

An Immunologically Cryptic Epitope of *Plasmodium falciparum* Circumsporozoite Protein Facilitates Liver Cell Recognition and Induces Protective Antibodies That Block Liver Cell Invasion*

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Circumsporozoite, a predominant surface protein, is involved in invasion of liver cells by *Plasmodium* sporozoites, which leads to malaria. We have previously reported that the amino terminus region (amino acids 27–117) of *P. falciparum* circumsporozoite protein plays a critical role in the invasion of liver cells by the parasite. Here we show that invasion-blocking antibodies are induced by a polypeptide encoding these 91 amino acids, only when it is presented in the absence of the rest of the protein. This suggests that when present in the whole protein, the amino terminus remains immunologically cryptic. A single reactive epitope was identified and mapped to a stretch of 21 amino acids from position 93 to 113. The epitope is configurational in nature, since its recognition was affected by deleting as little as 3 amino acids from either end of the 21-residue peptide. Lysine 104, the only known polymorphic position in the epitope, affected its recognition by the antibodies, and its conversion to leucine in the protein led to a substantial loss of binding activity of the protein to the hepatocytes. This indicated that in the protein, the epitope serves as a binding ligand and facilitates the interaction between sporozoite and hepatic cells. When considered along with the observation that in its native state this motif is immunologically unresponsive, we suggest that hiding functional moieties of the protein from the immune system is an evasion strategy to preserve liver cell binding function and may be of importance in designing anti-sporozoite vaccines.

Malaria is one of the parasitic diseases for which development of a vaccine has been vigorously pursued. The infection commences with the bite of an infectious mosquito that introduces sporozoites into the microvasculature. The surface of malaria sporozoite is covered by a protein termed circumsporozoite (CS)¹ (1, 2), which plays several roles in the parasite's life cycle, from the time of oocyst development in the

mosquito through the development of exoerythrocytic merozoites in the liver. Some of the biological functions of CS protein include maintenance of species specificity during infection (3), facilitating the passage of sporozoites into the salivary gland of the mosquito (4), attachment and invasion of the liver cells (5–8), and directly inhibiting protein synthesis in the host cells (9). To invade liver cells, the protein interacts with heparan sulfate proteoglycans expressed on the liver cell surface (7). CS protein has been widely investigated as a vaccine candidate, and several CS-based vaccines are currently undergoing clinical trials (10–13).

The protein is constituted of three modules (*viz.* the amino terminus, central repeat region, and carboxyl terminus region) of roughly equal sizes (Fig. 1). Immunologically, the central repeat region is the immunodominant segment of the protein, with 90% of the anti-protein antibodies directed against this region (14). Unfortunately, this immune response does not lead to protection and is now believed to be a “smoke screen effect” developed by the parasite to sidetrack the attention of the host immune mechanism, which prevents the recognition of other parasitic components, whose recognition could be deleterious for the parasite (15). The carboxyl terminus region of the protein encodes a cytotoxic T cell epitope (16), and most of the CS-based vaccine studies currently being performed include either the complete or selected regions of carboxyl terminus domain (10–12). In contrast, the amino terminus region of the protein is widely believed to be immunologically unresponsive (17) and is not being utilized for vaccine purposes.

We have recently demonstrated that the amino terminus of the protein plays a crucial role in the pathogenesis process by helping the sporozoite in the attachment and invasion of liver cells (6) and suggested that structural configuration of the amino terminus region is critical for this activity. We have now performed an immunological and structural investigation of the amino terminus and demonstrate that a 21-amino acid segment is involved in binding; antibodies against this segment are protective in nature and can successfully prevent the invasion of liver cells by the sporozoites. Furthermore, we show that substitution of a single lysine residue in this domain with leucine affects the binding of the protein to liver cells.

MATERIALS AND METHODS

Recombinant Protein Expression and Purification—Construction of plasmid pCS27 encoding DNA for amino acids 27–117 of *Plasmodium falciparum* circumsporozoite protein (N-terPfCSP), its expression, and its purification has been described elsewhere (6). The construct encodes two extra amino acids (methionine and arginine) preceding the coding sequence, for translation initiation and cloning purposes, as described

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¹ The abbreviations used are: CS, circumsporozoite; ELISA, enzyme-linked immunosorbent assay.

