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Passive Immunoprotection of *Plasmodium falciparum*-Infected Mice Designates the CyRPA as Candidate Malaria Vaccine Antigen

Anita M. Dreyer,*† Hugues Matile,‡ Petros Papastogiannidis,*† Jolanda Kamber,*† Paola Favuzza,*† Till S. Voss,*† Sergio Wittlin,*† and Gerd Pluschke*†

An effective malaria vaccine could prove to be the most cost-effective and efficacious means of preventing severe disease and death from malaria. In an endeavor to identify novel vaccine targets, we tested predicted *Plasmodium falciparum* open reading frames for proteins that elicit parasite-inhibitory Abs. This has led to the identification of the cysteine-rich protective Ag (CyRPA). CyRPA is a cysteine-rich protein harboring a predicted signal sequence. The stage-specific expression of CyRPA in late schizonts resembles that of proteins known to be involved in merozoite invasion. Immunofluorescence staining localized CyRPA at the apex of merozoites. The entire protein is conserved as shown by screening of the CyRPA encoding gene from a diverse range of *P. falciparum* isolates. CyRPA-specific mAbs substantially inhibited parasite growth in vitro as well as in a *P. falciparum* animal model based on NOD-scid IL2Rγnull mice engrafted with human erythrocytes. In contrast to other *P. falciparum* mouse models, this system generated very consistent results and evinced a dose-response relationship and therefore represents an unprecedented in vivo model for quantitative comparison of the functional potencies of malaria-specific Abs. Our data suggest a role for CyRPA in erythrocyte invasion by the merozoite. Inhibition of merozoite invasion by CyRPA-specific mAbs in vitro and in vivo renders this protein a promising malaria asexual blood-stage vaccine candidate Ag. The Journal of Immunology, 2012, 188: 6225–6237.

Although preventable and curable, malaria was estimated to have claimed 781,000 lives in 2009 (1). Besides other control measures, implementation of an effective vaccine against malaria is regarded as a cost-effective measure to reduce mortality and morbidity. Three different malaria vaccine strategies can be distinguished: infection-blocking vaccines targeting pre-erythrocytic stages, anti-morbidity vaccines targeting the erythrocytic stages, and transmission-blocking vaccines targeting the sexual stages. It is assumed that a highly effective malaria vaccine needs to target multiple stages of the parasite life cycle (2, 3).

The importance of a parasite blood-stage component in a malaria subunit vaccine is evident, as clinical symptoms of malaria are mainly attributed to asexual blood stages. In exposed humans, protection from symptomatic disease is acquired after repeated exposure over years (4). This naturally acquired immunity is attributed at least in part to Ab responses targeting blood-stage Ags. This was demonstrated by passive immunotherapy studies, in which transfer of Igs from immune individuals to malaria patients led to very substantial reductions of parasitemia and clinical symptoms (5–7).

The feasibility of an asexual blood-stage vaccine is supported by studies in humans and animal models (8–13). To date, research in this field has focused on a few protein candidates including merozoite surface protein (MSP)-1 (14), MSP-2 (8), MSP-3 (15–18), apical membrane Ag 1 (AMA-1) (19), erythrocyte binding Ag 175 (EBA-175) (20), glutamate-rich protein (GLURP) (15, 21), and serine repeat Ag 5 (SERA5) (22). These Ags are all merozoite proteins, either located on the merozoite surface or contained within apical invasion organelles. In contrast to intraerythrocytic stages, which are largely hidden within the RBCs, free merozoites are directly accessible to Abs. Abs are thought to interfere with the invasion of erythrocytes by binding to merozoite surface proteins or proteins released from apical organelles. Merozoite invasion involves a complex series of orchestrated molecular interactions but nevertheless takes less than a minute to be completed (23, 24). After low-affinity attachment of a freshly released merozoite to an erythrocyte, the merozoite reorients to its apical pole. Released microneme and rhoptry neck resident proteins mediate high-affinity attachment by the establishment of a tight junction. This in turn initiates the release of rhoptry bulb resident proteins, which are involved in the formation of a membrane to surround the parasitophorous vacuole. Mediated by an actomyosin motor complex, the tight junction is then moved toward the anterior pole, terminating with the sealing of the parasitophorous vacuole membrane at the posterior pole of the merozoite (for a review, see reference 25). Most proteins currently regarded as key malaria blood-stage vaccine candidate Ags were shown or are thought to be involved in the invasion process. These Ags are targets of invasion inhibitory Abs, which interfere with different steps of the invasion process. Parasite growth inhibitory Abs specific for MSP-1, the most abundant protein on the surface of merozoites assumed to be involved in the initial attachment, were for example shown to inhibit merozoite invasion either by preventing MSP-1 processing or by agglutination.
preventing the dispersal of released merozoites (26, 27). By contrast, inhibitory Abs specific for AMA-1, a micronemal protein and constituent of the tight junction, were shown to interfere sterically with the assembly of the protein complex forming the tight junction (28). The modes of action of all invasion inhibitory Abs have not been identified, but suggested mechanisms also include opsonization and destruction of merozoites by phagocytic cells (29, 30), complement activation (31), or neutrophil respiratory bursts (32).

To date, relatively few blood-stage Ags are in clinical development as vaccine components (33). Unfortunately, the most advanced blood-stage vaccine Ags, AMA-1 and MSP-1, have not demonstrated efficacy in African children to date (14, 34, 35). However, a multistage vaccine comprising an AMA-1 component showed a 50% reduced incidence rate of clinical malaria episodes in child vaccinees compared with that of control children (36). But whether protection is associated with AMA-1–specific responses remains to be shown. The extensive polymorphisms of current candidate Ags is deemed to be a major hurdle for blood-stage vaccine development (37–41). Hence, although a couple of blood-stage Ags are under vaccine development, it is possible to search for more vaccine candidates. The comparison of a large range of protein Ags in preclinical assays would allow a more rational prioritization of candidates for inclusion into a vaccine. Since the availability of the *Plasmodium falciparum* genome in 2002, reverse vaccinology is considered an opportunity to identify novel vaccine candidates in a more rational way (42–50). On the basis of the genome-wide transcriptomic and proteomic information generated since 2002, we selected the *P. falciparum* open reading frame (ORF) PFD1130w for further characterization. PFD1130w was selected because the available information suggested that the protein is a merozoite protein involved in erythrocyte invasion: 1) transcription has been shown to be upregulated in late asexual blood stages (46, 47); 2) the encoded protein is predicted to contain a N-terminal secretion signal peptide (51); 3) a genome-wide in silico study based on gene coexpression, sequence homology, and domain–domain and yeast two-hybrid interaction data showed that PFD1130w clustered into an interaction network implicated in merozoite invasion (48); and 4) the *pfd1130w* gene lies in close proximity to genes encoding proteins known to be involved in RBC invasion, such as reticulocyte-binding homolog 4 (RH4) and reticulocyte-binding homolog 5 (RH5) proteins, SURFIN4.2, and glideosome-associated protein with multiple membrane spans 2 (GAPM2) (Fig. 1) (52–55). On the basis of these data, we selected PFD1130w for generation of mouse mAbs (56), which allowed us further to characterize the protein and to perform functional assays.

