminal domain of MAHRP-1 (3D7 strain, amino acids 131–242), referred to as MAHRP-1c, was expressed as a 41-kDa GST fusion protein in E. coli (Fig. 3A), and the purified protein MAHRP-1c was used to raise antibodies in mice. The serum recognized the recombinant MAHRP-1c-GST fusion protein but not recombinant GST on a Western blot (Fig. 3B). The antiserum recognized a single band of the predicted size at ≈30 kDa in an extract of parasitized erythrocytes (Fig. 3C, lane a). The antiserum does not appear to cross-react with other proteins. In particular, it does not recognize bands corresponding to the ≈60-kDa protein recognized by an antiserum against HRP2; Fig. 3C (lane b) or the ≈90-kDa band obtained with serum against the KAHRP (Fig. 3C, lane c). In addition, the anti-MAHRP-1c serum gave no signal on protein preparations from uninfected erythrocytes (data not shown).

Infected erythrocytes were treated with detergents to elucidate the physical organization of MAHRP-1. Most of the MAHRP-1 signal was associated with the saponin-insoluble pellet (Fig. 4A, lane a). Further partitioning of the pelleted fraction using the detergent Triton X-114 yielded three fractions, i.e., a detergent-soluble fraction, an aqueous (detergent-depleted) fraction, and an insoluble fraction. MAHRP-1 appeared to be largely associated with the detergent-soluble fraction, which is consistent with it being a membrane protein (Fig. 4B, lane c).

Subcellular Location of MAHRP-1—We have used indirect immunofluorescence microscopy to examine the subcellular location of MAHRP-1 in P. falciparum-infected erythrocytes. MAHRP-1c antibodies reacted with punctuate structures within the cytoplasm of the host erythrocytes (Fig. 5A). The pattern obtained resembles the pattern obtained for Pf322 (17), PfSBP1 (18), and PfEMP3 (14, 16), which has been shown to be located in parasite-derived membranous structures in the erythrocyte cytosol, referred to as Maurer’s clefts.

MAHRP-1 was first observed in vesicle-like structures within the erythrocyte cytosol approximately 10 h post-invasion. As the parasite matured, the labeled structures increased in number. In the schizont stage, a diffuse staining of the entire erythrocyte appeared to underlie the punctuate foci. To confirm the Maurer’s cleft location, we performed dual labeling studies using an antiserum against PfEMP3 (16), which has previously been described to be Maurer’s cleft-located (Fig. 5B). A high degree of colocalization between PfEMP3 and MAHRP-1 was observed. Dual labeling with EXP-1, a parasite protein that is exported into the parasitophorous vacuolar membrane, indicated that MAHRP-1 is mainly located outside the parasite confines and within the confines of the erythrocyte membrane. We also used antiserum against PfEMP1, a protein also transiently located in Maurer’s clefts (15). As shown in Fig. 5C, there is a tight colocalization of the erythrocyte cytosol population of PfEMP1 with MAHRP-1 in early trophozoite stages. In mature stages, PfEMP1 is partly...
FIG. 5. Immunofluorescence analysis of MAHRP-1 in *P. falciparum* (3D7) infected erythrocytes. *A*, a young trophozoite stage-infected erythrocyte showing distinct punctuate staining within the erythrocyte cytosol (panel 1) and a mature, multinucleated schizont showing punctuate staining overlaying a more generalized staining (panel 3). The same infected erythrocytes were 4,6-diamidino-2-phenylindole-stained to visualize the nuclei (2 and 4). *B*, dual labeling studies with PfEMP3 and EXP-1. Panels 1–4, a young trophozoite-infected erythrocyte examined by bright field imaging (panel 1) or labeled with MAHRP-1c antiserum (panel 2, red) and PfEMP3 antiserum (panel 3, green). Panel 5–8, a young trophozoite-infected erythrocyte examined by bright field imaging (panel 5) or for labeling with MAHRP-1c antiserum (panel 6, red) and EXP-1.
MAHRP-1, a Novel P. falciparum Histidine-rich Protein

redistributed to the erythrocyte membrane, whereas MAHRP-1 remains largely associated with the Maurer's clefts.