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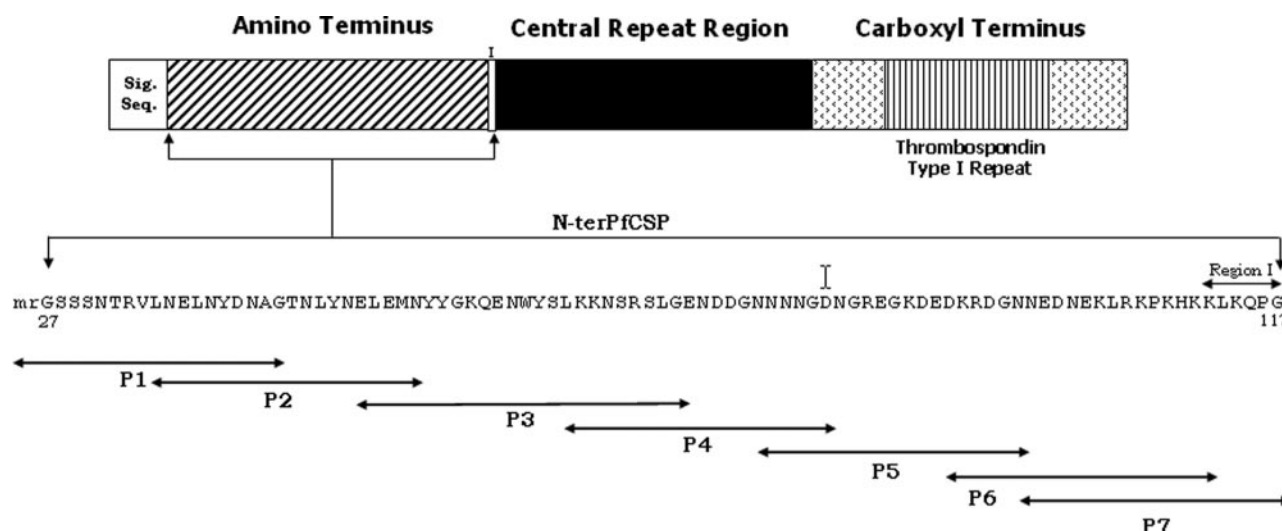


FIG. 1. Schematic representation of CS protein, the amino acid sequence of N-terPfCSP, and the location of the seven synthesized peptides spanning N-terPfCSP. The first two residues in the protein (methionine and arginine) were introduced as part of the cloning strategy.

previously (5). For expression, the construct was transformed in the BL21 strain of *Escherichia coli*, and the expression was induced with isopropyl 1-thio- β -D-galactopyranoside at a final concentration of 1 mM. The recombinant protein was secreted into the periplasm. The periplasmic fluid containing the recombinant protein was loaded onto a heparin-Sepharose affinity column (Amersham Biosciences), and the bound CS protein was eluted using a 0–1 M NaCl gradient. The fractions containing the recombinant protein were pooled, and the protein was purified to apparent homogeneity using gel filtration chromatography.

Peptide Synthesis and Purification—Peptides representing the amino terminus region of CS protein were synthesized using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry on chlorotrityl resins. Subsequent to synthesis, the peptides were purified by reversed phase chromatography on a Luna C18 (50 $\times$ 2 mm) column (Phenomenex, Torrance, CA) by a gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid, to a purity of >90%. Authentication was performed by electrospray mass spectrometry using an Agilent 1100 mass spectrometer.

Generation of Antibodies—BALB/c mice were subcutaneously immunized with 10 μ g of recombinant N-terPfCSP in complete Freund's adjuvant. Three weeks after the first immunization, mice received two subsequent injections at 3-week intervals with 10 μ g of protein delivered in incomplete Freund's adjuvant. The mice were bled 2 weeks after each immunization, and serum samples were collected.