In this study we carried out an in-depth characterization of PFD1130w and demonstrate its potential as a malaria asexual blood-stage vaccine candidate. PFD1130w is a conserved cysteine-rich protein that we designated cysteine-rich protective Ag (CyRPA). Our data on localization, stage-specific expression pattern, and functional assays suggest a role of CyRPA in erythrocyte invasion by the merozoite. Importantly, CyRPA elicits Abs that inhibit merozoite invasion in vitro and in vivo. Furthermore, our passive immunization studies in *P. falciparum*-infected NOD-scid IL2R alpha mice with anti-CyRPA mAbs identified this animal model as an unprecedented system to quantitatively evaluate functional potentials of malaria-specific Abs.

**Materials and Methods**

**Ethics statement**

This study was carried out in strict accordance with the Rules and Regulations for the Protection of Animal Rights (Tierschutzverordnung) of the Swiss Bundesamt für Veterinärwesen. The protocol was ethically approved by the Ethikkommission beider Basel ( Permit Number 2375). Human sera were obtained from adult volunteers after receiving written informed consent. Ethical clearance was obtained from the institutional ethical review board of the Navrongo Health Research Center (Navrongo, Ghana).

**Culture of parasites**

*P. falciparum* strains 3D7, 7G8, D6, W2mef, and K1 were cultured essentially as described previously (57). The culture medium was supplemented with 0.5% AlbuMAX (Life Technologies) as a substitute for human serum (58). Cultures were synchronized by sorbitol treatment (59). Erythrocytes for passages were obtained from the Swiss Red Cross (Basel, Switzerland).

**Isolation of free merozoites**

To obtain *P. falciparum* merozoites, erythrocytes infected with highly synchronous schizont-stage parasites were centrifuged at 700 × g for 5 min to separate released merozoites from unruptured schizonts and uninfected erythrocytes. Supernatants containing free merozoites were centrifuged at 3000 × g for 10 min to collect merozoites.

**Western blot analysis**

Blood-stage parasite lysates were prepared essentially as described previously by saponin lysis of *P. falciparum* 3D7-infected erythrocytes (57). In brief, cultured parasites were washed once with PBS. Pelleted infected RBCs were lysed in 20 volumes of 0.06% (w/v) saponin in PBS for 20 min. Parasites were washed, and the final pellet was resuspended in 3 volumes of PBS and stored at −80°C until further use.

Cell lysate of transfected HEK cells was prepared as described previously (56).

For SDS-PAGE, cell or parasite lysate was resolved on precast 4–12% gradient gels (NuPAGE Novex 4–12% Bis-Tris Gel; Invitrogen) with MES running buffer according to the manufacturer’s directions. The proteins were electrophoretically transferred to nitrocellulose membrane using a dry-blotting system (iBlot; Invitrogen). After blocking the membrane, specific proteins were detected with appropriate dilutions of anti-CyRPA mAbs (56) or anti-hexa-his tag mAb (56) followed by HRP-conjugated goat anti-mouse IgG mAb (Kirkgaard & Perry Laboratories). Blots were developed using ECL Western blotting detection reagents (ECL Western Blotting Substrate; Pierce).

**Immunofluorescence staining of infected erythrocytes and free merozoites**

For indirect immunofluorescence microscopy, smears of infected RBCs were fixed in 60% methanol and 40% acetone for 2 min at −20°C and blocked with 1% BSA in PBS. Isolated free merozoites were spotted and fixed onto 1-lysine–coated multilayer glass slides as described previously (60). Cells were probed with the following primary or secondary Abs: biotin-labeled mouse anti-CyRPA mAb c06 (56), Alexa 488-labeled mouse anti-RAp-1 5-2 mAb (61), Alexa 488-labeled mouse anti–AMA-1 DV5a mAb (60), Alexa 488-labeled mouse anti–MSP-1 1C7.2 mAb (G. Pluschke, unpublished observations), Alexa 488-labeled mouse anti-GAPDH 1.4a mAb (62), anti–MSP-2 rabbit serum (63), anti–MSP-5 rabbit serum (64), Alexa 488-labeled mouse anti-CyRPA mAb c06 (56), Alexa 488-labeled mouse anti-RAp-1 5-2 mAb (61), Alexa 488-labeled mouse anti–AMA-1 DV5a mAb (60), Alexa 488-labeled mouse anti–MSP-1 1C7.2 mAb (G. Pluschke, unpublished observations), Alexa 488-labeled mouse anti-GAPDH 1.4a mAb (62), anti–MSP-2 rabbit serum (63), anti–MSP-5 rabbit serum (64), Alexa 568-labeled streptavidin (Invitrogen), and Alexa 488-labeled chicken anti-rabbit IgG (H+L) (Invitrogen). The slides were mounted in mounting medium containing DAPI (ProLong Gold antifade reagent with DAPI; Invitrogen). Fluorescence microscopy was performed on a Leica DM-5000B using a ×60 oil immersion objective lens and documented with a Leica DFC300FX digital camera system. Images were processed using Leica Application Suite and Adobe Photoshop CS5.

**Immunofluorescence staining of live versus fixed merozoites**

Isolated merozoites were incubated with Alexa 488-labeled mouse anti–MSP-1 1C7.2 mAb (G. Pluschke, unpublished observations). Alexa 488-labeled mouse anti-GAPDH 1.4a mAb (62), or biotin-labeled mouse anti-CyRPA mAb c06 (56) and Alexa 568-labeled streptavidin (Invitrogen) diluted in parasite culture medium. Thereafter, cells were washed two times with PBS and then fixed 30 min in 4% PFA in PBS. For staining of fixed merozoites, cells were first incubated for 30 min in 4% PFA and thereafter immunofluorescence-stained as described earlier. Cells were mounted in mounting medium containing DAPI (ProLong Gold antifade reagent with DAPI; Invitrogen). Fluorescence microscopy was performed as described earlier.
In vitro growth inhibition assay

In vitro growth inhibition assays with *P. falciparum* strains 3D7, 7G8, D6, W2meF, and K1 were conducted as described (60). Each culture was set up in triplicate in 96-well flat-bottom culture plates. Viable parasites were stained with hydroethidine and analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software. A total of 30,000 cells per sample were analyzed. Percent inhibition was calculated from the mean parasitemia of triplicate test and control wells as follows: percent inhibition = (control – test)/(control) x 100.

In vivo growth inhibition assay

Abs were tested in the murine *P. falciparum* model essentially as described (65). The only modification implemented was that daily human blood injections (0.75 ml) were administered by the i.v. route instead of the i.p. route. Once parasitemia reached >0.5%, mice received a single dose of 0.5, 2.5, or 5 mg mAb in PBS by i.v. injection. Thereafter, parasitemia was measured for the next 5 d. To monitor serum levels of administered Abs, serum samples were taken just before, 1, 2, and 5 d after mAb injection.

For the transfer experiment, blood cells of mice having previously received 2.5 mg anti-CyRPA mAb c12 were washed with 0.9% NaCl and used to infect naive human erythrocytes engrafted NOD-scid IL2Rγc−/− mice.