MAHRP-1 Interacts with FP—Because of the high histidine content of MAHRP-1, we examined the ability of recombinant 3D7 MAHRP-1c to bind to an immobilized metal column. We found that MAHRP-1c-GST fusion protein bound strongly to a Ni²⁺-chelate column and was eluted with imidazole, thus providing potentially an additional step for purification of the protein (data not shown).

Given the ability of MAHRP-1 to bind to metal ions and the proposed function of HRP s in FP detoxification, we examined the interaction between the protein and FP. Use was made of the visible absorption spectrum of FP to examine its interaction with protein. As shown in Fig. 6A, FP exhibits a characteristic absorption peak known as the Soret band with a maximum at 390 nm and a major shoulder at 350 nm and smaller (α and β) bands at wavelengths greater than 500 nm. Quantitative spectroscopic measurements of FP in aqueous solution are complicated by the presence of an equilibrium between the monomer and μ-oxo dimeric form of the FP, which is both pH- and concentration-dependent (24, 25). The FP dimer is favored under basic conditions and at high concentrations. Under acidic conditions, the monomer is favored; however, FP also tends to aggregate and to form β-hematin crystals (6).

The maximum wavelengths and extinction coefficients for the FP absorption bands are sensitive to the presence of bound ligand and can be used to examine an interaction between FP and FP binding molecules (26). In this work, we examined the interaction of recombinant MAHRP-1c with FP. We performed FP binding assays at pH 7.4 in an effort to resemble the physiological environment of the erythrocyte cytosol. Under these conditions, FP exhibits a broad Soret band of relatively low intensity and another much lower intensity band at ~620 nm. These features are characteristic of the μ-oxo dimer under basic conditions and indicate that the FP is largely present as a dimer, probably in equilibrium with some monomeric and aggregated species. This absorption spectrum is dramatically altered in the presence of 3D7 MAHRP-1c. There is a sharpening of the Soret band and a 25-nm blue shift to a wavelength maximum at 410 nm. In addition, the 620-nm peak disappears, and two peaks become apparent at 530 and 570 nm. These results indicate that the FP is present in a monomeric form.

The interaction between the 3D7 MAHRP-1c and FP was examined in greater detail by titrating 2.5 μM 3D7 MAHRP-1c with FP. Fig. 6B shows the normalized absorption spectra of the Soret and α and β bands as a function of the FP:protein ratio. There is little change in the FP spectrum when it is present at up to a 4× excess relative to MAHRP-1c. However, the spectra exhibit a blue shift and a broadening of the Soret band and the appearance of the 620 nm band at the higher ratios examined, indicating the presence of unbound FP. An examination of the Soret absorbance as a function of added FP shows an increased absorbance compared with that for the addition of FP to PBS, with an inflection point corresponding to a FP:MAHRP-1c ratio of 5 (Fig. 6C, open circles). This indicates that the stoichiometry is about 5 FP molecules per 3D7 MAHRP-1c, with an affinity in the submicromolar range. This stoichiometry is roughly consistent with the number of DHGH motifs in the primary sequence. We also examined binding of MAHRP-1c from the ITG2F6 strain to FP. Similar spectra were obtained (not shown); however, the stoichiometric point occurred at a FP:protein ratio of ~11:1 (Fig. 6C, open squares). This is consistent with the increased number of DHGH motifs present in this protein and supports our hypothesis that DHGH

antiserum (panel 7, green). Merges of the red and green channels are shown in panels 4 and 8. C, dual labeling studies with PfEMP1. Erythrocytes infected with parasites of increasing maturity (top to bottom) were examined by bright field imaging (left-hand panels) or for labeling with MAHRP-1c antiserum (second column, red) and PfEMP1 antiserum (third column, green). Merges of the red and green channels are shown in the right-hand panels. Yellow areas represent regions of co-localization. Bars represent 5 μm.
represents the FP binding motif.