ELISA—For protein ELISA, 250 ng of N-terPfCSP in 10 mM carbonate-bicarbonate buffer (pH 9.6) were coated onto the wells of an Immulon 4 microtiter plate and incubated at 37 $^{\circ}$ C for 2 h. For peptide ELISA, 2 nmol of each peptide was used for coating the wells. Uncoated sites were blocked with 1% nonfat dry milk in 50 mM Tris-buffered saline, pH 7.4. Different dilutions of anti-N-terPfCSP serum were added to the wells, and the plate was incubated at 37 $^{\circ}$ C for 1 h. For peptide competition experiments, antiserum was incubated in the presence of 4 nmol of each peptide. Unbound reagents were removed by washing the wells with 50 mM Tris-buffered saline, pH 7.4, containing 0.1% Tween 20, followed by the addition of anti-mouse alkaline phosphatase conjugate and incubation for 60 min at 37 $^{\circ}$ C. The wells were thoroughly washed to remove unbound conjugate and followed by the addition of *p*-nitrophenyl phosphate as substrate. The absorbance was measured at 405 nm.

Immunofluorescence—Immunofluorescence analysis was performed as previously described (18). Briefly, 2-fold diluted test sera or monoclonal antibody (50 μ g/ml) was reacted with air-dried *P. falciparum* sporozoites on a glass slide. Bound antibodies were detected using fluorescein isothiocyanate-labeled anti-mouse IgG (Southern Biotechnology, Birmingham, AL). Slides were mounted using VectaShield mounting medium for fluorescence (Vector Laboratories Inc., Burlingame, CA) and evaluated using a fluorescent microscope.

Sporozoite Invasion Assay—HepG2 (human hepatoma) cells were collected, washed, and resuspended in complete minimal essential medium and were subsequently plated at a density of 50,000 cells/0.3 ml in ECL-coated Labtek glass slides and incubated overnight at 37 $^{\circ}$ C in a CO₂ incubator. The next day, the medium was removed, and different dilutions of anti-N-terPfCSP serum, N-terPfCSP, peptide P6, or anti-CS

repeat monoclonal antibody was added per well (in triplicates). This was immediately followed by the addition of 20,000 sporozoites in 50 μ l of medium to each well. *P. falciparum* (strain NF54) sporozoites were obtained from the salivary glands of *Anopheles stephensi* mosquitoes as described by Ozaki (1). The sporozoites were allowed to invade liver cells for 3 h followed by the washing of cells with phosphate-buffered saline at pH 7.4. Subsequently, the cells were fixed with cold methanol. Sporozoites were visualized by immunostaining by using NFS1 as primary antibody and anti-mouse IgG-peroxidase conjugate. Diaminobenzidine was used as substrate. The slides were mounted with Paramount, and intracellular sporozoites were identified and counted. Percentage inhibition of invasion was calculated with the formula, ((control – test)/control) $\times$ 100.

Construction, Expression, and Purification of K104L—Plasmid pCS1 encoding the native CS protein sequence under the control of a T7 promoter (19) was used as template to introduce a single amino acid (Lys¹⁰⁴ $\rightarrow$ Leu) change using QuikChange, a PCR-based site directed mutagenesis kit (Stratagene), as previously described (20). This gave rise to plasmid pK104L. The authenticity of the mutant construct was verified by DNA sequencing. Expression and purification of the recombinant protein was performed as described above.

Binding Activity of CS Protein Mutant—HepG2, a hepatoma human cell line, was used, and the assay was performed as described (6). Briefly, 50,000 cells/well were plated in a 96-well plate, 36 h before the experiment. Cells were fixed with 4% paraformaldehyde followed by blocking with Tris-buffered saline containing 1% bovine serum albumin. Different concentrations of recombinantly expressed CS proteins were added to the cells for 1 h followed by a 30-min incubation with a monoclonal antibody that recognizes the central repeat region of the protein. Unbound material was removed, and anti-mouse alkaline phosphatase-coupled conjugate was added. 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.

RESULTS

Antibody Responses to N-terPfCSP in BALB/c Mice—Six mice were immunized with three subcutaneous injections of recombinantly expressed amino terminus of *P. falciparum* circumsporozoite protein (N-terPfCSP). Fig. 1 depicts the amino acid sequence of the polypeptide that was used for immunization. The serum samples were collected 2 weeks after each immunization and screened for anti-N-terPfCSP immune responses. Antibodies against the N-terPfCSP could be detected after the first immunization (data not shown). On subsequent boosting, anti-N-terPfCSP antibody titers increased significantly. Fig. 2 depicts the recognition of N-terPfCSP in serum from individual animals by ELISA. Serum from each animal recognized the antigen in a dose-dependent manner. Potent anti-N-terPfCSP antibody titers were generated as the antigen

