# Structural and Functional Properties of Region II-Plus of the Malaria Circumsporozoite Protein

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## Summary

During feeding, infected mosquitos inject malaria sporozoites into the host circulation. Within minutes, the parasites are found in the liver where they initiate the first stage of malaria infection. All species of malaria sporozoites are uniformly covered by the circumsporozoite protein (CS), which contains a conserved COOH-terminal sequence called region II-plus. We have previously shown that region II-plus is the parasite's hepatocyte-binding ligand and that this ligand binds to heparan sulfate proteoglycans (HSPGs) on the hepatocyte membrane. Using a series of substituted region II-plus peptides, we show here that the downstream basic amino acids as well as the interdispersed hydrophobic residues are required for binding of CS to hepatocyte HSPGs. We also show that this positively charged stretch of amino acids must be aggregated in order to bind to the receptor. On the basis of this information, we have synthesized a multiple antigen peptide that mimics the hepatocyte-binding ligand. This construct inhibits both CS binding to HepG2 cells in vitro as well as CS clearance in mice.

Malaria is transmitted when *Anopheles* mosquitos inject sporozoites into susceptible vertebrate hosts. Within minutes after injection, the sporozoites are cleared from the bloodstream and found in the liver, where they cross the space of Disse and invade hepatocytes. The rapidity of invasion and the uniqueness of the cellular target strongly suggest that the parasite is arrested in the liver by specific receptors. Several lines of evidence indicate that the initial event is the recognition of the parasite's coat protein (circumsporozoite protein [CS][1]) by heparan sulfate proteoglycans (HSPGs) on the membrane of hepatocytes (1, 2). The ligand is contained in the COOH-terminal of CS, in a region highly conserved among all species of mammalian malaria parasites. Here we designate the conserved motif as region II-plus because it encompasses part of the previously defined region II (3), as well as a stretch of positively charged amino acids at the 3′ end (see Fig. 1).

A similar motif is found in several host proteins, such as thrombospondin, the complement proteins properdin and complement protein 6 (C6) (4–6), F-spondin (7), UNC-5, a molecule that guides pioneering axon migration in *Caenorhabditus elegans* (8), thrombospondin-related anonymous protein (TRAP) or sporozoite surface protein 2 (SSP-2), another malaria surface protein (4, 9, 10), and EtHL6, a protein from the coccidian parasite *Eimeria tenella* (11) (see Fig. 1). Although the function of the motif in this diverse group of proteins is unknown, synthetic peptides representing the motif, or subregions of the motif, are biologically active in vitro and in vivo. Some of these peptides inhibit platelet aggregation and melanoma cell metastasis (12), whereas others have antiangiogenic activity (13) and display cell-adhesive properties (14–16). In view of the postulated importance of the motif in the initial phases of malaria infection as well as in a variety of cellular interactions in the mammalian host, we have determined some of the structural features of region-II plus required for activity in a malaria model, i.e., for adhesion of CS to hepatocytes in vitro and its clearance by hepatocytes in vivo.

## Materials and Methods

*Recombinant Proteins.* The *Escherichia coli*–derived recombinant CS27IVC (27-123[NANPNVDP]$_3$[NANP]$_{21}$300-411) represents the complete *Plasmodium falciparum* CS sequence from the T4 isolate, except that the hydrophobic $NH_2$- and COOH-terminal amino acids 1-26 and 412-424 have been deleted and five histidine residues have been added to the COOH-terminus to facilitate purification (17). The recombinant protein used in these studies was kindly provided by Dr. Bela Takacs (F. Hoffmann-La Roche Ltd., Basel, Switzerland).

*Antibodies.* The mAb 2A10 (18) is directed against an epitope contained in the (NANP)$_n$ repeat domain of *P. falciparum* CS. The

[1] *Abbreviations used in this paper:* C6, complement protein 6; CS, circumsporozoite protein; HSPG, heparan sulfate proteoglycan; MAP, multiple antigen peptide; SSP-2, sporozoite surface protein 2; TBS, Tris-buffered saline; TRAP, thrombospondin-related anonymous protein.

mAb 3D11 (19) reacts with the repeat-containing domain of *P. berghei* CS. The mAb 2E6, a gift from Dr. M. Tsuji (New York University Medical Center) reacts with the liver stage of *P. berghei*.

*Peptides.* Peptides were synthesized by Boc chemistry, using the multiple peptide synthesis method described by Houghten (20). Cleavage from the resin was performed with low-high hydrofluoric acid. Purity was verified by HPLC and amino acid analysis, and in some cases, by mass spectrometry. Multiple antigen peptides (MAPs) were made as described by Tam et al. (21). Each MAP contains four copies of its respective peptide linked to a core of three lysines prepared from branching two levels of Boc-Lys (Boc) and using $\gamma$-butyric acid and $\beta$-alanine as spacers. The cysteines in the region II-plus MAPs were blocked with acetamide groups. Three MAPs were synthesized utilizing the following peptides: QCNVTCGSGIRVRKRKGSN (*P. berghei* region II-plus), PCSVTCGNGIQVRIKPGSA (*P. falciparum* region II-plus), and NANPNANPNANPNANP (*P. falciparum* repeat). Peptide CSVTCG was a gift from Dr. George Tuszynski (Medical College of Pennsylvania, Philadelphia, PA). Cysteine-containing peptides, with the exception of the MAPs, contained free sulfhydryl groups as detected by the Ellman reaction (22), but were Ellman negative after oxidation by long-term storage at −20°C, or by stirring in 10 mM Tris, pH 8, overnight, at concentrations from 1 to 10 mg/ml.

*Mice.* BALB/C females from Taconic Farms Inc. (Germantown, NY) weighing between 15 and 20 g were used.

