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Molecular Mechanism of Host Specificity in *Plasmodium falciparum* Infection

ROLE OF CIRCUMSPOROZOITE PROTEIN*[S]

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***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 (PfCSP). 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[1] 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 trails (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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[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. S1 and S2.

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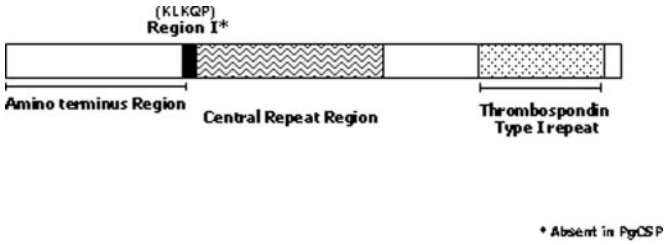
[1] The abbreviations used are: CSP, circumsporozoite protein; PfCSP, *P. falciparum* CSP; PgCSP, *P. gallinaceum* CSP; PkCSP, *P. knowlesi* CSP; PyCSP, *P. yoelii* CSP; HSPG, heparan sulfate proteoglycans; SPR, surface plasmon resonance; TSR, thrombospondin type I repeat; MOPS, 4-morpholinepropanesulfonic acid; RU, response unit.



Species	Host	Basic Repeat Unit
P. falciparum	Human	NANP
P. gallinaceum	Avian	QPAGGNGGV
P. knowlesi	Simian	NAGQPQAQGDGA
P. yoelii	Rodent	QGPGAP

FIG. 1. **Schematic representation of *Plasmodium* CS protein.** Basic repeat units of each of the CS proteins used in this study have been shown in the table.

is involved in the binding and invasion process of the sporozoites. The amino terminus has regions of homology that are conserved across the species (3). In the present study we have investigated the role of CSP in maintaining the host specificity during the invasion of liver cells by the *P. falciparum* parasite, a critical step in initiating the malaria infection in humans.

EXPERIMENTAL PROCEDURES

Materials

The sensor chip used in the SPR studies was a Pioneer Chip C1, which was obtained from Biacore (Biacore AB, Uppsala, Sweden) along with *N*-hydroxysuccinimide, *N*-ethyl-*N*-(dimethyaminopropyl)carbodiimide, and the sodium acetate immobilization buffer. Bovine serum albumin was from Amresco (Solon, OH). The albumin-heparin conjugate, HEPES, NaCl, Tween 20, EDTA, glycine, and guanidine were acquired through Sigma-Aldrich (St. Louis, MO).

Cloning, Expression, and Purification of CS Protein of Plasmodium Species

Cloning, expression, and purification of recombinant CS protein of *P. falciparum* (16) and *P. knowlesi* (17) has been described previously. DNA encoding *P. gallinaceum* mature CS protein was amplified as a *Nde*I-*Eco*RI fragment by PCR and cloned in pET11a, a T7 promoter-based *Escherichia coli* expression vector. Protein expression was done using *E. coli* strain BL21(λDE3). Cells were transformed and grown in super broth (pH 7.2), containing 100 μg/ml ampicillin, at 37 °C with shaking. At an A_{600} of 2.0, the cells were induced with 2 mM isopropyl-1-thio-β-D-galactopyranoside, and 4 h later, they were harvested by centrifugation at 4000 × *g* for 20 min. Total cell pellet was resuspended in washing buffer (50 mM Tris, pH 7.5, 20 mM EDTA) containing lysozyme at a concentration of 0.5 mg/ml. Cell suspension was incubated at room temperature, for 1 h with intermittent shaking. NaCl and Triton X-100 were added to obtain a final concentration of 0.5 M and 2.5%, respectively, and the suspension was further incubated at room temperature for 30 min, with vigorous shaking. This suspension was then spun at 13,000 × *g* at 4 °C, for 50 min, and the resultant pellet was resuspended in washing buffer containing 1% Triton X-100, using a Tissuemizer, and spun at 13,000 × *g* for 50 min. The pellet was washed repeatedly in washing buffer, without Triton X-100. After four washings, the pellet containing inclusion bodies was dissolved in 6 M guanidine hydrochloride and incubated for 2 h at room temperature followed by centrifugation at 50,000 × *g* at 4 °C, for 30 min. Supernatant, containing denatured protein, was collected, and the protein concentration was adjusted to 10 mg/ml with 6 M guanidine hydrochloride. Denatured protein was reduced by adding dithioerythritol, to a final concentration of 65 mM, and incubating at room temperature for 2 h. Protein was renatured by diluting 100-fold, in refolding buffer (100 mM Tris, pH 8.0, 0.5 M L-arginine-HCl, 2 mM EDTA, and 0.9 mM oxidized glutathione) (18). After incubating at 10 °C for 36 h, renatured material was dialyzed against 20 mM Tris, pH 8.0, containing 100 mM urea. The renatured protein was loaded onto a Q-Sepharose column, and eluted by 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.[2] The construct was expressed in *Saccharomyces cerevisiae* cell line VK1. The protein was purified on a nickel-nitrilotriacetic 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 secondary 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 antiserum 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 nM 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 (NFS1) 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.