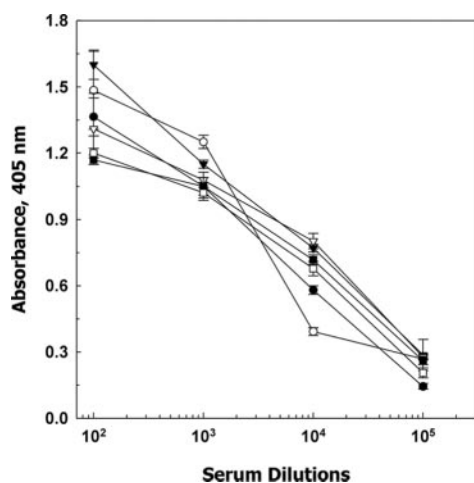


FIG. 2. Antibody titers against the amino terminus region of CS protein in mice. 250 ng of N-terPfCSP was coated in ELISA plates followed by blocking with 1% nonfat dry milk in Tris-buffered saline. Log dilutions of serum were evaluated for the recognition of N-terPfCSP followed by the addition of anti-mouse-alkaline phosphatase conjugate. Absorbance was measured at 405 nm using an ELISA reader. Symbol types represent titers from individual animals at different dilutions.

was recognized even at a 1:100,000 dilution of the serum. The recognition was specific, since (i) anti-N-terPfCSP serum did not recognize a polypeptide representing the carboxyl terminus of PfCSP and (ii) normal mouse serum did not recognize N-terPfCSP (data not shown).

Identification of the Recognized Epitope—To identify the epitope(s) recognized by the antiserum, seven overlapping peptides spanning the entire N-terPfCSP were synthesized and purified by reversed phase chromatography (Fig. 1). These synthetic peptides were individually screened for their recognition by anti-N-terPfCSP serum by ELISA. The analyses revealed that the antibodies are predominantly directed against a 21-amino acid peptide P6, representing amino acids 93–113 of N-terPfCSP. Peptide P7 (amino acids 99–117), which significantly overlapped (75%) with peptide P6, was also recognized, although its recognition was reduced by 50% (Fig. 3), suggesting that exclusively amino acids 93–113 are recognized by the anti-N-terPfCSP serum. This recognition (of the P6 peptide) by the antiserum was specific, since incubation with antibodies in the presence of soluble N-terPfCSP protein led to a >80% loss of reactivity in an ELISA assay (Fig. 4). That the antiserum preferentially recognizes amino acids 93–113 was further verified by performing a converse experiment where N-terPfCSP was coated onto the plate and the peptides were used as competitor to the binding of anti-N-terPfCSP antibodies to the protein. Only peptides P6 and P7 could compete the binding of antibodies to the protein (Table I).

Effects of Polymorphism on the Recognition of Peptide P6 by Anti-N-terPfCSP Antibodies—A comparison of the P6 epitope sequence from 107 *P. falciparum* CS protein sequence, representing isolates from Asia, Africa, and South America available in GenBankTM, revealed a single polymorphic site in the epitope at position 104, represented by a lysine residue. In isolates from Thailand (accession number AAA29555), lysine has been substituted by a threonine (K104T) (21). In a second isolate from Brazil, lysine has been converted to an asparagine (K104N) (accession number AAN87594) (22). Historically, polymorphisms, especially in an epitope, are associated with immune evasion (23). To investigate whether these polymorphisms can attribute to a change in recognition of the peptide P6, two variant P6 peptides, P6N and P6T, where Lys¹⁰⁴ was substituted by an asparagine and threonine, respectively, were