ELISA

Detection of CyRPA-specific and MSP-1-specific Abs in human sera by ELISA. Human serum samples were derived from healthy Swiss adults with no history of malaria exposure (66) and from healthy individuals between 5 and 20 y of age living in the malaria endemic (67) Kassena-Nankana District in northern Ghana. ELISA plates (Maxisorp; Nunc) were coated with 10 μg/ml purified recombinant CyRPA protein produced in HEK cells or purified recombinant MSP-1 (aa 34–593) or MSP-1 (aa 1250–1563) produced in Escherichia coli (68). After blocking, plates were incubated with dilutions of human serum. Alkaline phosphatase-conjugated goat anti-human IgG Fab(‘)2 (Jackson ImmunoResearch Laboratories) was used as secondary Ab and p-nitrophenyl phosphate was used as substrate (Sigma). The OD of the reaction product was recorded at 405 nm with a microplate absorbance reader (Sunrise Absorbance Reader; Tecan).

Ab competition ELISA. Plates were coated with 10 μg/ml purified recombinant CyRPA protein produced in *E. coli*. After blocking, plates were incubated with 1 mg/ml, 1 μg/ml, or 0.1 μg/ml of different anti-CyRPA mAbs (c02, c04, c05, c06, c08, c09, c10, c12, c13). After 30 min, different biotinylated anti-CyRPA mAbs (c02, c04, c05, c06, c08, c09, c10, c12, c13) were added to each well resulting in a concentration of 1 μg/ml of labeled Abs. HRP-conjugated streptavidin (GenScript) was used as detecting agent. After washing, a Pierce (western blotting substrate; Pierce) was used for development.

Culture of eukaryotic cells

The human embryonic kidney cell line 293 HEK was obtained from the American Type Culture Collection (ATCC CRL-1573). 293 HEK cells were cultured in IMDM supplemented with 10% FCS, glutamine, and penicillin/streptomycin at 37°C in a humidified incubator.

Expression of CyRPA protein fragments on HEK cell surface

DNA sequences that encode fragments of CyRPA protein were amplified by PCR from a plasmid containing the codon-optimized sequence of PFD1130w. DNA sequences for amplification were as follows: Fr26-251 and 3910 (5'-CCAAATTgctagGCATATAATTG-3') and 3913 (5'-ATAAGAGGTccggcTTTTCAATTGATGTGTTCTCA-3'); Fr26-127, 3912 (5'-ATATgACTagcGGTAAAGAGATAGTACTGTTACG-3') and 3905 (5'-TTCGGTACCTCGGGAAATGTC-3'); Fr26-142, 3910 and 3911 (5'-ATAAGAGGcgcggcCTTTACATTGATGTGTTCTCA-3'); Fr26-295, 3912, 3913; Fr26-352, 3885 (5'-gctagcGACCTCCTTCTTCAATTGT-3') and 3887 (5'-gagccggcGTGTTAAGATCCGCGGTAG-3'); Fr26-181, 3910 and 3939 (5'-ATAAGAGGTccggcTTTTCAATTGATGTGTTCTCA-3'); Fr74-181, 3938 (5'-ATATgACTagcGGTAAAGAGATAGTACTGTTACG-3') and 3939; Fr74-251, 3938 and 3939. Amplifications were performed using REDPol DNA polymerase (Solis Bioody) according to the manufacturer’s protocol with the following profile: 5 min, 95°C; 30 × (30 s, 95°C; 30 s, 60°C; 2 min, 72°C); 6 min, 72°C. The amplicons were digested with MluI, XhoI, and NotI (New England Biolabs) and then ligated into a pCDNA3.1-based expression vector (56). This expression vector allows surface expression of a protein of interest. It contains the secretion signal of bee-venom melittin, a cloning site for the protein of interest, a FLAG tag, a transmembrane domain of mouse growth hormone-A, and a hexa-his tag. The 293 HEK cells were transfected with the different expression vectors using JetPEI transfection reagent (PolyPlus) according to the manufacturer’s protocol. One to two days later, transient transfectants were used for Western blot analysis or immunofluorescence staining.

Immunofluorescence staining of living transfected HEK cells

Immunofluorescence staining of live HEK cells was performed in chamber slides (8-well chamber-slide, Lab-Tek; Nunc). HEK cells (12,000 cells/well) were seeded and 4 d later transfected with different vectors. The following day, immunofluorescence stainings were performed essentially as described with the wells with 100 μg/ml anti-CyRPA mAb diluted in IMDM for 30 min on ice. After washing, cells were fixed for 30 min with 4% formaldehyde in PBS. After washing, cells were incubated for 30 min with Cy3-labeled goat anti-mouse IgG Fab(‘)2 (Jackson ImmunoResearch Laboratories) diluted in PBS and washed four times with PBS. Slides were mounted with mounting solution containing DAPI (ProLong Gold antifade reagent with DAPI; Invitrogen). Stainings were assessed by fluorescence microscopy as described earlier.

Bacterial strains and media

*E. coli* strain Top10 (TOP10 Chemically Competent *E. coli* Cells; Invitrogen) was used for the amplification of plasmids. *E. coli* strain BL 21 Star (DE3) (Invitrogen) was used for recombinant expression of CyRPA protein. Bacteria were grown in lysogen broth medium containing 100 μg/ml ampicillin.

Recombinant protein expression and purification

CyRPA (aa 26–352) was recombinantly expressed in *E. coli* using the pET28a expression system (Novagen, modified to contain an ampicillin selection cassette). Briefly, a PCR product of CyRPA was generated from a plasmid containing the sequence of PFD1130w using primers 5'-ATAGCACCGTGATAATTGACGC-3' and 5'-CCCGCAGGAGGTTGTAATGCGCTTGGGA-3'. The amplicon was digested with restriction endonucleases NcoI and XhoI (New England Biolabs) and cloned into NcoI and XhoI sites of pET28a. Protein expression in *E. coli* BL 21 Star (DE3) (Invitrogen) was induced by addition of 1 mM isopropyl thio-galactoside (Calbiochem) for 2 h at 37°C. The recombinant protein was purified from inclusion bodies using denaturing conditions (8 M urea, 500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole) by Ni-NTA chromatography. The purity and integrity of the purified protein was analyzed by SDS-PAGE, and the protein concentration was determined by measuring OD590. The purified recombinant protein was identified as the expected CyRPA protein by Western blot analysis with CyRPA-specific mAbs.

Hexa-his-tagged recombinant proteins corresponding to aa 34–593 and aa 1250–1563 of MSP-1 of *P. falciparum* MAD20 were a kind gift of Bela Takacs (Pharma Research Basel, F. Hoffmann-La Roche, Basel, Switzerland [retired]). Proteins were produced in *E. coli* and purified by Ni-NTA chromatography (68).