We also examined the effects of BSA, a known FP-binding protein, and GST, the fusion moiety present in MAHRP-1c, on the spectral properties of FP. Fig. 6A shows that both GST and BSA cause an increase in the absorption of the Soret band, with only a small red shift and slight sharpening of the band. Similarly, the \( \alpha \) and \( \beta \) bands have an increased absorption, but their relative positions are not affected. The different effects on the spectrum suggest a different mode of FP binding for these proteins compared with MAHRP-1c. It is likely that the FP binds to BSA (27) and GST via nonspecific hydrophobic interactions. The increase in absorbance may therefore reflect the increased solubility or decreased aggregation of the FP in the presence of these proteins.

Effect of MAHRP-1c on the Redox Properties of FP—FP can react with \( \text{H}_2\text{O}_2 \) to form a ferryl (Fe(IV)) intermediate that can participate in a number of enzyme-like reactions. These reactions can destroy FP and convert \( \text{H}_2\text{O}_2 \) to the innocuous compounds water and oxygen (1, 28). In earlier studies, we have shown that the antimalarial drug chloroquine inhibits \( \text{H}_2\text{O}_2 \)-mediated FP destruction under conditions designed to mimic the food vacuole (1). We have also shown that HRP2 can modulate the redox properties of FP (8, 13). In this work, we have examined the interaction of FP with \( \text{H}_2\text{O}_2 \) at pH 7.4, the presumed pH of the erythrocyte cytosol. We find that about 20% of FP is degraded by \( \text{H}_2\text{O}_2 \) within 20 min at pH 7.4 (Fig. 7A). Interestingly, we found that MAHRP-1c (2 \( \mu \text{M} \)) enhances the initial rate of destruction of 5 \( \mu \text{M} \) FP by reaction with \( \text{H}_2\text{O}_2 \) by 10-fold (Fig. 7A). By contrast, equivalent concentrations of GST or BSA had no effect on the rate of degradation of FP by \( \text{H}_2\text{O}_2 \).

We also examined the ability of FP to catalyze \( \text{H}_2\text{O}_2 \)-mediated oxidation of OPD. At pH 7.4, FP (5 \( \mu \text{M} \)) greatly increases the level of oxidation of OPD (5 \( \text{mM} \)) by \( \text{H}_2\text{O}_2 \) (2 \( \text{mM} \)) (Fig. 7B, open columns). Varying protein concentrations (0.1–2 \( \mu \text{M} \)) were added to determine the effect of protein on this oxidation process. MAHRP-1c substantially increased the level of oxidation of OPD. It is noteworthy that the 2.2-fold increase in OPD oxidation with 2 \( \mu \text{M} \) MAHRP-1c is comparable with the 1.8-fold increase in OPD oxidization observed with the same amount of HRP2 at pH 7.4 (8). BSA or GST had relatively little effect on the level of oxidation of OPD (Fig. 7B).

DISCUSSION

Invasion of mature human erythrocytes, which are devoid of all subcellular organelles, presents a major challenge to \textit{P. falciparum}. To survive and to multiply the parasite needs to modify the properties of the host membrane. To do this it needs to establish the machinery for trafficking of proteins beyond its own boundaries. Shortly after invasion, the parasite begins the process of exporting proteins to the erythrocyte cytosol. The initial set of exported proteins may represent the machinery for the subsequent trafficking of important cargo molecules. Indeed maturation of the ring stage parasite is associated with the development of membranous extensions of the parasitophorous vacuole and characteristic Maurer’s clefts with electron-
dense coats that can be observed by electron microscopy in the host cytosol. By the late ring stage, a number of parasite-encoded proteins are present in the erythrocyte cytosol, bound to the cytoskeleton, or inserted into the erythrocyte membrane (29–31). The Maurer’s clefts are thought to be an important intermediate compartment in the trafficking of proteins to the red blood cell membrane (15); however, very little is known about the molecular components of the Maurer’s clefts or the molecular basis of the function of these parasite-derived structures.

In an attempt to identify crucial processes and possibly targets for intervention, we have generated a stage-specific cDNA library from early erythrocytic stages (19). Among a small set of genes exclusively expressed during the ring stage, we have identified MAHRP-1. Its tightly regulated early expression, a putative transmembrane domain, and the presence of a histidine-rich domain made it a prime candidate for further investigation. Several histidine-rich proteins have been described in *P. falciparum* with interesting structural and functional properties (7, 8, 13, 32–34), but MAHRP-1 is the first membrane-associated HRP.