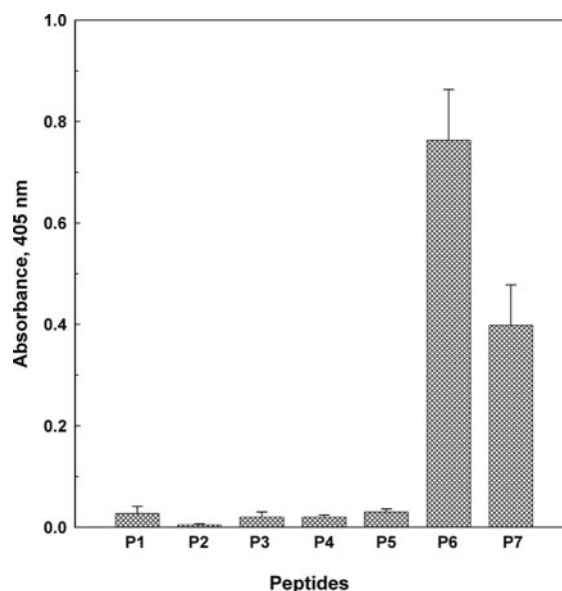


FIG. 3. Identification of immunogenic segments in the amino terminus region of CS protein. Equimolar (2-nmol) amounts of each of the seven peptides were coated onto an ELISA plate followed by the addition of anti-N terminus CS serum at a 1:100 dilution. Values shown here are the mean absorbance from individual animals.

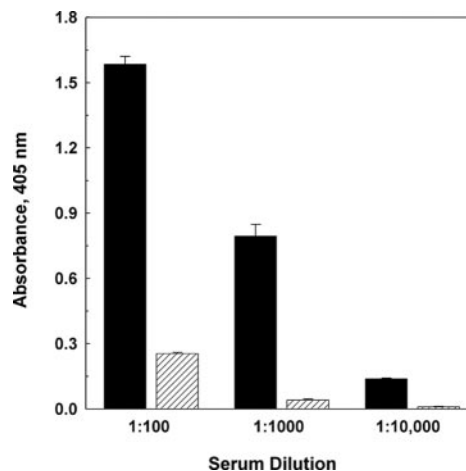


FIG. 4. Recognition of peptide P6 by anti-N-terPfCSP serum is specific in nature. Recognition of peptide P6 by different dilutions of pooled antisera was evaluated in the absence (black bars) or presence of 2.5 µg of N-terPfCSP protein (hatched bars) in an ELISA. Absorbance was measured at 405 nm using an ELISA reader.

synthesized and probed for their recognition by anti-N-terPfCSP serum. Both of the variants showed a 40% decrease in recognition in comparison with the native sequence (Table II), suggesting that these changes in the field isolates are most likely associated with immune evasion.

Fine Mapping of the Recognized Epitope—To identify the exact epitope, six variants of peptide P6, 9–18 amino acids in length, were synthesized and used as an antigen in ELISA. In peptides P6N3, P6N6, and P6N9, 3 amino acids each were serially deleted from the amino terminus. Conversely, peptides P6C3, P6C6, and P6C9 were missing 3, 6, and 9 amino acids, respectively, from the carboxyl terminus. Deletions of as few as 3 amino acids from either end led to a 50% decrease in the recognition of the epitope by the antibodies (Table II). Loss of 6 amino acids from the amino terminus (residues 93–98, peptide P6N6) led to a 65% loss, whereas any further deletions led to a nearly complete loss of epitope recognition. This suggested that the epitope encoded in residues 93–113 is configurational in

TABLE I
Peptide P6 and P7 could compete the binding of
antibodies to N-terPfCSP in an ELISA

Recognition of N-terPfCSP by the antisera was examined in the absence or presence of 4 nmol of each peptide by ELISA. Percentage decrease represents a change in absorbance with respect to the recognition of N-terPfCSP in the absence of the peptides.

Protein/Peptide	A ₄₀₅	Decrease %
N-terPfCSP	1.35 ± 0.07	
P1	1.50 ± 0.01	
P2	1.40 ± 0.01	
P3	1.30 ± 0.02	3.8
P4	1.32 ± .025	2.3
P5	1.28 ± 0.01	5.2
P6	0.57 ± 0.01	57.7
P7	0.76 ± 0.02	43.4

nature and that its tertiary structure is important for its recognition by the antibodies.

Antibodies Recognize CS Protein Expressed on Sporozoite Surface—Anti-N-terPfCSP serum was evaluated for the recognition of CS protein expressed on *P. falciparum* (NF54 strain) sporozoite surface and was compared with the recognition of the central repeat region by anti-repeat monoclonal antibody NFS1. The antibodies recognized the expression of CS protein expressed on the sporozoite surface (Fig. 5A), but its recognition was weaker in comparison with the recognition of the repeat region by NFS1 (Fig. 5B).