CyRPA was recombinantly expressed in HEK cells. The codon-optimized sequence of PFD1130w corresponding to aa 22–362 was cloned into a pCDNA3.1-based expression vector. This expression vector allows secretion of the protein of interest. It contains the secretion signal of bee-venom melittin, a cloning site for the protein of interest, and a C-terminal hexa-his tag. 293 HEK cells were stably transfected with the expression vector using JetPEI transfection reagent (PolyPlus) according to the manufacturer’s protocol, and clones were generated by limiting dilution. For recombinant protein production, transfected cells were cultured in serum-free medium (Pangaea 293A; PAN Biotech). The recombinant protein was purified from culture supernatant by Ni-NTA chromatography. The purity and integrity of the purified protein was analyzed by SDS-PAGE. The purified recombinant protein was identified as the expected CyRPA protein by Western blot analysis with CyRPA-specific mAbs.

Analysis of binding of soluble CyRPA to RBCs by flow cytometry

Human RBCs were incubated with 100, 20, 4, or 0 μg/ml purified recombinant CyRPA (produced in HEK cells) diluted in PBS, 1% BSA. Surface-bound protein was detected by incubating cells with 100 μg/ml anti-hexa-his tag goat (Ab 609 (56) and subsequently with Alexa Fluor 680 donkey anti-mouse IgG (H+L) conjugate. FACS analysis was performed on a FACSscan (Becton Dickinson) using CellQuest software (Becton Dickinson), and 30,000 events were collected for each sample.
Analysis for binding of human RBCs to CyRPA expressed on HEK cells

Human RBCs were incubated with 50% confluent stably transfected HEK cells expressing recombinant CyRPA on the cell surface (56) for 2 or 24 h. After repeated washing with culture medium, RBCs attached to HEK cells were assessed by microscopy.

Surface plasmon resonance analysis of anti-CyRPA mAb–CyRPA interaction

The affinities of the anti-CyRPA mAbs for CyRPA were determined with an optical biosensor using the real-time surface plasmon resonance (SPR) technology (BIArrayc 3000; Pharmacia Biacore) at 35˚C. About 2000 response units of goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) were coupled to CM5 sensor chip using N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Approximately 350 response units of anti-CyRPA mAbs in PBS were captured onto the surface. The purified recombinant CyRPA produced in HEK cells at concentrations ranging from 400 to 3.1 nM was injected for 2 min using a flow rate of 50 μl/min. Dissociation of bound Ag in PBS was followed for 5 min. The surfaces were regenerated after each cycle. Equilibrium dissociation constant (Kd) was calculated from the ratio of the dissociation and association rate constants obtained with the BIACore evaluation 2.1 software using a 1:1 binding model with mass transfer.

Sequencing the CyRPA gene of different strains

Genomic DNA was prepared from P. falciparum strains 3D7, MAD20, FC27, RFC3, W2met, Hb3, Ro-33, 7G8, K1, FC3R, ITG2F6, FVO, IF4, IA6, IA10, IA12, IA18, and IFA9 and used for PCR amplification of PFD1130w. PFD1130w was amplified in three overlapping fragments with the following primer combinations: 5'-AAAATTTTTGAGAAATGGTTGAGAA-3' and 5'-AAAAGGATGACAGCAGGCATATTCC-3'; 5'-TGTTCTCTTTGTTCATGATGTTGTTT-3' and 5'-TCTCATTATGTTGGAAACTCATTGGA-3'; 5'-TGACAACTTCAAAATTAGGTGTGCAA-3' and 5'-AAATATGATGATTGTTGAATGGTT-3'. Amplifications were performed using DreamTaq DNA polymerase (Fermentas Life Sciences) according to the manufacturer’s protocol with the following profile: 5 min, 95˚C; 35 × (30 s, 95˚C; 30 s, 60˚C; 2 min, 72˚C); 6 min, 72˚C. Amplicons were purified and subjected to direct sequencing (Macrogen, Seoul, South Korea). Sequences were analyzed using AutoAssembler 1.4.0 software (Applied Biosystems, PerkinElmer).

Accession numbers for all genes and proteins mentioned in this study

Sequences of genes and proteins mentioned in this study can be found on PlasmoDB (http://plasmoDB.org/plasmo/) with the following accession numbers: P. falciparum CyRPA (PFD1130w), P. vivax CyRPA (PVX_090240), P. knowlesi CyRPA (PKH_052740), P. falciparum MSP-1 (PF1475w), P. falciparum MSP-2 (PFB0300c), P. falciparum MSP-3 (PF10_0345), P. falciparum MSP-4 (PFB0310c), P. falciparum MSP-5 (PFBO350c), P. falciparum AMA-1 (PF11_0344), P. falciparum EBA-175 (MALP1_176), P. falciparum GLURP (PF10_0344), P. falciparum SERA5 (PFBO340c), P. falciparum RH4 (PFD1150c), P. falciparum RH5 (PFD1145c), P. falciparum SURF1N42 (PFD1160c), P. falciparum GAPDH (PF14_0598).

Results

Sequence analysis of P. falciparum CyRPA

pfd1130w/cyra is 1188 bp long with a 99-bp intron and is localized in the subtelomeric region of chromosome 4 in close proximity to genes encoding proteins known to be involved in RBC invasion, such as RH4 and RH5, SURF1N42, and GAPM2 (Fig. 1) (52–55). The hypothetical protein encoded by pfd1130w is predicted to contain an N-terminal secretion signal peptide. Orthologs of CyRPA are only present in the genomes of P. knowlesi (PKH_052740) and P. vivax (PVX_090240) and are absent in Plasmodium species infecting rodents. Comparison of these protein sequences revealed conservation with 36% and 38% identity of P. falciparum CyRPA with the P. knowlesi and the P. vivax orthologs, respectively. Furthermore, cysteine residues in the orthologs are positionally conserved (Fig. 2). BLAST search identified the mosquito-stage protein PSOAP12 (PFE0680) as paralog of CyRPA (69). This protein shares nine positionally conserved cysteine residues in the homologous N-terminal part, but additionally has a 6-cysteine-protein domain at the C terminus (70).

CyRPA is nonpolymorphic

To assess potential sequence diversity of the CyRPA protein, cyra genes of 12 P. falciparum standard strains from different geographical origin (3D7, K1, MAD20, FC27, RFC3, RFC3R, W2met, Hb3, Ro-33, 7G8, ITG2F6, and FVO) and six field isolates from Tanzania (IFA4, IFA6, IFA10, IFA12, IFA18, and IFA19) were amplified by PCR and sequenced. Apart from one nonsynonymous single nucleotide polymorphism (SNP) at base pair position 1116 in P. falciparum strains K1, FC3R, ITG2F6, and FVO, all sequences obtained were identical to the P. falciparum 3D7 reference sequence. In the protein sequence, this SNP results in an arginine/serine dimorphism at amino acid position 339 (Fig. 2).

Stage-specific expression of CyRPA in schizonts and free merozoites

The PFD1130w gene encodes a 362-aa-long protein with a predicted molecular mass of 42.8 kDa. Using highly synchronized asexual blood-stage parasite cultures, we assessed the expression profile of CyRPA in P. falciparum across the intraerythrocytic developmental cycle at the protein level by Western blot analysis with CyRPA-specific mAbs at 8-h intervals. A discrete band of ~36 kDa was detected in schizont stages, free merozoites, and very early ring stages but not in late ring and early trophozoite stages (Fig. 3A). Stage-specific expression of CyRPA in schizont stages and free merozoites was affirmed by indirect immunofluorescence staining of synchronized blood-stage parasites with anti-CyRPA mAbs (Fig. 3B). These results are in agreement with transcriptional data for PFD1130w, showing elevated transcript levels in late stages of the asexual blood cycle of P. falciparum with maximal expression measured at 40 to 48 h postinvasion (46, 47, 71). Additionally, mass spectrometry-based evidence for expression of PFD1130w was reported in schizont stages (72).