*Binding of CS to HepG2 Cells.* HepG2 cells, a hepatoma cell line, (ATCC HB8065; American Type Culture Collection, Rockville, MD) were maintained in MEM (GIBCO BRL, Gaithersburg, MD), 10% FCS (Hyclone Laboratories, Logan, UT), 1 mM glutamine (GIBCO BRL), 3 mg/ml glucose (Sigma Chemical Co., St. Louis, MO), and 1× nonessential amino acids (GIBCO BRL) (FCS-MEM). Cells ($10^5$) were deposited in 96-well plates (Removawell tissue culture plates; Dynatech Laboratories, Inc., Chantilly, VA), allowed to grow overnight, fixed with 4% paraformaldehyde (Eastman Kodak Co., Rochester, NY) for 10 min, rinsed three times with Tris-buffered saline (TBS; 130 mM NaCl, 50 mM Tris, pH 7.4), and then blocked for 2 h at 37°C with 1% BSA in 130 mM NaCl, 50 mM Tris-HCl, and 0.02% sodium azide, pH 7.4 (TBS/BSA). The cells were incubated with various concentrations of peptide in TBS/BSA for 1 h at 37°C, washed three times with TBS, incubated with recombinant CS at 2–5 $\mu$g/ml in TBS/BSA for 1 h at 37°C, washed three times with TBS, and finally incubated with $^{125}$I-labeled mAb 2A10 ($2 \times 10^5$ cpm/well) for 30 min, washed three times with TBS, and then counted in a LKB $\gamma$-counter (model 1260; Pharmacia, Inc., Piscataway, NJ). The inhibitory ability of peptide fractions obtained after gel filtration chromatography was tested either in column buffer (TBS, pH 6.45), or in TBS, pH 7.45, with identical results.

*Molecular Sieving Chromatography.* To separate peptide monomers from aggregates, peptides were filtered through columns containing Sephadex superfine G-50 (Pharmacia, Inc.) equilibrated in TBS, pH 6.45, except as noted in the text. Peptides containing 20 or more amino acids were dissolved in 200 $\mu$l of column buffer, loaded onto a 30-ml column (C16/20, Pharmacia), and eluted with a flow rate of 5 cm/h. 0.8-ml fractions were collected and peptide concentration was measured in a spectrophotometer by absorbance at 220 nm. Separation of aggregates from monomers of smaller peptides (15 amino acids) was performed in a C16/70 column (Pharmacia) with a bed volume of 120 ml.

*Mass Spectrometry.* Mass spectra of different fractions of the region II-plus peptide were measured using a time-of-flight mass spectrometer constructed at the Rockefeller University (23, 24). 1 $\mu$l of 20 $\mu$M of each fraction in water was mixed with 9 $\mu$l of $\alpha$-cyano-4-hydroxycinnamic acid (10 g/liter in formic acid/water/isopropanol [1:3:2, vol/vol/vol]), and 0.5 $\mu$l of this solution was placed on the mass spectrometer probe tip and dried with a stream of cold air. The sample was irradiated with 10-ns laser pulses (wavelength 355 nm) from a Nd (YAG) laser. The ions created were accelerated in an electrostatic field and their time-of-flight was measured with a transient digitizer (model 8828D; LeCroy Research Systems, Corp., Spring Valley, NY). To improve the statistics, 200 individual spectra were added together. The time-of-flight spectra were converted into mass spectra using dynorphin 1-17 as an internal calibrant.

*Reduction and Alkylation of P. berghei Sporozoites.* $10^5$ sporozoites were resuspended in 4.8 M urea, 1 mM EDTA, 50 mM dithiothreitol (DTT; Sigma Chemical Co.), and incubated at 25°C for 1 h. *N*-ethylmalemide (Sigma Chemical Co.) was then added to a final concentration of 100 mM, and incubation proceeded for 30 min at 25°C.

*SDS-PAGE and Western Blotting.* SDS-PAGE was performed in 7.5% slab gels. Approximately $10^4$ sporozoites were loaded per lane in either reducing or nonreducing conditions. For Western blotting, the gel contents were electrophoretically transferred to an Immobilon membrane (Millipore Corp., Bedford, MA). The membrane was blocked for 1 h with TBS/BSA, incubated for 1 h with 5 $\mu$g/ml of mAb 3D11 in TBS/BSA, washed three times with TBS/0.05% Tween, and incubated with rabbit anti–mouse Ig coupled to alkaline-phosphatase (Sigma Chemical Co.) for 1 h. The membrane was then washed three times with TBS/0.05% Tween, and the bound enzyme was revealed with bromochlorophenol blue and nitrotetrazolium blue (both from Sigma Chemical Co.) in 100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.0.

*Assay for Sporozoite Infectivity.* This was performed according to the methods described by Hollingdale et al. (25) and Sinden et al. (26). HepG2 cells were plated in FCS-MEM at a density of $10^5$ cells/ml in eight-chamber slides (model 4808; Lab-tek, Naperville, IL) 48 h before each experiment. *P. berghei* sporozoites were dissected from mosquito salivary glands and resuspended in TBS, pH 7.45, containing either *P. berghei* region II-plus peptide aggregates, monomers, or buffer alone. In the experiment with the MAPs, the parasites were resuspended in FCS-MEM alone, with the repeat region MAPS, or with *P. berghei* region II-plus MAPs. Sporozoites ($10^5$) were then added to each well and the parasites were incubated with the HepG2 cells for 2 h at 37°C. The medium was removed after 2 h and replenished with fresh FCS-MEM. The medium was changed again at 18 and 28 h, and after 48 h the cultures were fixed with cold methanol containing 0.3% $H_2O_2$. Wells were blocked with TBS/BSA (without azide) for 45 min at 37°C, incubated for 45 min at 37°C with 10 $\mu$g/ml mAb 2E6, washed six times with TBS/0.05% Tween, incubated for 45 min at 37°C with goat anti–mouse Ig conjugated to horseradish peroxidase (Accurate Chemical & Scientific Corp., Westbury, NY), and washed six times with TBS/0.05% Tween. The bound enzyme was revealed with 1 mg/ml 3,3'-diaminobenzidine (Sigma Chemical Co.) in 0.05 M Tris, pH 7.6, 0.1% $H_2O_2$. Slides were mounted in 50% glycerol in PBS and the number of exoerythrocytic forms in each well was counted under a ×20 objective.