MAHRP-1 is a small (28.9 kDa) protein with no significant homology to any known protein. It has no predicted N-terminal signal sequence but has a 16-amino acid long putative transmembrane domain (Fig. 1). Its C-terminal domain contains a histidine-rich region comprising a variable number of DHGH repeats. We have shown that polymorphism exists between different *P. falciparum* strains, which is confined to the histidine-rich domain, and in particular to the number of DHGH repeats.

Specific antisera were generated against the MAHRP-1 C-terminal domain. These antisera recognized a 29-kDa protein in parasite extracts and did not cross-react with two other parasite-derived histidine-rich proteins, KAHRP and HRP2. The antisera were used to study the location and organization of MAHRP-1. Immunofluorescence microscopy studies indicated that MAHRP-1 is located in the erythrocyte cytosol as clearly defined foci in a similar pattern to that observed for PISBP1, P332, and PIEMP3 (16–18). Indeed, in dual-labeling studies we found a high degree of colocalization of MAHRP-1 with PIEMP3. Maurer’s clefts have been visualized by electron microscopy as long, slender structures coated with electron-dense material (35, 36). It is likely that MAHRP-1 is anchored via its transmembrane domain in the bilayer that delineates these structures. Very little MAHRP-1 is detected inside the parasite’s cytosol or in the parasitophorous vacuole, indicating that the protein is rapidly translocated into the erythrocyte cytosol upon expression.

The Maurer’s clefts appear to be a transit depot in the export of PIEMP1 to the erythrocyte membrane (15). PIEMP1 is a family of proteins with a variable extracellular segment containing cysteine-rich binding domains that are responsible for cytoadherence to the vascular endothelium. PIEMP1 is produced in ring stage parasites and trafficked to the erythrocyte surface (via the Maurer’s clefts) about 20 h after invasion (15, 16, 37, 38). In this work, dual labeling studies were used to confirm that PIEMP1 is located in the same compartment as MAHRP-1 at least for part of the intraerythrocytic cycle. Thus, MAHRP-1 might play a direct or indirect role in the trafficking of this critical virulence factor.

Although KAHRP and PIEMP1 are transiently associated with the Maurer’s clefts (16), MAHRP-1 appears to reside in these organelles throughout the parasite’s development. Upon maturation of the parasite, the Maurer’s clefts increase in number and are often observed in close apposition to the periphery of the host cell; however, they do not appear to fuse with the erythrocyte membrane. It is possible that MAHRP-1 has a structural role in the Maurer’s clefts. Indeed it is possible that the electron-dense appearance of the Maurer’s clefts in electron microscopy studies is due to binding of the heavy metal electron microscopy stain to the histidine moieties in MAHRP-1. However, it is also possible that MAHRP-1 plays an active role in protein trafficking, such as in the sorting or chaperoning of proteins such as PIEMP1.

It is interesting to note that MAHRP-1 is slightly acidic (pI = 5.92). This could allow an interaction with basic residues within KAHRP (pI = 9.2). In this regard, it is useful to note that PIEMP1 has a highly acidic cytoplasmic domain (pI = 4.50), which has been shown to interact with domains of KAHRP (39).

We also performed detergent solubility studies that are consistent with MAHRP-1 being located in Maurer’s cleft membranes. Saponin is a cholesterol binding detergent that permeates the erythrocyte membrane, releasing soluble, cytosolic proteins such as hemoglobin. MAHRP-1 was found in the pellet fraction after saponin treatment, which is consistent with previous studies suggesting that the Maurer’s clefts remain associated with the erythrocyte membrane upon host cell lysis (18, 35). Further fractionation of the parasite samples using two phase-forming Triton X-114/water mixtures revealed that the protein is largely soluble in Triton-X-114. This is consistent with MAHRP-1 being a membrane-embedded protein.