CyRPA is localized at the merozoite apex

CyRPA has a putative N-terminal secretion signal sequence and is cysteine-rich (Fig. 2). In contrast to fixed merozoites, CyRPA could not be stained with specific mAbs in live merozoites, indi-
cating that the Ag is located intracellularly in free merozoites (Supplemental Fig. 1). To determine the localization of CyRPA in schizont stages and free merozoites more accurately, we performed colocalization studies using Abs specific for the cytosol (GAPDH), the micronemes (AMA-1) (73), the rhoptry bulb (RAP-1) (74), and the merozoite surface [MSP-1 (75) and MSP-5 (64)] (Fig. 4). The pattern of CyRPA staining in free merozoites included a dot toward the merozoite apical end and a weaker staining dispersed over the anterior pole of the merozoite. The apical dot did not colocalize with micronemes or rhoptry bulbs. Api
cal dots of MSP-5 and CyRPA largely overlayed, but the degree of additional faint staining of the merozoite body differed, being spread over the anterior part for CyRPA and just at the apical pole for MSP-5. Accordingly, schizonts were stained by CyRPA-spe
cific mAbs in a patchy to dotty manner, not colocalizing with rhoptry bulbs or micronemes. CyRPA-specific and MSP-5–spe
cific stainings showed a relatively similar pattern. Hence, CyRPA localizes to an apical structure distinct from rhoptry bulbs and micronemes.

Amino acid sequence alignment of P. falciparum CyRPA with orthologs in P. knowlesi and P. vivax. Full-length putative orthologs of P. falciparum CyRPA are only found in the genomes of P. vivax (PVX_090240) and P. knowlesi (PKH_052740). Sequence identities are 38 and 36%, respectively. Asterisk (*), identical; colon (:), conservation between groups of strongly similar properties; period (.), conservation between groups of weakly similar properties substitution. Ten of twelve cysteine residues are positionally conserved (shaded in gray). Amino acid residues of the predicted secretion signal sequences are indicated in boldface. DNA-sequencing of the cyrpa gene of a set of P. falciparum strains revealed one nonsynonymous SNP at base pair position 1116. Hence, at amino acid residue 339 (framed), the strains 3D7 (airport malaria), MAD20 (PNG), FC27 (PNG), RFCR3 (The Gambia), W2met (Indochina), Hb3 (Honduras), Ro-33 (Ghana), 7G8 (Brazil), IFA4 (Tanzania), IFA6 (Tanzania), IFA10 (Tanzania), IFA12 (Tanzania), IFA18 (Tanzania), and IFA19 (Tanzania) encode an arginine residue, whereas K1 (Thai land), FCR3 (The Gambia), ITG26 (Brazil), and FVO (Vietnam) encode a serine residue.

Stage-specific expression of CyRPA in late asexual blood-stage parasites. (A) Western blot analysis with lysates of tightly synchronized P. falciparum 3D7 blood-stage parasites with anti-CyRPA mAb c12 (upper panel). The blot was probed for equal loading with an anti-GAPDH mAb (lower panel). 1-4, 4-12, 12-20, 20-28, 28-36, 36-44, 44-52: hours postinvasion. M: free merozoites. (B) Indirect immunofluorescence stainings of asexual blood-stage parasites confirmed stage-specific expression in schizont stages and free merozoites. Methanol/acetone fixed P. falciparum 3D7 parasites were probed with anti-CyRPA mAb c06 (red) and anti-GAPDH mAb (green). Exposure times were identical for all pictures of the same channel. Nuclei were stained with DAPI (blue). Original magnification X1008.
Anti-CyRPA mAbs inhibit merozoite invasion in vitro

Considering the apical localization of CyRPA, and as CyRPA was predicted to be implicated in merozoite invasion (48), we assessed anti-CyRPA mAbs for in vitro parasite growth inhibitory activity. Growth inhibition assays were conducted for two cycles of merozoite invasion. Of the anti-CyRPA mAbs tested, seven out of nine consistently inhibited growth of all five tested *P. falciparum* strains in a concentration-dependent manner (Fig. 5A, Supplemental Fig. 2). At a concentration of 1000 μg/ml, the anti-CyRPA mAb c12 inhibited parasite growth by 58.0 ± 2.8%. In contrast, anti-CyRPA mAb c05 had no effect on parasite growth, and anti-CyRPA mAb c13 exerted only marginal growth inhibitory activity even at high concentrations (Fig. 5A). All mAbs tested were produced and purified in parallel in the same manner, and results were reproducible with independent mAb production batches (data not shown).

To identify the step at which the anti-CyRPA mAbs exert their effect, parasite growth was compared in the presence or absence of inhibitory mAbs during one blood-stage cycle of highly synchronized parasites. Anti-CyRPA mAb c12 (250 μg/ml) was added to early ring stages, and parasitemia was monitored after 13, 27, and 31 h by flow cytometry. No significant difference in the development of trophozoites from ring stages was observed at any time point compared with the PBS control (Fig. 5C). When the anti-CyRPA mAb c12 was added to a synchronized parasite culture at the schizont stage and parasitemia was monitored during development into new ring and subsequently into trophozoites, a significant reduction in parasitemia compared with the PBS control was measured (Fig. 5C). This reduction emerged as soon as the parasitized erythrocytes had ruptured and the released merozoites had infected new erythrocytes (Fig. 6A). In mice that had received the control mAb, a sigmoidal increase in parasitemia was measured, reaching 21.0 ± 3.6% on day 6. Parasitemia in mice having received 2.5 mg anti-CyRPA mAb 13, which has shown a very weak in vitro growth inhibitory effect, increased similarly. In contrast, parasitemia of mice having received 2.5 or 5 mg anti-CyRPA mAb c12 increased only marginally, reaching 3.5 ± 1.2% and 3.7 ± 1.1% on day 6. The difference in parasitemia on day 6 in mice receiving 2.5 mg anti-CyRPA mAb c12 compared with the negative control group was highly significant (two-sided test; \( p = 0.0013 \)). Also, a five times lower dose of mAb c12 (0.5 mg) still reduced parasite growth (15.0 ± 1.2% parasitemia on day 6). Titration of mAbs in the circulation of the passively protected mice by ELISA showed that Ab levels remained high over the entire study period, ranging from 57 to 117% on day 6 compared with day 2, and immunofluorescence staining of persisting parasites showed that they still expressed CyRPA (data not shown).