*Radiolabeling of Proteins.* The mAb 2A10 was labeled with $^{125}$I (Amersham, Arlington Heights, IL) to a sp act of $\sim 3 \times 10^6$ cpm/$\mu$g using iodogen (Pierce, Rockford, IL), according to the manufacturer's instructions. Recombinant CS used for in vivo studies was labeled to a lower specific activity ($\sim 2.5 \times 10^5$ cpm/$\mu$g) with a milder procedure in order to reduce oxidative damage to the protein. 1 mCi of $^{125}$I was oxidized in 25 $\mu$l of 0.1 M sodium phosphate buffer, pH 7.4, for 5 min in a glass tube precoated with io-

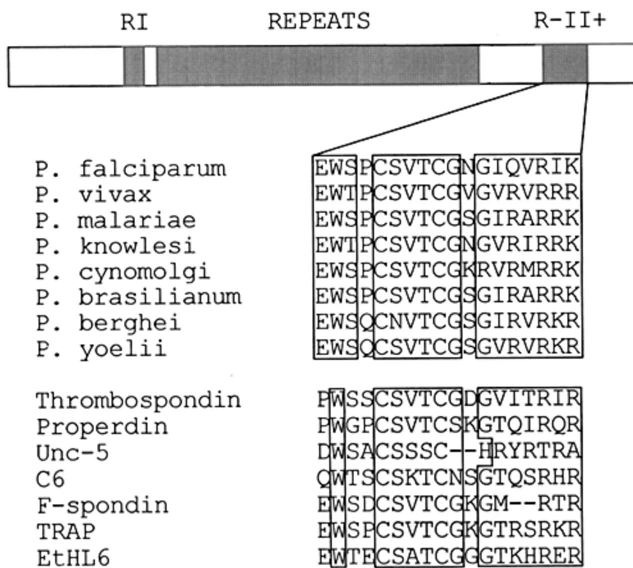



**Figure 1.** Schematic representation of the CS with region II-plus sequences and homologous sequences from other proteins. (*REPEATS*) Species-specific tandem repeats of amino acids. Region I (*RI*) A conserved group of amino acids, KLKQP, $NH_2$-terminal to the repeats. Region II-plus (R-II$^+$) A conserved group of amino acids COOH-terminal to the repeats (3). Below are region II-plus sequences from a variety of malaria parasites. (*Boxes*) Highlight different portions of this motif: an upstream tryptophan residue, a cysteine-containing region, and a downstream "plus" region containing basic and hydrophobic residues. Also shown are homologous sequences from thrombospondin, properdin, the *C. elegans* protein UNC-5, complement protein 6 (*C6*), the neuronal adhesion molecule F-spondin, another malaria surface protein TRAP, and the *E. tenella* protein EtHL6. The amino acid sequences derive from the following sources: *P. falciparum* (42); *P. vivax* (43), *P. malariae* (44); *P. knowlesi* (45); *P. cynomolgi* (46); *P. brasilianum* (47); *P. berghei* (48); *P. yoelii* (49); thrombospondin (4); complement proteins properdin and C6 (5, 6); UNC-5 (8); F-spondin (7); TRAP (4); and EtHL6 (11).

dogen. This solution was then transferred to another tube and incubated for 5 min on ice with 20 μl of CS27IVC (1.28 mg/ml). The free iodide was removed by filtration in Sephadex G-25 (Isolab, Inc., Akron, OH). The labeled CS was then subjected to chromatography in a heparin–Sepharose column (Sigma Chemical Co.) in order to separate CS aggregates (active fraction) from monomers (inactive fraction). For this purpose, the heparin-Sepharose was equilibrated with TBS/BSA (without azide), the iodinated protein was added, and the column was washed with five-column volumes of buffer. The bound, aggregated CS was eluted with 1 ml of 50 mM Tris, 1.5 M NaCl, pH 7.4, and stored at 4°C until use. Under these conditions, the labeled aggregates maintained their heparan-binding activity for about 3 d and decayed gradually thereafter.

*Clearance Studies.* Mice were anesthetized with ether and injected with 500 μg of peptide in PBS, or with PBS alone via the periorbital sinus. 5 min after injection, they were injected via the opposite periorbital sinus with $10^5$ cpm of radiolabeled CS in PBS. The mice were killed 2 min after this last injection. They were exsanguinated and their organs were removed, rinsed in TBS, blotted dry, weighed, and counted in an LKB γ-counter.

## Results

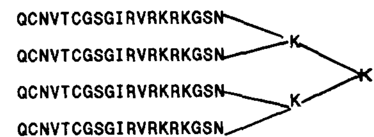
In a previous publication (1), we reported that a region II-plus peptide, containing a COOH-terminal extension of

**A**

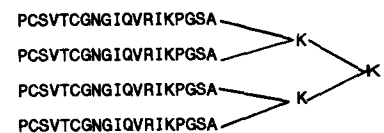
| Sequence | Peptide |
|---|---|
| PCSVTCGNGIQVRIKPGSAN | (Ia) |
| PCSVTCGNGIQVRIKPSAN | (Ib) |
| PCSVTCGNGIQVRIK | (II) |
| PCSVTCGNGIQVE*I*EPGSAN | (III) |
| PCSVTCGNGIQVE*IKPGSAN | (IV) |
| PCSVTCGNGIQVN*IN* | (V) |
| PCSVTCGNGS*QS*RIK | (VI) |
| CSVTCG | (VII) |
| GNGIQVRIKPGSAN | (VIII) |
| CGNGIQVRIKPGSAN | (IX) |
| QCNVTCGSGIRVRKRKGSNKKAEDL | (X) |

**B**

P. berghei region II plus




P. falciparum region II plus




P. falciparum repeat region

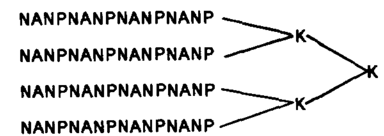



**Figure 2.** Linear and multiple antigen peptides (MAPs). (*A*) Peptide Ia was used in our original studies (1) and represents the *P. falciparum* region II-plus with a five amino acid extension at the COOH-terminal end. Peptides Ib–IX are truncated and modified versions of this peptide (asterisks indicate the changed residues). Peptide X is *P. berghei* region II-plus used in the sporozoite invasion assays. (*B*) A schematic representation of MAPs made from *P. berghei* and *P. falciparum* region II-plus and from *P. falciparum* central repeats.