Preparation of the Sensor Chip—Albumin-heparin was immobilized to the sensor surface using a previously published procedure (20). Briefly, the sensor chip surface was activated with an equimolar mix of *N*-hydroxysuccinimide/*N*-ethyl-*N*-(dimethyaminopropyl)carbodiimide followed by the 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 (RUs) 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 M HEPES, 150 mM NaCl, 1.5 mM EDTA,

[2] S. Kumar, unpublished data.

TABLE I
Secondary structure estimation of CS protein from different species

	P. falciparum	*P. gallinaceum*	*P. knowlesi*	*P. yoelii*
Helical	0.0	10.0	0.0	0.0
β-Sheet	27.3	37.1	47.0	29.6
Turn	7.4	0.0	0.0	15.8
Coil	65.3	53.0	53.0	54.6

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_D values) were calculated by taking the inverse of the estimated affinity constants ($K_D = 1/K = (k_{d1}/k_{a1}) * (k_{d2}/k_{a2})$), and the reported K_D 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 PfCSP, PkCSP, and PgCSP were in pET11a, a T7 promoter-based *E. coli* expression vector. Both PfCSP 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. PyCSP 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.

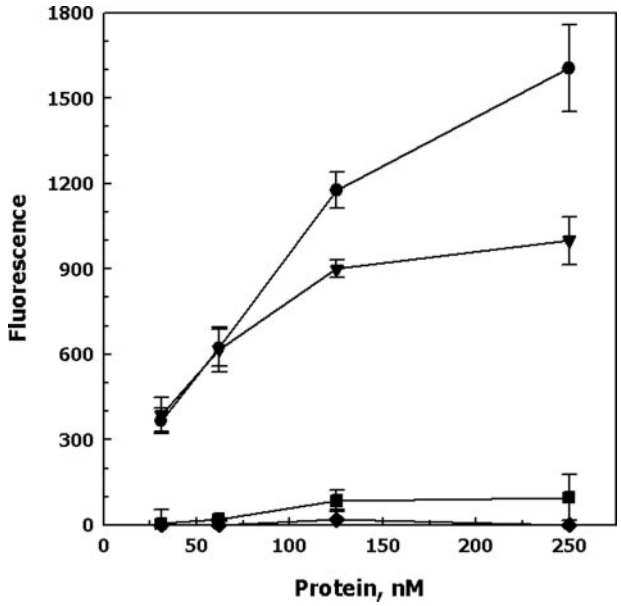


FIG. 2. **Binding activity of CS protein from four different species of *Plasmodium* on liver cells.** Equimolar concentrations of recombinant CS proteins of *P. falciparum* (●), *P. gallinaceum* (▼), *P. knowlesi* (■), and *P. yoelii* (♦) were incubated with HepG2 cells followed by the addition of respective anti-CS antibodies and anti-host antibody coupled to alkaline phosphatase.