On day 6, blood cells of mice having received 2.5 mg anti-CyRPA mAb c12 were used in a follow-up experiment to infect three naive groups of NOD-scid IL2Rγnull mice engrafted with human erythrocytes to allow the growth of *P. falciparum* (65). Groups of three mice with a parasitemia of 0.87 ± 0.12% were injected once with 0.5, 2.5, or 5 mg anti-CyRPA mAb c12, respectively. The control group received 2.5 mg subclass-matched control mAb (hexa-his tag-specific). Parasitemia of all mice was monitored for the next 5 d (Fig. 6A). In mice that had received the control mAb, a sigmoidal increase in parasitemia was measured, reaching 21.0 ± 3.6% on day 6. Parasitemia in mice having received 2.5 mg anti-CyRPA mAb 13, which has shown a very weak in vitro growth inhibitory effect, increased similarly. In contrast, parasitemia of mice having received 2.5 or 5 mg anti-CyRPA mAb c12 increased only marginally, reaching 3.5 ± 1.2% and 3.7 ± 1.1% on day 6. The difference in parasitemia on day 6 in mice receiving 2.5 mg anti-CyRPA mAb c12 compared with the negative control group was highly significant (two-sided test; \( p = 0.0013 \)). Also, a five times lower dose of mAb c12 (0.5 mg) still reduced parasite growth (15.0 ± 1.2% parasitemia on day 6). Titration of mAbs in the circulation of the passively protected mice by ELISA showed that Ab levels remained high over the entire study period, ranging from 57 to 117% on day 6 compared with day 2, and immunofluorescence staining of persisting parasites showed that they still expressed CyRPA (data not shown).

Fine specificity of anti-CyRPA mAbs

Of nine CyRPA-specific mAbs analyzed, two (c05 and c13) showed no or borderline growth inhibitory effect, and seven (c02, c04, c06, c08, c09, c10, and c12) showed inhibitory activity. To explain these differences in biological activity, affinities as well as fine specificities of the anti-CyRPA mAbs were assessed.

SPR with immobilized anti-CyRPA mAbs and recombinant CyRPA injected at various concentrations was used to analyze binding association and dissociation (Supplemental Fig. 3). All mAbs showed similar on-rates and slightly varying off-rates.
resulting in $K_D$s ranging from low nanomolar to low picomolar. However, no correlation between affinity and growth inhibitory activity was observed.

Additionally, the fine specificities of the different mAbs were analyzed. First, Ab–Ab competition experiments were performed by ELISA using plates coated with recombinant CyRPA protein expressed in E. coli (Table I). mAbs c02, c04, c06, c08, c09, and c12 competed against each other. These mAbs did not compete with and were not competed by mAbs c05, c10, and c13. Whereas mAbs c05 and c13 competed with each other, mAb c10 did not compete with and was not competed by any of the other anti-CyRPA mAbs. According to these results, the nine mAbs were
grouped into three epitope groups (I, II, III), with the two non-
inhibitory mAbs forming epitope group III.

Second, the anti-CyRPA mAbs were tested for their reactivity with overlapping protein fragments of CyRPA. For this purpose, HEK cells transiently transfected with expression plasmids encoding fragments of CyRPA comprising aa 26–352, 26–251, 26–181, 127–352, 236–352, 74–251, 74–181, 26–142, and 127–251 were analyzed by Western blot analysis and life cell staining. In Western blot analysis, CyRPA-specific mAbs bound to fragments 26–181, 26–251, and 26–352 but not to the two overlapping subfragments 26–141 and 74–181. This indicates that all nine mAbs recognize conformational epitopes that are present in fragment 26–181 but not formed in subfragments 26–141 and 74–181 (Supplemental Fig. 4 and data not shown). Live cell staining of HEK cells expressing the protein fragments on their cell surface with different anti-CyRPA mAbs revealed five distinctive reactivity patterns (A, B, C, D, E) (Table I). When combining results from Ab–Ab competition experiments and epitope mapping, the seven growth inhibitory anti-CyRPA mAbs were classified into three fine specificity groups. These were distinctive from the fine specificities of the two noninhibitory mAbs c05 and c13, which were the only mAbs reacting with the N-terminally truncated fragment comprising aa 74–251 (Table I). The identified dimorphism at amino acid position 339 does not lie within the sequence stretch aa 26–181 relevant for binding of the analyzed anti-CyRPA mAbs (Table I). Accordingly, anti-CyRPA mAb 12 inhibited growth of P. falciparum strains expressing either variant (3D7 versus K1) (Supplemental Fig. 2B).

Synergistic inhibitory activity of anti-CyRPA mAbs

In vitro growth inhibition assays with combinations of anti-CyRPA mAbs were performed to assess whether different mAbs functionally interfere with each other (Fig. 5B). The functional activity of inhibitory anti-CyRPA mAbs was not affected by the addition of the noninhibitory anti-CyRPA mAb c05 or a malaria-unrelated control mAb. A synergistic inhibitory activity was measured when combining two inhibitory anti-CyRPA mAbs, which bind to distinct epitopes: 46.0 ± 1.1% and 52.1 ± 3.4% growth inhibition was measured for 500 μg/ml of anti-CyRPA mAb c10 or c12, respectively, but combining 250 μg/ml of either mAb inhibited parasite growth by 81.6 ± 0.3%. In contrast, when combining two inhibitory anti-CyRPA mAbs with the same fine specificity, only an additive but no synergistic inhibitory effect was observed.

Natural immunogenicity of CyRPA

To examine if natural exposure to P. falciparum leads to the development of anti-CyRPA Abs, human sera were analyzed by ELISA for their reactivity with purified recombinant CyRPA

![FIGURE 6. CyRPA-specific mAbs inhibit parasite growth in the NOD-scid IL2Rγnull mouse model. (A) P. falciparum-infected NOD-scid IL2Rγnull mice received purified anti-CyRPA mAbs or isotype/subclass-matched control mAbs by i.v. injection. The arrow indicates the day of mAb injection, and values are the mean parasitemia in peripheral blood of three mice per group. Data are means ± SD. (B) On day 6, blood cells of mice having received 2.5 mg anti-CyRPA mAb c12 were used to infect fresh NOD-scid IL2Rγnull mice. Once parasitemia exceeded 0.5%, mice received a single dose of 2.5 mg anti-CyRPA mAb c12 or control mAb by i.v. injection. Parasitemia was monitored over the next 5 d. Data are mean parasitemia of three mice per group ± SD.](http://www.jimmunol.org/Download)
expressed in HEK cells (Fig. 7). Sera were collected from healthy individuals between 5 and 20 y of age living in the Kassena-Nankana District in northern Ghana where *P. falciparum* is highly endemic (67). Sera from healthy Swiss adults without any history of malaria served as negative control. Whereas sera from malaria-exposed individuals readily contained IgG specific for two MSP-1–derived recombinant protein fragments (mean OD 0.61 ± 0.03 and mean OD 0.50 ± 0.02, respectively, at 1:200 serum dilution), basically no CyRPA-specific IgG was measured (mean OD 0.07 ± 0.00 at 1:200 serum dilution).