five amino acids (peptide Ia, Fig. 2), inhibited the binding of recombinant *P. falciparum* CS to HepG2 cells. Here we used a series of substituted and truncated peptides (peptides Ib–IX, Fig. 2) to study the structural requirements for the interaction of region II-plus with hepatocytes. As shown in Fig. 3, removal of the COOH-terminal glycine (peptide Ib, Fig. 2) or of the five COOH-terminal amino acids from the original peptide (peptide II, Fig. 2), have no effect on the inhibitory activity of the peptide. We also made substitutions in the cluster of positively charged amino acids at the COOH-terminal end of the motif. A peptide in which only the arginine is replaced with glutamic acid (peptide IV, Fig. 2) is less active than the original peptide. When both lysine and arginine are replaced by either glutamic acid or asparagine (peptides III and V, Fig. 2), the peptides are inactive (Fig. 3). Adjoining the positively charged amino acids, all CS have two to three hydrophobic residues (Fig. 1), e.g., isoleucine and valine in *P. falciparum*. If these are replaced by serine (peptide VI, Fig. 2), most inhibitory activity is lost (Fig. 3). Despite the importance of the downstream basic/hydrophobic residues, when this portion of region II-plus (peptide VIII, Fig. 2) is separated from the cysteine-containing portion of the motif (peptide VII, Fig. 2), neither peptide has inhibitory activity (Fig. 3).

Previously it has been shown that if the two cysteines are replaced by alanines or chemically modified to prevent disulfide bond formation, the region II-plus peptide loses activity (1). To examine the possibility that inhibitory activity is associated with disulfide-linked aggregates, the region II-plus peptide (peptide Ib, Fig. 2) was subjected to molecular sieving chromatography through Sephadex G-50. The profile obtained is complex, indicating the presence of molecules of various sizes (Fig. 4). The Sephadex fractions also differ in their contents of free sulfhydryl groups as detected by the Ellman reaction. Fractions 24–30 from the low molecular weight peak are Ellman positive, whereas the fractions 14–23 from the first peak and from the preceding shoulder are negative. Analysis of the Sephadex fractions by mass spectrometry is shown in Fig. 5. Fraction 25 from the low molecular weight peak contains monomers almost exclusively (Fig. 5 *A*). Fraction 22 from the high molecular weight peak contains dimers and probably noncovalently associated monomers (Fig. 5 *B*). Fraction 17 from the shoulder is a mixed aggregate of covalently bound tetramers, trimers, and probably dimers and noncovalently associated monomers (Fig. 5 *C*). Because monomers and double-charged dimers have similar profiles by mass spectrometry (Fig. 5, *B* and *C*) we cannot determine the proportion of noncovalently associated monomers in the dimer and aggregate fractions (frx 22 and 17).

The various fractions were then assayed for activity. As shown in Fig. 4, only those fractions containing dimers and higher molecular weight aggregates inhibit binding of CS to HepG2 cells. The monomeric species is totally inactive. To examine the possibility that the lack of activity in the monomers is due to the presence of free sulfhydryls, we air-

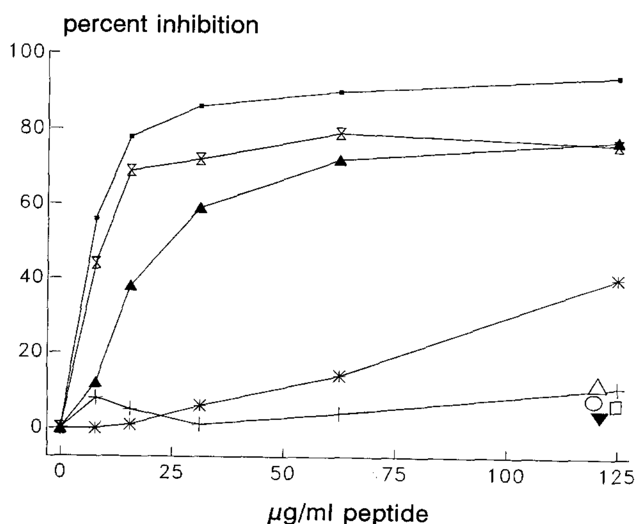



**Figure 3.** Peptide inhibition of CS binding to HepG2 cells. Paraformaldehyde-fixed HepG2 cells were preincubated with peptides at concentrations between 0 and 125 μg/ml, washed, and incubated with CS27IVC at 2.5 to 5 μg/ml. After washing, cells were incubated with iodinated mAb 2A10, washed, and bound antibody was counted in a γ-counter. Shown is percent inhibition of binding of CS27IVC to HepG2 cells in the presence of peptide compared with results obtained in the absence of peptide. Each peptide concentration was assayed in triplicate, and standard deviations were not greater than 5%. Changed amino acids are underlined: (⧗) PCSVTCGNGIQVRIKPSAN; (■) PCSVTCGNGIQVRIK; (▲) PCSVTCGNGIQVEIKPGSAN; (✳) PCSVTCGNGSQSRIK; (△) GNGIQVRIKPGSAN; (□) CSVTCTGNGIQVEIEPGSAN; (○) PCSVTCGNGIQVNIN; (+) CSVTCG; and (▼) CGNGIQVRIKPGSAN.

