Molecular Mechanism of Host Specificity in Plasmodium falciparum Infection

ROLE OF CIRCUMSPOROZOITE PROTEIN

Dharmendar Rathore‡§, Sybil C. L. Hrstka, John B. Sacci, Jr., Patricia De la Vega**, Robert J. Linhardt¶, Sanjay Kumar‡‡, and Thomas F. McCutchan$$

From the §Growth and Development Section, Laboratory of Malaria and Vector Research, NIAID, National Institutes of Health, Bethesda, Maryland 20892, the ¶Virginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, the §§Departments of Medicinal and Natural Products Chemistry, Chemistry, Chemical and Biochemical Engineering, University of Iowa, Iowa City, Iowa 52242, the ¶Department of Microbiology and Immunology, School of Medicine, University of Maryland, Baltimore, Maryland 21201, the **Malaria Program, Naval Medical Research Center, Silver Spring, Maryland 20910, and the $$$Bacterial and Parasitic Diseases Section, Division of Emerging, Transfusion, and Transmitted Diseases, Center for Biologies Evaluation and Research, Food and Drug Administration, Rockville, Maryland 20852

Plasmodium falciparum sporozoites invade liver cells in humans and set the stage for malaria infection. Circumsporozoite protein (CSP), a predominant surface antigen on sporozoite surface, has been associated with the binding and invasion of liver cells by the sporozoites. Although CSP across the Plasmodium genus has homology and conserved structural organization, infection of a non-natural host by a species is rare. We investigated the role of CSP in providing the host specificity in P. falciparum infection. CSP from P. falciparum, P. gallinaceum, P. knowlesi, and P. yoelii species representing human, avian, simian, and rodent malaria species were recombinantly expressed, and the proteins were purified to homogeneity. The recombinant proteins were evaluated for their capacity to bind to human liver cell line HepG2 and to prevent P. falciparum sporozoites from invading these cells. The proteins showed significant differences in the binding and sporozoite invasion inhibition activity. Differences among proteins directly correlate with changes in the binding affinity to the sporozoite receptor on liver cells. P. knowlesi CSP (PkCSP) and P. yoelii CSP (PyCSP) had 4,790- and 17,800-fold lower affinity for heparin in comparison to P. falciparum CSP (PF CSP). We suggest that a difference in the binding affinity for the liver cell receptor is a mechanism involved in maintaining the host specificity by the malaria parasite.

Malaria infection in humans is initiated with the bite of an infectious female mosquito, which inject sporozoites of Plasmodium species into the circulation. These sporozoites rapidly bind and invade liver cells and undergo rapid multiplication, leading to the release of thousands of infective merozoites (1). Out of more than 20 well documented and characterized species of Plasmodium that cause malaria in various vertebrates, only four species viz., P. falciparum, P. vivax, P. malariae, and P. ovale infect humans. It is intriguing that, although numerous parasite surface antigens involved in infectivity and pathogenesis possess inter-species homology and have been identified in numerous humans, rodents, and simian Plasmodium species (2–5), the parasite maintains its host specificity and non-natural host infections are rare. In laboratory conditions, it is possible to infect Aotus monkeys with human parasites (6, 7). Most of these infections have been induced by inoculating erythrocytic stage parasites or salivary gland-isolated sporozoites in splenectomized animals and with a parasite load not seen in malaria endemic areas (7, 8). Initiation of a human malaria infection by sporozoites in Aotus monkeys through the natural course (bites of infected mosquitoes) has not been successful.

Maintenance of host specificity is an important aspect of pathogenicity and sustenance of an infection. It generally involves a complex interplay between pathogen and host factors, which are influenced by various evolutionary and genetic determinants. Understanding which parasite components play a role in host specificity can provide information about the pathogenesis and can also help in disease control.