**Discussion**

With the release of the fully annotated genome of *P. falciparum* in 2002, genome-wide searches for potential vaccine candidate Ags have become possible (44, 45). On the basis of published transcriptome and proteome data, suggesting expression in extracellular stages and surface localization, we have selected sets of uncharacterized ORFs for evaluation of their potential as vaccine candidate Ags (Refs. 46–48, 51, 56 and A. Dreyer, G. Pluschke, unpublished observations). Among the proteins characterized, CyRPA exhibited outstanding properties, demonstrating the utility of systematic genome-wide approaches for vaccine Ag selection. However, for each individual Ag, detailed evaluations, including functional assays, remain indispensable to evaluate its potential as a vaccine candidate.

One major problem in the development of a malaria asexual blood-stage vaccine is the lack of functional assays with proven predictive potential. Established assays only assess certain potential Ab-mediated effector functions against blood-stage parasites (18, 76–78); that is, growth inhibition by Abs alone or Ab-dependent cellular inhibition. Still, these assays can provide valuable information on the protein function. Abs against CyRPA

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(A) Ab–Ab Competition ELISA

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(B) Reactivity of Anti-CyRPA mAbs with CyRPA Fragments

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(A) Ab–Ab competition ELISA results of nine anti-CyRPA mAbs. According to their competition pattern, mAbs were assigned to epitope groups I, II, or III. x, no Ab competition.

(B) Capability of nine anti-CyRPA mAbs to bind to fragments of CyRPA expressed on the cell surface of HEK cells assessed by live-cell immunofluorescence staining. According to their reactivity pattern, mAbs were assigned to epitope groups A, B, C, D, or E. x, Staining; x, no staining.

*No reactivity in Western blot analysis of HEK cell lysates (data not shown).*
on its own showed a strong parasite growth inhibitory activity. This activity targeted the process of merozite invasion into RBCs but not the development of intraerythrocytic stages. These results point toward a complement- and leukocyte-independent mode of action, such as blocking of molecular interactions involved in merozite invasion (26, 28).

*Plasmodium* in vitro cultures are very sensitive to physiological changes. Thus, the in vitro growth inhibition assay is criticized for being very sensitive to impurities of Ab preparations tested. We addressed this by testing more than one independently produced batch of the individual mAbs and obtained consistent results.

Importantly, the mouse in vivo model offers the advantage of a constant physiological environment given that factors like nutrient supply, electrolyte balance, and pH are very well controlled by the animal (79). Because *P. falciparum* cannot infect rodent erythrocytes and rodent malaria parasites lack many Ags of *P. falciparum*, including CyRPA, nonhuman primates or SCID-mice engrafted with human erythrocytes are the only in vivo models available to study growth inhibitory effects of CyRPA-specific Abs. It was previously shown that the *P. falciparum* model in NOD-SCID mice genetically deficient in IL-2Rγ-chain (NOD-Scid IL2Rγnull) engrafted with human erythrocytes is a reliable test system for drug evaluation in vivo (65, 80). Also in our hands, parasite growth in infected NOD-Scid IL2Rγnull mice was very consistent and reproducible. However, this model has never been used previously for passive immunization studies. Anti-CyRPA mAbs administered at the beginning of the exponential growth phase exerted a strong, dose-dependent parasite growth inhibitory effect. To the best of our knowledge, our study describes the first Ig transfer assay using a *P. falciparum* murine model in which a dose-response relationship has been reported. In contrast to the previously described model (79), this system therefore permits comparison of the relative inhibitory potency of malaria-specific Abs in vivo. Thus, we propose this model as a reliable in vivo model to test protective efficacy of vaccine Ags against *P. falciparum* blood stages.

The NOD/Scid-IL2Rγnull mouse model is restricted to passive immunization as mice lack the adaptive immune system and additionally have deficiencies in the innate immune system (83, 84). However, besides passive immunization with Abs, adoptive transfer of specific effector immune cells may potentially be studied in this model. Another question left unanswered is whether this model can be used for the assessment of the other Ab-mediated effector mechanisms apart from plain neutralization that have been described for blood-stage-specific Abs (26, 27, 29–32, 81, 82). Such studies may shed light into the relevance of different Ab-mediated effector mechanisms for in vivo protection.

Growth inhibitory capacities of different anti-CyRPA mAbs assessed in the in vivo model correlate with the results obtained in the in vitro assays, strengthening credibility of both systems. The inhibitory effect of CyRPA-specific Abs seen in vivo can well be ascribed to the same effector mechanisms as in vitro; namely, blocking of invasion-relevant processes. Yet, we cannot exclude involvement of additional immune effector mechanisms. NOD-Scid IL2Rγnull mice are incapable of mounting adaptive immune responses and additionally lack a functional common IL-2Rγ-chain, which is required for high-affinity binding of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 to their receptor (83). As a consequence, NOD-Scid IL2Rγnull mice have no NK cells and show additional defects in innate immunity. Involvement of complement or cellular effector mechanisms in the observed protection is nevertheless possible, as in these mice the percentage of macrophages/monocytes and granulocytes is normal or even increased (84).

Although it has been demonstrated that very high titers of specific Abs can be induced by appropriate vaccine formulations (85), the high Ab concentration required for in vivo protection may represent a hurdle for the development of a CyRPA-based vaccine. However, a substantial synergistic growth inhibitory effect was observed when combining two inhibitory anti-CyRPA mAbs with different fine specificity. Hence, stronger inhibitory activities may be achieved if active immunizations with CyRPA induce Abs specific for more than one inhibitory epitope. If so, lower titers of total CyRPA-specific Abs may be required to confer protection.

Anti-CyRPA mAbs inhibited, but did not completely block, parasite growth. The multiplication rate in vivo was much reduced but remained above one, leading to a very slow rise in parasitemia. Increasing the applied dose from 2.5 to 5 mg per mouse did not reduce multiplication further, indicating a saturation of the inhibitory mechanism. From our functional data, we deduce that anti-CyRPA mAbs reduce parasite growth by partially inhibiting some of the invasion-relevant processes crucial for invasion of erythrocytes by merozites, be it a specific protein interaction or processing of invasion-relevant proteins. However, RBCs did not bind to HEK cells that express CyRPA on their cell surface, and purified recombinant CyRPA did not specifically bind to the erythrocyte surface (data not shown). These results indicate that CyRPA, at least on its own, is no ligand for erythrocyte surface receptors.
*P. falciparum* merozoites are described to use alternative invasion pathways to evade Ab-mediated immunity (86). Use of a CyRPA-independent invasion pathway of limited efficiency could explain persistence of infection associated with a reduced multiplication rate. Because parasites that survived treatment with anti-CyRPA mAb showed normal multiplication rates when transferred into naïve mice and remained sensitive to re-exposure to anti-CyRPA mAbs, no rigid switch to an alternative invasion pathway seems to occur. The fact that, at least over a short time period, no CyRPA-Ab–resistant parasites were selected is of importance when considering the inclusion of CyRPA into a malaria subunit vaccine.