Invasion-inhibitory Activity of Anti-N-terPfCSP Antibodies—Since antibodies recognized the expression of CS protein on the sporozoite surface, we investigated the ability of these antibodies in inhibiting the invasion of liver cells by live *P. falciparum* (NF54 strain) sporozoites. Anti-N-terPfCSP antibodies were successful in preventing the invasion of liver cells in a dose-dependent manner. At 1:100 and 1:400 dilutions of serum, sporozoite invasion in HepG2 cells was inhibited by 90 and 80%, respectively. This inhibitory activity was comparable with the inhibitory activity of anti-central repeat region monoclonal antibody, which at two concentrations (100 and 25 µg/ml) inhibited the invasion by 92 and 77%, respectively (Table III). Similarly, protein N-terPfCSP and peptide P6 could also prevent the invasion of liver cells by the parasites, albeit at lower levels (Table III). This suggested that antibody responses against a short amino acid sequence can prevent the invasion of liver cells, and this epitope could be involved in receptor-ligand interactions between the host cells and the parasite.

Role of Lysine 104 in Malaria Pathogenesis—On hepatocytes, CS protein interacts with heparan sulfate proteoglycans expressed on their surface (7, 24). This interaction is electrostatic in nature and has been proposed to occur between positively charged (lysine and arginine) and polar residues of CSP and the negatively charged sulfate and carboxylate ions of heparan sulfate. If involved, lysine 104 and its known substituents (asparagine and threonine), all polar amino acids, will retain their capacity to interact with the host receptor, since all 3 residues have known heparin-interaction capabilities in other biological systems (25). This selective substitution suggested that the parasite has a limited degree of freedom for replacing lysine 104 and that the residue could be playing an important role in the biology of the parasite. To investigate this possibility, we designed a mutant CSP construct, where Lys¹⁰⁴ was converted to leucine, a nonpolar residue, by site-directed mutagenesis, giving rise to plasmid *pK104L*. The construct was expressed in *E. coli*, and the protein was purified to homogeneity by a two-step column chromatography. Whereas the protein indeed bound onto the heparin-Sepharose column, it eluted from the column at a much lower salt concentration in

comparison with the nonmutated protein (data not shown), suggesting that a single amino acid change has affected the affinity of the protein toward heparin. Binding analysis of K104L was performed on HepG2 cells and was compared with the binding activity of the nonmutated protein. The analysis revealed that the substitution of lysine 104 to leucine leads to a 75% decrease in binding activity of the protein to host cells (Fig. 6), suggesting that the residue plays a critical role in attachment of parasites to the liver cells.

DISCUSSION

In biological systems, concealing information until such a time when it is advantageous to reveal, is an intrinsic mechanism and has evolved with organisms as part of their complex architecture. In pathogens, this veil of secrecy could play a critical role in the onset of infection and associated pathogenicity. Therefore, in the case of intracellular pathogens like *Plasmodium*, safeguarding proteins or their domains involved in host infectivity from any kind of host-led attack could be crucial for initiating a successful infection.

We recently demonstrated that the amino terminus of circumsporozoite protein, a predominant parasite surface antigen, plays a critical role in the invasion of liver cells by the parasite, which sets the stage for malaria infection (6). This region is generally believed to be immunologically inert, since immunization with the complete protein generates antibodies that primarily recognize the immunodominant central repeat region of the protein (14) (Fig. 1) but do not recognize the amino terminus region of the protein. CS protein is one of the most promising vaccine candidates, and its carboxyl terminus fragment (Fig. 1) is currently being tried as a vaccine by several investigators (11, 13). The critical involvement of this segment in host-parasite interaction (6) but its nearly total oblivion by the host immune system (17, 26) led us to investigate this segment in isolation from the whole protein.

Mice were immunized with a recombinantly expressed 93-amino acid-long polypeptide representing the N terminus of *P. falciparum* CS protein, and the antibody responses were analyzed for both specificity and their potential to inhibit the invasion of liver cells by *P. falciparum* sporozoites. All of the immunized mice developed high antibody titers against the immunized polypeptide (Fig. 2). This suggested that lack of recognition of this portion of the molecule in the whole protein is not an inherent failure of the immune system to recognize or present the peptide but could be due to the crypticity of the region in the presence of the immunodominant central repeat region. Parallels for such behavior exist in biological systems, where cryptic epitopes are unmasked after the loss of immunodominant epitope (27).