It is interesting to speculate on the possible sequence signals for trafficking of proteins to the Maurer’s clefts. Apart from PIEMP1, only one Maurer’s cleft-associated membrane protein (PISBP1) has been described previously (18). Like MAHRP-1, PISBP1 and PIEMP1 have a single transmembrane domain and lack a classical N-terminal secretory signal (18, 40). For membrane proteins lacking an N-terminal signal, the transmembrane domain can act as a start transfer signal for translocation into the ER membrane. The transmembrane domain can also contain information for trafficking to particular compartments such as the Golgi (41). Thus, the transmembrane segments of MAHRP-1, PISBP1, and PIEMP1 may contain the signal for export to the Maurer’s clefts. By contrast, other parasite-encoded membrane proteins, such as EXP-1, EXP-2, and AMA-1, which are not exported past the parasitophorous vacuole membrane, have classical N-terminal signal sequences (42–44).

The mode of insertion of membrane-embedded proteins into the ER membrane determines their final orientation. Membrane proteins that lack an N-terminal signal sequence can be inserted into the ER with the N-terminal region facing the ER lumen (type Ib orientation) or the cytoplasm (type II orientation) (for review, see Ref. 45). Analysis of the MAHRP-1 sequence using the Psort algorithms (available at psort.nibb.ac.jp) suggests a type Ib orientation. PIEMP1 and PISBP1 are also predicted to be type Ib proteins and are known to be exported to the erythrocyte cytosol with their C-terminal regions facing the erythrocyte cytoplasm (18, 40). Thus, it appears likely that the histidine-rich C-terminal domain of MAHRP-1 also faces the host cytoplasm.

Given its location in the Maurer’s clefts and the prominent histidine-rich domain, we considered the possibility that MAHRP-1 might play a role in protecting the parasite from deleterious effects of FP. The parasite degrades hemoglobin as a source for amino acids, and the consequent byproducts such as FP and H2O2 are highly toxic (for review, see Ref. 46). Recently it has been reported that ~90% of the ~15 mM heme that is released from hemoglobin is sequestered as hemozoin (6). Nonetheless, it is likely that a fraction of the pool of released FP escapes sequestration and diffuses down the concentration gradient into the parasite cytosol. FP readily crosses membranes and would be expected to rapidly equilibrate throughout the cell. Indeed significant levels of free FP (~100 μM) are distributed between the
parasite and host compartments (1, 10). As a consequence of its toxic waste products, the parasite confers a high level of oxidative stress on the host cell (47). This leads to deposition of hemichromes at the cytoplasmic surface of the erythrocyte membrane and binding of autoantigens to the external surface (48). Indeed it has been suggested that P. falciparum alters the permeability of the erythrocyte by applying a controlled oxidative stress to the host cell membrane (49). It appears likely therefore that the parasite would produce proteins to modulate the effect of FP and reactive oxygen species on the host cell. For example, HRP2 has been suggested to play a role in detoxification of the byproducts of hemoglobin digestion (7, 8, 12, 10).

In this work we have examined the ability of the histidine-rich C-terminal domain of MAHRP-1 to interact with FP and to modulate its redox properties. A spectroscopic analysis showed that in the absence of protein, at pH 7.4 FP exhibited a broad Soret peak. This may reflect the dominance of FP μ-oxo dimers and the presence of some aggregates. Upon the addition of proteins such as BSA or GST that interact with FP by hydrophobic interactions, there was an increase in the Soret absorption indicative of increased solubilization of the dimeric form of FP. Upon the addition of MAHRP-1c, there was a sharpening and a red shift of the Soret band of FP as well as changes in the red region of the spectrum. Similar changes have also been reported with FP titrated with imidazole (50) or with HRP2 (8, 51). Thus, the results indicate that in the presence of MAHRP-1c, the FP is present in a monomeric form presumably coordinated to histidines within the protein in a similar fashion to that for HRP2.

Our stoichiometric analysis reveals that 3D7 MAHRP-1c binds FP at a ratio of 1:5, whereas the ITG2F6 MAHRP-1c binds with a stoichiometry of 1:11. This is consistent with the increased number of DHGH motifs in the ITG2F6 isoform and indicates that DHGH is the FP binding motif. Thus, it might be possible that MAHRP-1 isoforms with an increased number of repeats might be able to tolerate higher concentrations of FP, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, although this needs to be tested experimentally.
MAHRP-1, a Novel P. falciparum Histidine-rich Protein