Plasmodium sporozoites express CSP on the surface of all the Plasmodium species. CSP is an important molecule for the parasite, because it is involved in the development of infectious sporozoites in mosquitoes (9), plays a role in the invasion of salivary gland, and is essential to the binding and invasion of liver cells in the vertebrate host (1, 10). It is also a malaria vaccine candidate undergoing clinical trials (11–13). The general structural organization of CSP is conserved across the species. The protein can be divided into three regions of roughly equal sizes; the amino terminus, a central repeat segment, and a carboxyl terminus region containing a conserved TSR domain (schematic in Fig. 1). The TSR domain is present in a large number of proteins and performs diverse functions (14, 15). In contrast, the central repeat region is a species-specific, low complexity segment with no known function. We have recently demonstrated that in P. falciparum amino terminus of the CSP

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** To whom correspondence should be addressed: Laboratory of Malaria and Vector Research, NIAID, National Institutes of Health, Rm. 126, Bldg. 4, 4 Center Dr., MSC 0425, Bethesda, MD 20892-0425. Tel.: 301-496-6149; Fax: 301-402-0079; E-mail: tmccutchan@niaid.nih.gov.

† The abbreviations used are: CSP, circumsporozoite protein; PICSP, P. falciparum CSP; PgCSP, P. gallinaceum CSP; PkCSP, P. knowlesi CSP; PyCSP, P. yoelii CSP; HSPG, heparan sulfate proteoglycan; SPR, surface plasmon resonance; TSR, thrombospondin type I repeat; MOPS, 4-morpholinepropanesulfonic acid; RU, response unit.
a salt gradient using a fast-protein liquid chromatography system. Relevant fractions were pooled and purified to homogeneity by gel filtration chromatography on a TSK 3000 column. DNA encoding PyCSP was cloned in a Yeast expression vector with a histidine tag at its 3'-end. The construct was expressed in *Saccharomyces cerevisiae* cell line VK1. The protein was purified on a nickel-nitriilotriacetic acid agarose column using imidazole followed by size-exclusion chromatography.

### Structural Analysis of Recombinant CS Proteins

Circular dichroism polarimetry was utilized to investigate the secondary structure of recombinant CSP. Equimolar amounts of recombinant CSP in 10 mM sodium phosphate buffer, pH 7.0, were separately scanned in the far-UV range, at 25 °C, using a JASCO J710 spectropolarimeter. This second structure was calculated using Yang's reference parameters (19).

#### Cell Binding Assay

Hepatoma cell line HepG2, maintained in RPMI 1640 containing 2 mM glutamine, and 10% heat-inactivated fetal bovine serum was used, and the assay was performed as described previously (1). Briefly, cells at a density of 100,000 cells per well were fixed with 4% paraformaldehyde followed by blocking with Tris-buffered saline containing 1% bovine serum albumin. Proteins were incubated with cells for 1 h followed by anti-CS antibody for 45 min and alkaline phosphatase-coupled conjugate for 30 min. Because anti-CS antibody for each protein is different, a dilution of each of these antisera giving an optical density of 1 in an enzyme-linked immunosorbent assay was used for the analysis. 1 mM 4-methylumbelliferyl phosphate was used as substrate, and fluorescence was measured in a fluorometer with excitation at 350 nm and emission at 460 nm.

#### Effect of Heparinase I on the Binding Activity of CS Protein

HepG2 cells were treated with log dilutions of heparinase I or buffer (100 mM MOPS, pH 7.0, 10 mM calcium chloride) for 2 h at 37 °C. The cells were washed thrice with Tris-buffered saline followed by addition of 50 mM recombinant protein and incubation at 37 °C for 1 h. Fluorescence was measured as described above.

### Sporozoite Invasion Assay

HepG2 cells were collected, washed, and resuspended in complete medium. 50,000 cells were added to each well of ECL-coated Labtek slides and incubated overnight at 37 °C to allow them to adhere. *P. falciparum* (NF54) sporozoites were isolated from infected *Anopheles stephensi* mosquitoes. 20,000 sporozoites were added to the HepG2 cells immediately followed by the addition of different concentration of recombinant CS protein and incubated for 3 h. Cells were subsequently washed and fixed with cold methanol. *P. falciparum* CS anti-repeat region monoclonal antibody (NF51) was added followed by anti-mouse IgG peroxidase conjugate to stain the sporozoites. Intracellular sporozoites were identified and counted, and the percent inhibition of invasion was calculated with the following formula [(control − test)/control] × 100.