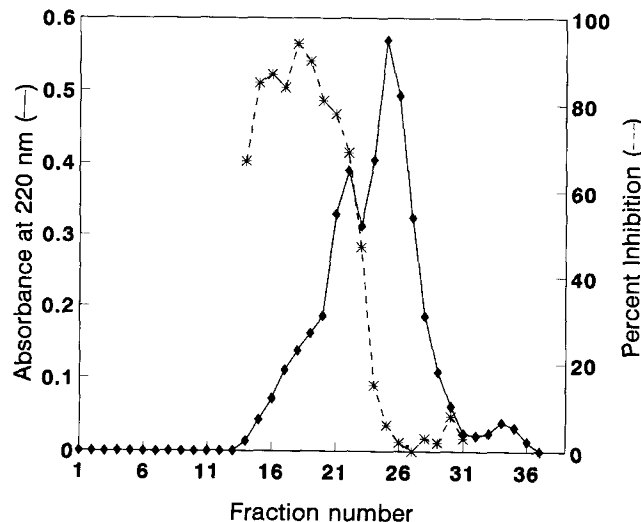



**Figure 4.** Size fractionation of region II-plus peptide with binding activity of different fractions. Crude region II-plus peptide (peptide Ib, Fig. 2) was run over a G-50 sizing column equilibrated in TBS. (*Solid line*) Absorbance at 220 nm of the fractions. Fractions 14-23 are Ellman negative and fractions 24-30 are Ellman positive. Each fraction was then tested for inhibition of binding of CS to HepG2 cells. Paraformaldehyde-fixed cells were incubated with the column fractions for 1 h, washed, and then incubated with CS27IVC at 2.5 μg/ml for 1 h, followed by iodinated mAb 2A10. (*Dotted line*) Percent inhibition of binding of CS to HepG2 cells. Each point was assayed in triplicate and standard deviations were within 5% of the mean. As shown, only the multimer containing fractions have inhibitory activity.

oxidized the crude peptide at pH 8.0 before subjecting it to size exclusion chromatography. The profile obtained was identical to the one shown in Fig. 4, except that none of the fractions were Ellman positive. The oxidized monomers from this experiment also lacked inhibitory activity (data not shown).

We have also analyzed the composition of two inactive peptides with modified COOH-terminal regions (peptides III and VI, Fig. 2). It could be argued that these peptides are not able to inhibit binding of CS to HepG2 cells (Fig. 3) because the substitutions rendered them incapable of forming aggregates rather than incapable of binding to HPSGs. As shown in Fig. 6, the chromatographic profiles of oxidized peptides PCSVTCGNGSQSRIK and PCSVTCGNGIQVEIEPGSAN are indistinguishable from the profile of the native region II-plus peptide (Fig. 3). Nevertheless, the fractions containing aggregates of the modified peptides had no inhibitory activity (data not shown).

In other experiments, we tested a peptide containing a single cysteine, CGNGIQVRIKPGSAN. This peptide can only form disulfide-linked dimers upon oxidation, and it is inactive (Fig. 3). In a previous publication (1) a longer peptide containing a single cysteine CGNGIQVRIKPGSANKPKDE had some inhibitory activity. The reason for this discrepancy is not known. However, the extra basic residues at the COOH terminus may increase the binding avidity of these longer dimers for the HSPGs of HepG2 cells.

Taken together, these experiments indicate that one of the functions of the region II-plus cysteines may be to assemble multimers of the downstream basic amino acids, thereby increasing the binding activity of CS for the negatively charged HSPG receptors on the hepatocyte surface. To obtain further support for this hypothesis, we synthesized region II-plus peptides in which the cysteines were blocked with acetamide groups and assembled them as the four arms of a MAP (Fig.

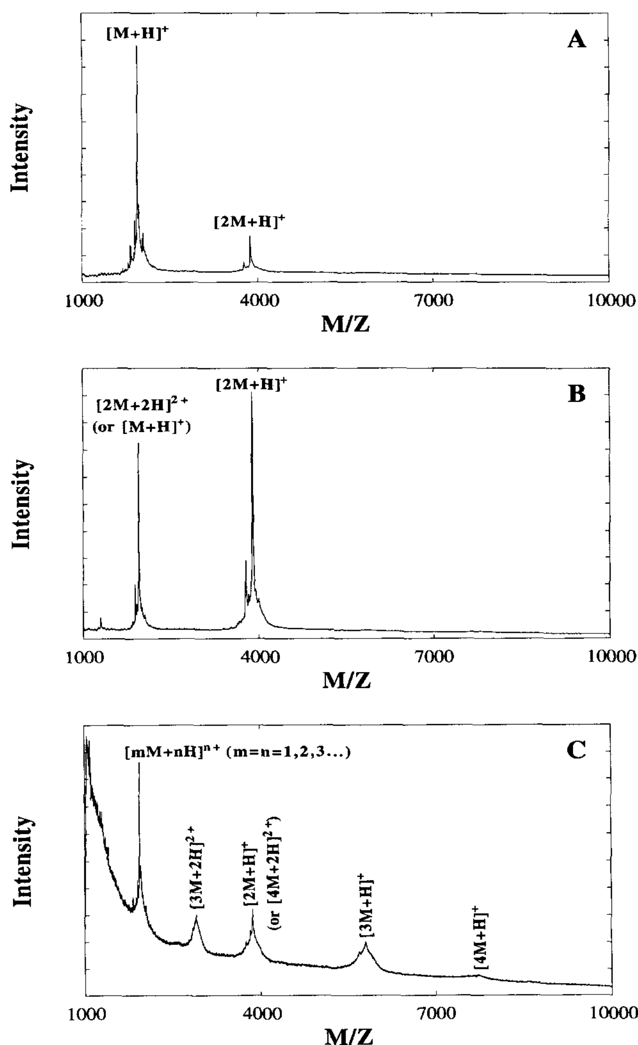



**Figure 5.** Mass spectrometry of region II-plus peptide fractions 17, 22, and 25. $[mM+nH]^{n+}$ designates the clusters of the intact molecule ($m=1$, monomer; $m=2$, dimer; $m=3$, trimer; $m=4$, tetramer) with $n$ protons attached to it. (*A*) The spectrum from fraction 25. (*B*) The spectrum from fraction 22. (*C*) The spectrum from fraction 17. As indicated, the first peak in *B* and *C* contains monomers and double-charged dimers which cannot be distinguished from one another by m/z. This is also the case for the dimer peak in *C* which may include double-charged tetramers. The exponential background, most evident in *C*, is due to fragmentation and/or clustering of matrix ions.