Recognition of the amino terminus was limited to a single site within the 93-amino acid peptide. It was mapped to a 21-amino acid region (peptide P6) of the protein from amino acid 93 to 113 (Fig. 3) and could be inhibited in the presence of free N-terPfCSP (Fig. 4). Alternately, peptides representing region 93–113 (P6) or part thereof (P7) could also inhibit the recognition of N-terPfCSP by the antisera (Table I), confirming the exclusivity of its recognition by the antibodies.

Nonetheless, this region is sensitive to deletion of residues in the CS protein. In some field isolates from areas of acute malaria transmission and laboratory clones, a 19-amino acid (residues 81–99) segment is deleted (28), which will result in a loss of amino acids 93–99 from peptide P6. Mimicking this deletion led to a significant but not a total loss of recognition of this epitope (Table II). This suggested that the integrity and the associated structural conformation of this epitope were important for its optimal recognition. Immunofluorescence analysis of *P. falciparum* sporozoite (NF 54 strain) expressing

TABLE II
Truncated and mutated P6 peptides are poorly recognized by the antibodies

2 nmol of each of the peptide was coated onto an ELISA plate, and their recognition by the anti-N-terPfCSP serum was measured by ELISA. Any decrease in recognition of the variant peptides was measured in comparison with peptide P6 (100% activity). Single amino acid changes in peptide P6N and P6T are shown in boldface type.

Peptide	Peptide size	Sequence	Decrease
	amino acids		%
P6	21	DKRDGNNEDNEKLRLKPKHKKL	
P6N	21	DKRDGNNEDNE N LRLKPKHKKL	39.5
P6T	21	DKRDGNNEDNE T LRLKPKHKKL	41.7
P6N3	18	DGNNEDNEKLRLKPKHKKL	55.8
P6N6	15	NEDNEKLRLKPKHKKL	65.4
P6N9	12	NEKLRLKPKHKKL	97.4
P6C3	18	DKRDGNNEDNEKLRLKPKH	59.0
P6C6	15	DKRDGNNEDNEKLRLK	89.7
P6C9	12	DKRDGNNEDNEK	96.1

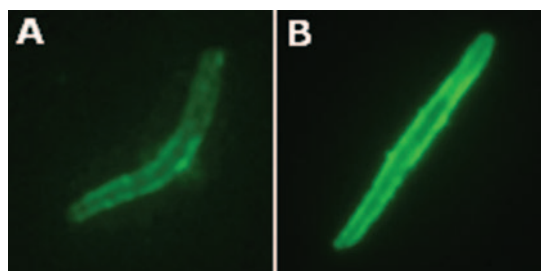


FIG. 5. Immunofluorescence analysis of *P. falciparum* sporozoites. Air-dried *P. falciparum* sporozoites were incubated with anti-N-terPfCSP serum (A) or anti-repeat monoclonal antibody (B) followed by the addition of anti-mouse-fluorescein isothiocyanate conjugate.

TABLE III

Effect of CS protein, peptides, and anti-CS antibodies on liver cell invasion by *P. falciparum* (NF54) sporozoites

Liver cells were invaded by *P. falciparum* sporozoites in the absence (control) or presence of different concentrations of peptide P6, protein N-terPfCSP, anti-CS repeat monoclonal antibody, or anti-N-terPfCSP serum. Percentage inhibition represents the decrease in the number of intracellular (invaded) sporozoites with respect to control that was incubated with the culture medium containing 10% fetal bovine serum.

Treatment	Concentration	Inhibition
		%
Anti-CS-monoclonal antibody	100 μ g/ml	91.9
	25 μ g/ml	76.5
Anti-N-terPfCSP serum	1:100	89.8
	1:400	78.9
N-terPfCSP	20 μ g/ml	61.2
	10 μ g/ml	37.1
Peptide P6	500 μ g/ml	62.6
	250 μ g/ml	51.0

the deleted variant of CS protein confirmed that the recognition of the truncated epitope is somewhat weaker in comparison with the recognition of the central repeat region of the protein (Fig. 5). Based on these results, we anticipated that sporozoites expressing the truncated version of CS protein might be able to evade these antibodies and successfully invade liver cells, thus providing an escape for the parasite.