### Binding Affinity of CSP to Heparin

Surface plasmon resonance (SPR) measurements were performed using a Biacore® 3000 biosensing system, which was operated with Biacore Control 3.2 software. All experiments were performed at 25 °C. Buffers were filtered and degassed prior to use in each experiment. The sensor chip surface was activated with a 1:1 mixture of N-hydroxysuccinimide/N-ethyl-N-(dimethylamino)proprylcarbodiimide followed by immobilization of albumin-heparin conjugate and blocking of unreacted sites with ethanolamine. The albumin-heparin conjugate was immobilized in two flow cells, one of which contained 80 response units (RU) of material and the other, 200 RU. Bovine serum albumin was immobilized to the sensor surface in a similar fashion to serve as a control. The sensor surface in the fourth flow cell was activated, and then deactivated, and it served as the reference surface for detecting changes in the bulk refractive index, injection noise, baseline drift, and nonspecific binding.

### Interaction Studies

Concentrated CS protein samples were serially diluted with HBS buffer (0.01 mM HEPES, 150 mM NaCl, 1.5 mM EDTA)
0.01% Tween 20, pH 7.4) yielding concentrations ranging from 5 nM to 8 μM. Samples were injected over the sensor surface at a flow rate of 50 μl/min for 1 to 2 min. The sensor surface was regenerated using a glycine-NaOH mixture (5 mM glycine, pH 11.5, 15 s) and an 80-μl injection of 2 M NaCl. Each prepared sample was injected over the sensor chip three times in random order to prevent bias in the data. All experiments were repeated at least three times to ensure reproducibility.

**Evaluation of Data**—The binding of CS protein to heparin was determined using double referencing (21). Although the albumin-CS protein interactions were small (∼2 RU), the data were further corrected for this component of the interaction. The kinetic rate constants (k$_{a}$ and k$_{d}$) and the affinity constant (K = k$_{a}$/k$_{d}$) were determined by applying global analysis to both the association and dissociation phases simultaneously according to a two stage reaction (A + B = AB → AB$^*$) using BIAevaluation software, version 3.1 (1999). Selection of a reasonable model for evaluating the interaction data was based on the ability to return consistent values for the estimated parameters for the two different levels of surface immobilized albumin-heparin conjugate. Only the two-stage reaction model proved to meet this criterion. The equilibrium constants (K$_{eq}$ values) were calculated by taking the inverse of the estimated affinity constants (K$_{eq} = 1/K = (k_{a}/k_{d})^* (k_{d}/k_{a})^{-1}$), and the reported K$_{eq}$ values are the averaged values obtained from both heparin-coated surfaces.

**RESULTS**

Cloning, Expression, and Purification of Recombinant CS Proteins—Recombinant CSP from four different species of *Plasmodium* representing human, rodent, simian, and avian malaria species were recombinantly expressed, and the proteins were purified to homogeneity. DNA encoding PICSP, PkCSP, and PgCSP were in pET11a, a T7 promoter-based *E. coli* expression vector. Both PICSP and PkCSP were secreted into the periplasm, which was used as a source to obtain the purified protein, as previously described (16, 17). Both the proteins were initially purified on a heparin-Sepharose affinity column followed by gel filtration chromatography, to obtain the purified protein (see online supplemental data). Recombinant PgCSP was predominantly present in the spheroplast as inclusion bodies (data not shown). Inclusion bodies were isolated from the spheroplast, solubilized using guanidine hydrochloride and reduced by adding dithioerythritol. The protein was subsequently renatured in *vitro* in a redox system using oxidized glutathione (18). The protein was purified to homogeneity by successive chromatography on anion exchange and gel filtration columns. PkCSP was initially purified on a nickel column followed by gel filtration chromatography.

**Structural Analysis of Purified CS Proteins**—The secondary structure of the recombinant proteins was evaluated by CD polarimetry. The far-UV CD spectra of purified CS proteins was obtained and compared. All four recombinant CS proteins had similar profiles with a major peak at 200 nm, suggesting a weakly ordered structure due to the predominant presence of random coil structures in the protein (see online supplemental data). Estimation of the secondary structure revealed that the all the four different CS proteins have inherently similar folding patterns. More than 50% of each of the protein has a random coil confirmation, whereas the remainder was primarily folded into beta sheet and turns (Table I). Similar results have been obtained by analyzing the CS polypeptides from several species (22, 23), which suggests that the proteins are able to obtain a proper conformation.