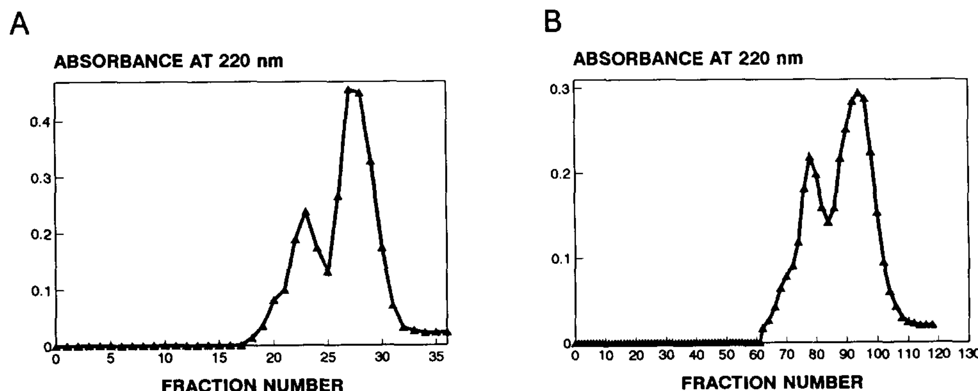



**Figure 6.** Size fractionation of region II-plus peptides with downstream substitutions. (*A*) 0.5 mg of peptide PCSVTCGNGIQVEIE PGSAN, with the changed residues underlined, was loaded onto a 30-ml Sephadex G-50 column and run with a linear flow rate of 5 cm/h. (*B*) 0.5 mg of peptide PCSVTC-GNGSQSRIK, with the changed residues underlined, was loaded onto a 60-ml Sephadex G-50 column and run with a linear flow rate of 5 cm/h. A larger column was necessary for adequate separation of the aggregates from the monomers of this smaller peptide. As shown, these profiles are virtually identical to that of the original peptide shown in Fig. 3.

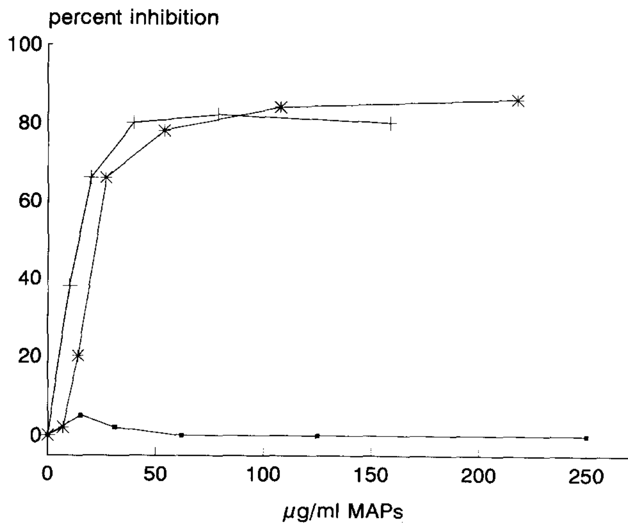



**Figure 7.** Region II-plus MAPs inhibition of CS protein binding to HepG2 cells. Paraformaldehyde-fixed HepG2 cells were incubated with the indicated concentrations of MAPs for 1 h, washed, and then incubated with 5 $\mu$g/ml of CS, followed by iodinated mAb 2A10. Shown is percent inhibition of binding of CS to HepG2 cells in the presence of MAPs compared with CS binding in the absence of MAPs. Each point represents the mean of triplicate samples. (+) *P. berghei* region II-plus MAPS; (✱) *P. falciparum* region II-plus MAPs; and (■) *P. falciparum* repeat-region MAPs.

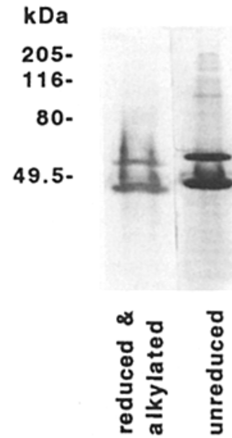
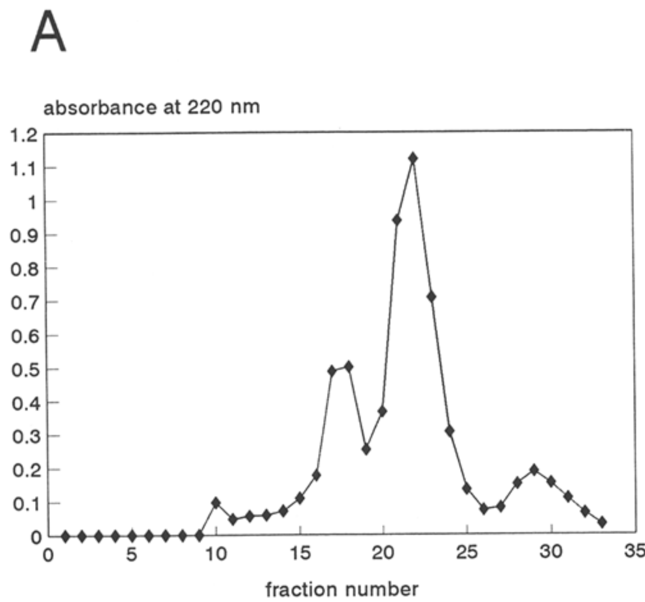



**Figure 8.** Western blot of native CS. (Lane *1*) $10^4$ freshly isolated sporozoites were reduced for 1 h in 4.8 M urea, 1 mM EDTA, 50 mM DTT, alkylated with 100 mM *N*-ethylmalemide, and then resuspended in sample buffer with 5% $\beta$-ME. (Lane *2*) An equal number of freshly isolated sporozoites were resuspended in nonreducing sample buffer. Both samples were subjected to SDS-PAGE, followed by Western blotting revealed with mAb 3D11. As shown, the native CS contains large aggregates as well as monomeric forms. These aggregates are no longer present after the parasites are reduced and alkylated. The two bands in the reduced and alkylated extract correspond to the membrane form of CS and the presumed intracellular precursor (27).