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. PfCSP 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 PfCSP. 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—PfCSP 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 PfCSP and PgCSP was investigated. Binding of both PgCSP and PfCSP was completely inhibited on heparinase I-treated HepG2 cells, indicating that the receptor specificity is maintained and, like PfCSP, 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 vivo* environment, because sporozoites are able to successfully invade and develop intracellular hepatic stages of

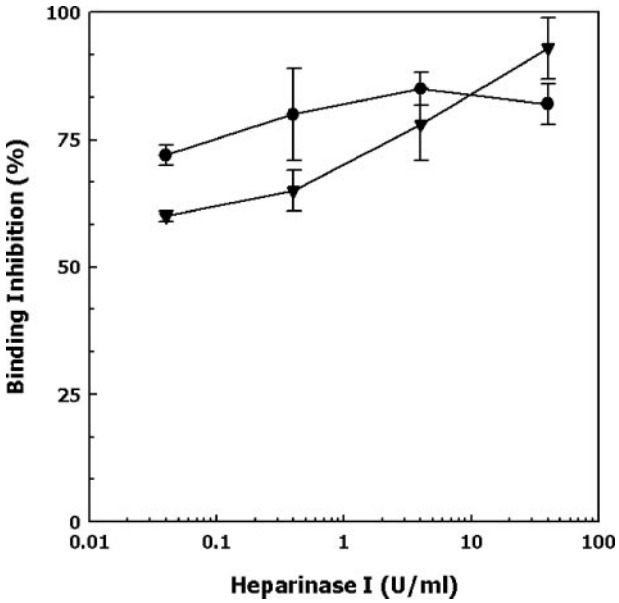


FIG. 3. **Binding specificity of *P. gallinaceum* and *P. falciparum* CS protein.** Binding of *P. falciparum* (●) and *P. gallinaceum* (▼) CS protein was evaluated on heparinase I-treated HepG2 cells.

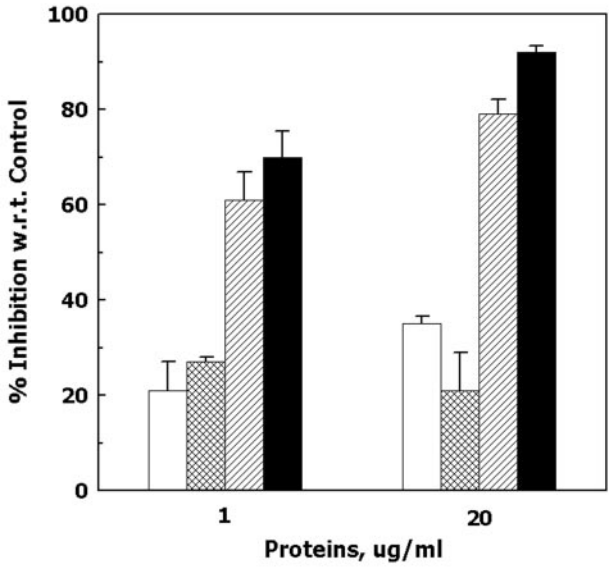


FIG. 4. **Inhibition of *P. falciparum* sporozoite invasion by recombinant CS protein from different species.** *P. falciparum* sporozoites were allowed to invade HepG2 cells in the presence of CSP from four different species. Percent inhibition of invasion was calculated as described.

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 ± 0.437 nM (Fig. 5*A* and Table II). PgCSP demonstrated a similar, but lower, affinity for heparin with an estimated K_D of 15.3 ± 7.28 nM (Fig. 5*B* and Table II). Association of PkCSP with heparin was measurable only at higher concentrations (>100 nM) (Fig. 5*C*) with a binding affinity of 1690 ± 537 nM (Table II). PyCSP showed minimal association with heparin (Fig. 5*D*) and consequently its binding affinity was 6060 ± 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.