**TABLE I**

|                | *P. falciparum* | *P. gallinaceum* | *P. knowlesi* | *P. yoelii* |
|----------------|-----------------|-----------------|---------------|------------|
| Helical        | 0.0             | 10.0            | 0.0           | 0.0        |
| β-Sheet        | 27.3            | 37.0            | 47.0          | 29.6       |
| Turn           | 7.4             | 0.0             | 0.0           | 15.8       |
| Coil           | 65.3            | 53.0            | 53.0          | 54.6       |

**Binding Analysis of CS Proteins on HepG2 Cells**—The binding activity of the four CS proteins was evaluated on HepG2 cells. An equimolar concentration of four different CS proteins was added onto the HepG2 cells followed by the addition of respective anti-CS antibodies. PICSP was then bound to HepG2 cells in a dose-dependent manner (Fig. 2). Like *P. falciparum* CSP, PgCSP, the CS protein from avian malaria parasite *P. gallinaceum*, also showed dose-dependent binding onto these cells, although its activity was 50% lower in comparison to PICSP. In contrast, PkCSP and PyCSP from simian and rodent malaria parasites, respectively, showed significantly reduced binding even at the highest concentration (Fig. 2).

**Specificity of PgCSP Binding to HepG2 Cells**—PICSP interacts with HSPG-based receptors on liver cells (24, 25). One possible reason for the binding of PgCSP to HepG2 cells could be the involvement of a different cell surface component that by coincidence can serve as a receptor. To investigate whether PgCSP utilized HSPG-based receptors on HepG2 cells for its interaction, binding was assayed on liver cells devoid of HSPG. HepG2 cells were pretreated with heparinase I, an enzyme that cleaves heparin and highly sulfated domains in heparan sulfate, and the binding activity of PICSP and PgCSP was investigated. Binding of both PgCSP and PICSP was completely inhibited on heparinase I-treated HepG2 cells, indicating that the receptor specificity is maintained and, like PICSP, PgCSP binding was also mediated through heparinase I-sensitive liver cells receptors (Fig. 3).

**Inhibition of Sporozoite Invasion by Recombinant Proteins**—To evaluate whether the differences in binding capacity of the CSP from four species toward the human liver cells is a mode of maintaining the biological specificity, the potential of these proteins to putatively compete for receptors and prevent the invasion of HepG2 cells by live *P. falciparum* sporozoites was investigated. *P. falciparum* sporozoites were freshly isolated from *A. stephensi* mosquitoes and were immediately incubated with HepG2 cells in the absence or presence of different concentrations of each of the four CSP. HepG2 cells closely mimics the *in vitro* environment, because sporozoites are able to successfully invade and develop intracellular hepatic stages of...
the life cycle. PfCSP inhibited sporozoite invasion by 70% at the lowest concentration tested and showed complete inhibition (>90%) at the higher concentration (Fig. 4). PgCSP corroborated the results of the binding assay and showed significant (60–70%) sporozoite invasion inhibition activity, which was comparable to the invasion inhibition activity of PfCSP. In contrast, PkCSP and PyCSP showed minimal inhibition (20–30%) even at the highest concentration. These results suggested that a difference in the liver cell invasion potential of different CSP is directly involved in maintaining the species specificity of infection.

Binding Affinity of CS Proteins Toward Its Receptor—To facilitate binding and invasion of liver cells, sporozoites exploit HSPG-based receptors on liver cells. We have recently quantified the binding affinity of PfCSP toward different glycosaminoglycans and shown that the protein has the highest affinity for heparin (26). The differences in the binding and sporozoite invasion inhibition activities of the four CSP proteins could be due to differences in binding affinity toward heparin. We investigated and quantified the interactions of CSP with heparin by surface plasmon resonance. An albumin-heparin chip was constructed by linking the primary amino groups in albumin to the activated carboxyl groups on the chip, to mimic a cell surface proteoglycan. PfCSP showed the highest affinity with significant binding at a concentration of 5 nM. At a concentration of 100 nM, the general shape of the binding curve changed, which suggested that protein-protein interactions became a significant contributing factor. For this reason, sample concentrations ranging up to 40 nM were selected for evaluation of the heparin and PfCSP interaction to minimize the contribution from PfCSP-PfCSP interactions. The apparent $K_D$ for this interaction was determined as $0.34 \pm 0.437$ nM (Fig. 5A and Table II). PgCSP demonstrated a similar, but lower, affinity for heparin with an estimated $K_D$ of $15.3 \pm 7.28$ nM (Fig. 5B and Table II). Association of PkCSP with heparin was measurable only at higher concentrations (>100 nM) (Fig. 5C) with a binding affinity of $1690 \pm 537$ nM (Table II). PyCSP showed minimal association with heparin (Fig. 5D) and consequently its binding affinity was $6060 \pm 2475$ nM (Table II). This clearly suggested that the differences in the binding affinity of these proteins toward the liver cell receptor are contributing toward maintaining the specificity of malaria infection.