2). As shown in Fig. 7, MAPs with peptide arms representing region II-plus of *P. berghei* and *P. falciparum* inhibit binding of CS to HepG2 cells in a dose-dependent manner, whereas control MAPs with $(NANP)_4$ arms are inactive.

Having established that region II-plus peptide aggregation is required for binding to HepG2 cells, we examined the structure of native CS from *P. berghei*. Sporozoites were isolated from salivary glands of mosquitos, suspended in sample buffer, and immediately subjected to SDS-PAGE under nonreducing conditions. A Western blot of this gel (Fig. 8) shows the




B

| | PEPTIDE | PERCENT INHIBITION |
|---|---|---|
| Expt. 1 | Aggregates 19 µg/ml | 46,43,49 |
| | Monomers 108 µg/ml | 9,9,26 |
| Expt. 2 | Aggregates 37 µg/ml | 24,27,34 |
| | Monomers 37 µg/ml | 4,7,14 |
| Expt. 3 | Aggregates 200 µg/ml | 30,34,35 |
| | Monomers 200 µg/ml | 15,12,0 |
| Expt. 4 | RII+ MAPs 500 µg/ml | 61,61,66 |
| | RII+ MAPs 250 µg/ml | 46,46,47 |
| | Repeat MAPs 500 µg/ml | 19,18,21 |
| | Repeat MAPs 250 µg/ml | 9,10,23 |

**Figure 9.** Peptide aggregates and region II-plus MAPs inhibit *P. berghei* sporozoite invasion of HepG2 cells. (*A*) A typical fractionation of the *P. berghei* region II-plus peptide using TBS, 7.4, as the column buffer. Fractions 17 (aggregates) and 22 (monomers) were then used in the HepG2 invasion assay with sporozoites. (*B*) Results from three invasion assays using *P. berghei* region II-plus fractions. In Exp. 4 the *P. berghei* region II-plus MAPs is used with the repeat region MAPs as a control. HepG2 cells were plated at a density of $5 \times 10^5$/ml, and incubated for 2 h with *P. berghei* sporozoites in the presence of peptide aggregates or monomers. Cells were grown for 2 d, fixed, and stained with mAb 2E6, followed by goat anti-mouse Ig conjugated to horseradish peroxidase. The exoerythrocytic forms of the parasite in each well were then counted under a $\times 20$ objective. Three samples of each peptide fraction were assayed in each experiment. Percent inhibition was calculated with reference to controls in which sporozoites were allowed to invade in the presence of column buffer or medium alone.

presence of the 44-kD surface molecule and the 54-kD intracellular precursor (27) as well as aggregates of both molecules. After reduction and alkylation, the higher molecular weight aggregates are no longer present (Fig. 8).

Next, using a Sephadex G-50 column, we separated high and low molecular weight fractions from oxidized *P. berghei* region II-plus (peptide X, Fig. 2) and assayed them for the ability to inhibit sporozoite invasion of HepG2 cells. At the concentrations tested, only the high molecular weight fractions inhibited sporozoite invasion (Fig. 9). We also tested the inhibitory activity of the *P. berghei* region II-plus MAPs on sporozoite invasion *in vitro* and as shown in Fig. 9, they are also active.

We have previously shown that recombinant CS aggregates (but not monomers) are rapidly cleared from the bloodstream of mice and accumulate on hepatocytes (28). To examine the effect of MAPs on CS clearance, mice were injected intravenously with 500 $\mu$g of thee *P. berghei* region II-plus MAPs and 5 min later with $^{125}$I-labeled CS aggregates. Control mice were injected with $(NANP)_4$ MAPs or with buffer alone, followed by a second injection with the CS aggregates. All mice were killed 2 min after CS injection, and their blood and organs were harvested and counted in a $\gamma$-counter. As seen in Fig. 10, the *P. berghei* region II-plus MAPs inhibited about 50% of the CS protein homing to the liver. Most of the CS that had not been cleared by the liver was still circulating in the blood. In other experiments, the same procedure was repeated except that the mice were killed after 15 min. At this time, there was no signficant difference between the results from the experimental and control mice (data not shown).

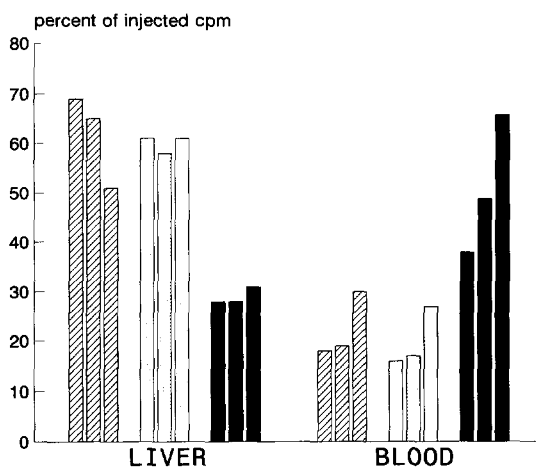



**Figure 10.** Peptide inhibition of CS homing to the liver. Mice were injected with either 0.5 mg of MAPs in PBS or with the same volume of PBS alone. 5 min later they were injected with 100,000 cpm of CS protein, and then killed 2 min after this second injection. At this time the mice were exsanguinated, their organs were harvested and washed, and radioactivity in each organ and in the blood was measured. Shown is the percent injected counts per minute found in the liver and blood of nine mice. Three mice preinjected with PBS alone (*slanted line bars*), three mice preinjected with repeat-region MAPs (*dotted bars*), and three mice preinjected with region II-plus MAPs (*solid bars*). The mice preinjected with the region II-plus MAPs have 50% fewer counts in their livers; most of the remaining counts are found in the blood.