Surprisingly, antibodies blocked the invasion activity of *P. falciparum* NF54 strain sporozoites, which express CS protein lacking residues 81–99 on its surface, by 90% (Table III). These results showed that the antibodies directed against the 21-amino acid sequence (residues 93–113) of the protein not only recognize the CS protein variant expressed on the sporozoite surface; they are also capable of interrupting the invasion process. This suggests that if “efficiently” recognized by the host immune system, antibodies against this region could be able to control the malaria infection. Similarly, both N-terPfCSP and peptide P6 could also prevent *P. falciparum* sporozoites from invading liver cells (Table III). Whereas antibodies

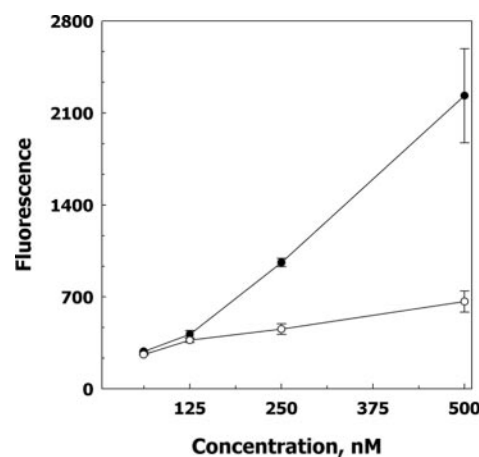


FIG. 6. Binding activity of the mutant protein on HepG2 cells. Different concentrations of unmutated CS protein (closed circle) and K104L (open circle) were incubated with HepG2 cells followed by the addition of anti-repeat monoclonal antibody. This was followed by the addition of anti-mouse alkaline phosphatase conjugate and a fluorescent substrate. Fluorescence was measured using a plate reader with excitation at 350 nm and emission at 460 nm.

against the central repeat region of the protein block invasion, this region does not play a role in host cell binding and invasion. As the central repeat region is present in close proximity to residues 93–113 (Fig. 1) and contains multiple copies of the recognized epitope, we suggest that binding of antibodies to the repeat region causes a steric hindrance, which interferes in the binding/invasion activity of the sporozoite, resulting in inhibition of invasion.

Not only is this 21-amino acid sequence sensitive to truncation; sequence analysis revealed that in field isolates from Thailand and Brazil it shows polymorphism, albeit only at position 104. In these isolates, the lysine residue at position 104 was either converted to an asparagine or a threonine residue. Whereas these variations cause a 40% decrease in recognition of the epitope by the antibodies (Table II), it was noticed that all 3 residues are polar in nature and are known for their interactions with heparin, the carbohydrate moiety of the host cell (hepatocyte) receptor (25). This suggested that this position, while under pressure to mutate, has limited options for residue substitution, which could be due to its involvement in host-pathogen interactions. We investigated this possibility by mutating lysine 104 to leucine in the full-length protein and comparing its propensity to interact with host cells *in vitro*. Lys¹⁰⁴ turned out to be an important residue for host cell binding, since its conversion to leucine, a nonpolar amino acid led to a >75% loss in the binding activity of the protein to liver cells (Fig. 6). Whereas lysine 104 is important in host-parasite interaction, it is most likely a part of a much bigger binding

domain whose residues are distributed throughout the amino terminus but are structurally aligned in the final conformation of the protein.

In conclusion, we have identified a 21-amino acid region in the amino terminus of CS protein, which participates in the host-parasite interaction and involves lysine 104 in this process. Part of this sequence has also been shown to be involved in the invasion of salivary glands (4), suggesting a broader role for this region. Antibodies directed against this region are protective in nature, since they effectively block the sporozoite-led invasion of liver cells. Although this epitope seems cryptic when presented as part of the whole protein, reported deletions and polymorphism in the epitope suggest that it could be under immune selection pressure. In pathogens, such events are associated with immune evasion (23). The recent report of a successful field trial of an antimalarial vaccine reemphasizes the importance of *P. falciparum* CS protein as a target antigen for protective immunity. Here we show evidence that leads us to believe that the response to CS protein can be potentiated by opening otherwise cryptic regions of the molecule to the host immune system.

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