DISCUSSION

Malaria sporozoite enters into a human body by the bite of an infected mosquito. Numerically, the sporozoite stage is the weakest link in the parasite lifecycle, because a very small population of the parasite enters into the host, makes its way to the host liver cells, and interacts with a specific host receptor for subsequent invasion and intra-hepatocytic development. One therefore assumes that this process of invasion must go efficiently in a natural environment to produce an infection.

We investigated the role of CSP from four well characterized
species of Plasmodium genus viz. P. falciparum (human), P. gallinaceum (avian), P. knowlesi (simian), and P. yoelii (rodent) in determining the host specificity of malaria infection in P. falciparum model. The proteins were expressed recombinantly, purified to homogeneity, and evaluated for their propensities to bind to HepG2 cells, a human hepatoma cell line, and stop the invasion of live P. falciparum sporozoites. PfCSP, being a human-parasite protein, effectively bound to HepG2 cells (Fig. 2) and completely prevented P. falciparum sporozoites from invading them (Fig. 4). PgCSP, although being an avian CSP, also bound to HepG2 cells (Fig. 2) and stopped P. falciparum sporozoites from invading these cells (Fig. 4), with an activity comparable to the PfCSP. PgCSP bound these cells using HS PG-based cell surface receptor, suggesting a receptor specificity identical to PkCSP (Fig. 3). In contrast, PkCSP and PyCSP were deficient in binding to these cells (Fig. 2) and showed low activity in sporozoite invasion inhibition assay (Fig. 4). Although these differences in binding and invasion inhibition activities were measured in vitro on a hepatic cell line, they most likely represent a true in vivo scenario as non-natural host infections are rare.

We quantified the binding affinity of each of the CSP toward heparin by SPR analysis. The result corroborated the outcome of binding and invasion assay, because binding affinity of PkCSP and PyCSP was considerably lower, 4,790- and 17,800-fold, respectively, in comparison to PfCSP (Fig. 5).

CSP-heparin interactions are charge-dependent, involving positively charged residues of the CSP and negatively charged sulfates and carboxylates groups on the sugar. We have recently shown that the CSP binds to the liver cells through its amino terminus, and the tertiary structure of the binding domain is extremely important for its activity (10). Within the Plasmodium genus, the amino terminus of the CSP shows 20–25% sequence identity (3), and, although all the four CSP have a preponderance toward beta sheets and turns, a structural conformation commonly found in heparin binding proteins (e.g. fibroblast growth factor) (27), we propose that sequence changes incorporated during the course of evolution have resulted in significant differences in the spatial arrangement of residues constituting the binding domain, without causing any significant change in the overall conformation. These changes have caused differences in binding affinities toward the receptor to provide a CSP-based mechanism for maintaining species specificity of infection. This CSP-based selection mechanism seems to work even at the mosquito stages as replacement of P. berghei CSP gene with P. falciparum CSP in P. berghei sporozoites results in a 90% decrease in the salivary gland sporozoite population in comparison to the wild type parasites (28).

Previously, using in silico phylogenetic analysis with numerous parasite DNA and protein sequences, we and others have proposed that P. falciparum and P. gallinaceum parasites have originated from a common progenitor, whereas the rodent and simian parasites have substantially diverged and form distinct phylogenetic clades (3, 29–31). The results from our study not only provide the first biological evidence of the close relationship between the P. gallinaceum and P. falciparum species; it also provides a biological correlation to the distribution of major phylogenetic divisions in the Plasmodium genus.

While changes in the CSP sequence between different species could contribute in maintaining the specificity, the host, on its part, could also be contributing by maintaining differences in the sulfated glycosaminoglycans component of the liver cell receptor across different species. It is known that every tissue of a mammalian species has a characteristic heparin composition, differing from other tissues, within and outside the spe-
cies, in the relative amount, type, and molecular size (32, 33). The presence of other host, vector, and parasite mechanisms for maintaining species specificity along different stages of the parasite lifecycle has not been ruled out.

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