## Discussion

Here we analyze some of the structural features of region II-plus of the malarial CS required for recognition of the HSPG receptors on the hepatocyte membrane. One of our main conclusions is that this interaction is dependent upon the cluster of positively charged amino acids and the adjoining hydrophobic residues found at the COOH-terminal of region II-plus. Since the hepatocyte receptors are HSPGs, it is likely that the positively charged amino acids form ionic bonds with the sulfated-glycosaminoglycan (GAG) chains of the receptor. In addition, the protein–carbohydrate interaction may be stabilized by interactions between side chains of the adjoining hydrophobic amino acids with nonpolar groups of the saccharides (29).

The present and previous findings (1, 28) also highlight the importance of aggregation of CS and of region II-plus for binding activity to hepatocytes in vitro and in vivo. The experiments depicted in Fig. 4 show that the inhibitory activity of the region II-plus peptides increases with the degree of aggregation. Mass spectrometry shows that the early fractions contain covalently linked tetramers, trimers, and dimers as well as noncovalently associated monomers. Although we do not know the precise molar proportions of the species in the aggregate fractions, if we assume that the average aggregate size in fraction 16 is tetramers and in fraction 19 is trimers (Fig. 4), then 50% inhibition of CS binding is achieved with an ~50-nM concentration of the tetramer and an ~500-nM of the trimer. In other words, the addition of a single monomer to a region II-plus aggregate leads to a 10-fold increase in binding avidity for the HSPG receptors. If this reasoning is correct, it would explain the remarkable speed and efficiency of sporozoite infectivity since the parasite surface coat contains a very high concentration of the region II-plus ligand.

We also provide evidence that native CS, in extracts of *P. berghi* sporozoites, contains disulfide-linked aggregates. This finding is in apparent contradiction to previous reports (for a review see reference 30). Most likely, these aggregates were not detected before because they are present at much lower concentrations than the CS monomers. Native CS contains two pairs of cysteines, one within region II-plus and the other further downstream, close to the COOH terminus. However, the position of the intra- and interchain disulfide bonds is not known. Nevertheless, the tightly woven coat created by these disulfide-linked aggregates on the parasite surface is continuously released by the parasite (31, 32) and can be seen as a morphologically distinct sheath when sporozoites are treated with antibodies to the repeat-containing domain of CS (circumsporozoite reaction). As suggested elsewhere (33), the release of the CS sheath may be a necessary event during hepatocyte invasion. The high binding avidity between the surface coat of the parasite and the hepatocyte receptors would require the parasite to leave its CS coat behind, attached to the HSPGs, in order to enter the hepatocyte.

The high copy number of the region II-plus ligand on the surface of the parasite and the large number of receptor sites in the liver (and perhaps also on HepG2 cells), make it difficult to compete for the HSPG receptors with region II-plus peptides or MAPs. In our clearance studies with CS, we inhibited its homing to hepatocytes by 50% in mice pretreated with $\sim$10 $\mu$M region II-plus MAPs, but only when the animals were killed 2 min after CS injection. This inhibitory effect disappeared if the mice were killed 15 min after CS injection. In addition, sporozoite invasion in vitro could be inhibited only 30–50% with synthetic peptides. Better region II-plus mimics have to be developed in order to effectively prevent the attachment of the Velcro-like surface structure of the parasite to the liver HSPGs.

Since the region II-plus motif of CS is found in several mammalian and nematode proteins (Fig. 1), the identification of amino acid residues that are critical for binding to HSPGs may be of relevance outside the malaria field. In addition, the requirement for multimerization that we have demonstrated for CS may be important for these other molecules, as most of these proteins have multiple copies of the conserved motif. Thrombospondin is made of three identical polypeptide chains, and each chain has three copies of region II-plus (34). UNC-5 has two copies of the motif per polypeptide chain (8). F-spondin (7) and properdin (5) have six copies in close proximity to each other, and in addition, properdin is aggregated in serum. Proteins of the terminal complement cascade C6-9 contain two to three copies of the motif (35), and these proteins assemble during formation of the membrane attack complex. Although the receptors for the motif in the non-CS proteins have yet to be identified, properdin, thrombospondin, and TRAP bind to sulfated glycoconjugates (36–38). Most likely the presence of several copies of the region II-plus motif increases the binding avidity of these proteins for their receptors, which may include cell surface and/or extracellular matrix proteoglycans.

It seems unlikely that only the cysteines are functionally relevant in the stretch of highly conserved, mostly hydrophobic amino acids EWSPCSVTCG at the $NH_2$-terminus of region II-plus. Others have shown that the CSVTCG sequence mediates cell adhesion in vitro (14–16), platelet aggregation in vitro, and melanoma metastasis in mice (12). In addition, the oxidation state of the cysteines is not important in these studies since the cysteines can be replaced by alanines without a loss of function.

Perhaps the $NH_2$-terminal portion of region II-plus, containing mostly hydrophobic amino acids, is recognized during sporozoite invasion by a different, yet to be identified, hepatocyte receptor. Alternatively, the side chains of these amino acids may also serve to align the cationic side chains of the downstream lysine and arginine residues on the same face of the CS for interaction with the anionic HSPGs. The importance of hydrophobic residues in the formation of structures in which the basic residues from different monomers are aligned with one another has been demonstrated for other heparin-binding proteins whose crystal stuctures have been resolved or inferred. For example, bovine platelet factor IV is a tetramer of identical dimers, and the lysines involved in binding heparin are found on $\alpha$-helices of each monomer. The $\alpha$-helix of one monomer is aligned with that of another monomer when a dimer is formed, and the dimer is stabilized by a cluster of hydrophobic residues near the helix (39). Thrombin is another heparin-binding molecule whose three-dimensional structure reveals two continuous positively charged grooves separated by a central wall that is also made up of basic residues (40). Lipoprotein lipase, which also binds to heparin, is thought to be active only as a dimer, and the segments involved in dimer formation appear to form a pocket flanked by basic residues that is capable of accommodating the heparin GAG chain (41). It is not known if the aggregated CS molecules on the parasite surface also contain binding pockets with defined three-dimensional structures for binding specific sulfated oligosaccharides from hepatocyte HSPGs. Additional work aimed at defining the structure of the CS ligand and the hepatocyte GAG chain receptors should facilitate the generation of more potent inhibitors of sporozoite invasion.

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