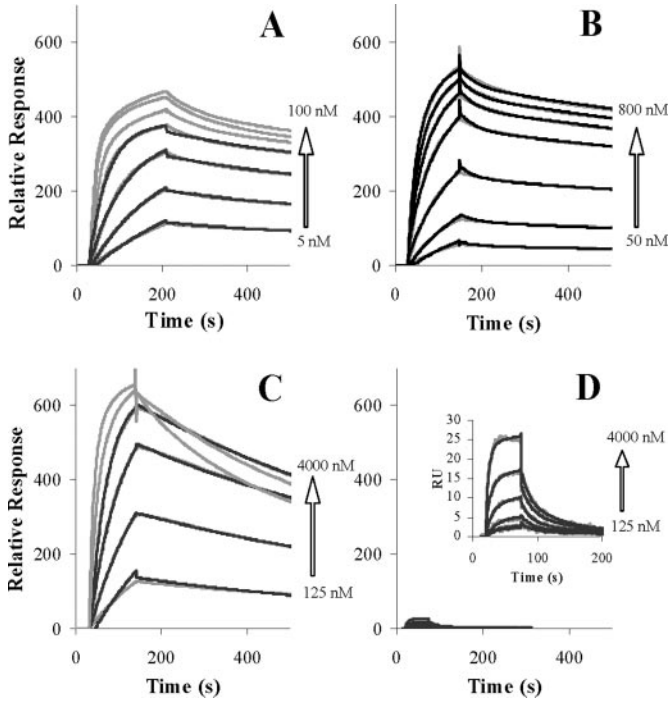


FIG. 5. **Surface plasmon resonance analysis of CSP.** Different concentrations of CSP were allowed to interact with the heparin immobilized on a chip. *A–D* show the interactions of PfCSP, PgCSP, PkCSP, and PyCSP with heparin, respectively.

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 HSPG-based cell surface receptor, suggesting a receptor specificity identical to PfCSP (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-

TABLE II
Binding affinity of CSP from different species to heparin

CSP	k_{a1}	k_{d1}	k_{p2}	k_{d2}	K	K_D	$K_{D\text{average}}$	Ch^2
	$1/M{\cdot}s$	$1/s$	$1/s$	$1/s$	$1/M$	M	M	
P. falciparum								
LCS	543,000 ± 2,370	9.14E − 04 ± 8.08E − 06	0.00153 ± 1.39E − 05	2.83E − 05 ± 2.60E − 05	3.21E + 10	3.12E − 11	3.40E − 10 ± 4.37E − 10	12
HCS	318,000 ± 2,330	0.00170 ± 2.07E − 04	0.00725 ± 7.84E − 04	8.78E − 04 ± 8.09E − 05	9.95E + 07	6.49E − 10		41.6
P. gallinaceum								
LCS	31,500 ± 166	0.01110 ± 2.73E − 04	0.01620 ± 1.71E − 04	0.00105 ± 1.16E − 05	4.89E + 07	2.04E − 08	1.53E − 08 ± 7.28E − 09	7.91
HCS	26,800 ± 87.5	1.87E − 03 ± 3.5E − 05	6.05E − 03 ± 1.18E − 04	8.74E − 04 ± 3.27E − 05	9.95E + 07	1.01E − 08		18.3
P. knowlesi								
LCS	24,100 ± 19.3	0.00114 ± 9.88E − 07	2.30E − 05 ± 2.63E − 08	0.00101 ± 1.17E − 06	4.83E + 05	2.07E − 06	1.69E − 06 ± 5.37E − 07	19.6
HCS	24,400 ± 102	4.78E − 04 ± 2.80E − 06	1.36E − 06 ± 5.84E − 06	9.06E − 05 ± 7.16E − 05	7.64E + 05	1.31E − 06		21.6
P. yoelii								
LCS	24,700 ± 626	0.0665 ± 0.00284	0.0128 ± 0.000898	0.0198 ± 9.55E − 04	2.32E + 05	4.31E − 06	6.06E − 06 ± 2.47E − 06	0.107
HCS	5,870 ± 107	0.0339 ± 0.00105	0.00909 ± 4.88E − 04	0.0123 ± 3.87E − 04	1.28E + 05	7.81E − 06		0.235

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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