Notably, among a set of nine mAbs specific for CyRPA, seven mAbs showed a parasite growth inhibiting effect whereas two did not. The growth inhibitory activity of the Abs was not determined by their affinity for the target Ag, but by their fine specificity. No confined CyRPA sequence stretch could be defined as epitope, as all mAbs seem to recognize conformational epitopes not present in short CyRPA sequence stretches. However, the fine specificity of inhibitory anti-CyRPA mAbs was distinct from the fine specificity of noninhibitory anti-CyRPA mAbs. The fact that only anti-CyRPA Abs of a certain fine specificity showed inhibition activity demonstrates once more the shortcoming of assessing immuno or vaccine potential of Ags by solely measuring Ab titers by ELISA. Besides CyRPA, a range of other blood-stage Ags, including AMA-1 and MSP-1, were shown to induce inhibitory Abs a predominantly apical distribution. Consistent with this finding, we showed by indirect immunofluorescence staining with CyRPA-specific mAbs that CyRPA localizes intracellularly at the merozoite apex. Additionally, we could show that CyRPA does not localize to micronemes or rhoptry bulbs. The CyRPA-specific staining showed similarity to the staining of MSP-5, possibly indicating that CyRPA shares the same subcellular localization as MSP-5, a protein considered as a blood-stage vaccine candidate (89, 90). Although MSP-5 is described as a merozoite surface protein, its exact localization is sparsely characterized, and immunofluorescence staining of schizonts with MSP-5–specific Abs was shown previously to result in a rather dotty staining pattern (64, 91). The exact subcellular structure at the merozoite apex to which CyRPA localizes and how this structure is implicated in merozoite invasion remain to be investigated.

*P. falciparum* is a highly polymorphic organism. This is particularly the case for many surface Ags as a result of natural selective pressure by the human immune responses (92). Most of the asexual blood-stage vaccine candidates evaluated to date, including AMA-1, MSP-1, and MSP-2, therefore have substantial polymorphisms (39–42). Immunodominance of the polymorphic and variant epitopes may explain the need for repeated exposure over several years to achieve clinical immunity against the natural polymorphic parasite populations (93). Malaria vaccines based on polymorphic Ags run the risk of compromised efficacy due to selection of vaccine-resistant variants (8). Therefore, it may be desirable to focus on Ags and protein domains with limited polymorphism. Sera of malaria-exposed adults tested here basically contained no CyRPA-specific Abs; natural immunogenicity of CyRPA thus appears to be very low. Our sequence analysis indicates that CyRPA has no substantial sequence polymorphisms. Only a single nonsynonymous SNP was detected among a range of *P. falciparum* isolates. This, together with the low immunogenicity, is an indication that CyRPA is not a major target of naturally acquired immune responses. A possible explanation for the low immunogenicity of CyRPA may be the intracellular localization of CyRPA in released merozoites. Hence, CyRPA may only be accessible to the humoral immune surveillance during the short period of invasion. Other reasons may be a critical function of CyRPA prohibiting variation or hindered accessibility of CyRPA in its native context.

Furthermore, we could demonstrate that the identified SNP-associated amino acid dimorphism is not located within the epitope of the growth inhibitory anti-CyRPA mAbs and that parasites harboring sequence variants are likewise affected by growth inhibitory anti-CyRPA mAbs, indicating that this dimorphism is of no functional importance. From these results, we deduce that the protection-associated epitopes of CyRPA are likely to be free of sequence polymorphisms, suggesting that a CyRPA-based vaccine would target the entire *P. falciparum* population. This represents a clear advantage compared with polymorphic vaccine Ags like AMA-1, where several studies showed significant allele specificity in the inhibitory activity of anti–AMA-1 Abs (94–96).

Our data show that CyRPA clearly fulfills three key criteria applied to select asexual blood-stage Ags as vaccine candidates (97): 1) the protein is conserved; 2) Abs against the Ag inhibit parasite growth in vivo and 3) are protective in animal models. To validate further CyRPA as a blood-stage vaccine Ag, one ought to demonstrate that 1) CyRPA is essential for parasite survival, 2) growth inhibitory anti-CyRPA Abs can be induced by active immunization with recombinant CyRPA, 3) immunization is safe, and 4) growth inhibitory anti-CyRPA Abs confer protection against clinical malaria in humans.

In summary, we have identified a conserved merozoite protein that induces Abs that inhibit parasite growth in vitro and in vivo. We suggest evaluating its suitability as a candidate Ag for inclusion into a multivalent malaria subunit vaccine. In addition, we adopted the improved *P. falciparum* mouse model based on NOD- scid IL2Rgnull mice for functional analysis of malaria blood-stage–specific Abs. This model now allows a more systematic and quantitative comparison of the in vivo functionality of malaria Ag-specific Abs.

**Acknowledgments**

We thank Bernard Rutten for Ab purification, Dr. Walter Huber for SPR analysis, Bela Takacs for providing recombinant MSP-1, and Dr. Caroline Kulangara for providing extracted DNA from different *P. falciparum* strains.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


**Supplementary figure 1.** CyRPA resides intracellular in free merozoites. Indirect immunofluorescence staining of fixed (left) and un-fixed (right) merozoites with mAbs specific for GAPDH (green), MSP-1 (green) and CyRPA (red). Nuclei were stained with DAPI.

**Supplementary Figure 2.** Some, but not all anti-CyRPA mAbs inhibit parasite growth of various strains. (A) Synchronous *P. falciparum* 3D7 blood stage parasites were cultured for two cycles in the presence of different concentrations of different purified anti-CyRPA mAbs. Percent parasite growth inhibition was calculated against the parasitemia of PBS control wells. Anti-6xHis-tag mAb was used as negative control mAb. Each symbol represents the mean of a triplicate experiment, and error bars indicate the standard deviation. (B) Synchronous blood stage parasites of *P. falciparum* strain 3D7, 7G8, D6, W2mef and K1 were cultured for two cycles in the presence of different concentrations of purified anti-CyRPA mAb c12 or c05. Percent parasite growth inhibition was calculated against the parasitemia of PBS control wells. Anti-6xHis-tag mAb was used as negative control mAb. Each symbol represents the mean of a triplicate experiment, and error bars indicate the standard deviation.
Supplementary figure 3. Surface Plasmon Resonance analysis of CyRPA binding to anti-CyRPA mAbs. (A) SPR sensograms showing binding kinetics of CyRPA and anti-CyRPA mAbs. A CM5 chip with immobilized anti-mouse IgG Abs was used to capture anti-CyRPA mAbs. Purified recombinant CyRPA was injected at concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.13 nM (coloured lines, from top to bottom). Fitted curves for a 1:1 binding model is superimposed in black. RU, response units. (B) summary of affinity constants for anti-CyRPA mAbs. ka; on-rate, kd; off-rate, Rmax; response at complete saturation, KA; association constant, KD; dissociation constant. No fit could be obtained for mAb c13.
Supplementary Figure 4. Reactivity pattern of anti-CyRPA mAb c12 with fragments of the target antigen. Lysates of HEK cells transiently transfected with expression plasmids encoding different CyRPA protein fragments were probed with anti-CyRPA mAbs. All nine mAbs recognized fragment 26-352 (dark pink), and all except mAb c10 recognized fragment 26-251 and fragment 26-181 (light pink). As an example, Western blots with anti-CyRPA mAb c12 are shown in the middle and right panel. As positive control lysates were probed with anti-6xHis-tag mAb (left panel). Relative molecular masses in kDa are indicated on the left.