

# The Binding of the Circumsporozoite Protein to Cell Surface Heparan Sulfate Proteoglycans Is Required for *Plasmodium* Sporozoite Attachment to Target Cells\*

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**The major surface protein of malaria sporozoites, the circumsporozoite protein, binds to heparan sulfate proteoglycans on the surface of hepatocytes. It has been proposed that this binding event is responsible for the rapid and specific localization of sporozoites to the liver after their injection into the skin by an infected anopheline mosquito. Previous *in vitro* studies performed under static conditions have failed to demonstrate a significant role for heparan sulfate proteoglycans during sporozoite invasion of cells. We performed sporozoite attachment and invasion assays under more dynamic conditions and found a dramatic decrease in sporozoite attachment to cells in the presence of heparin. In contrast to its effect on attachment, heparin does not appear to have an effect on sporozoite invasion of cells. When substituted heparins were used as competitive inhibitors of sporozoite attachment, we found that sulfation of the glycosaminoglycan chains at both the N- and O-positions was important for sporozoite adhesion to cells. We conclude that the binding of the circumsporozoite protein to hepatic heparan sulfate proteoglycans is likely to function during sporozoite attachment in the liver and that this adhesion event depends on the sulfated glycosaminoglycan chains of the proteoglycans.**

Protozoans of the genus *Plasmodium* are the causative agents of malaria. Malaria infection is initiated when an infected anopheline mosquito injects sporozoites during a blood meal. The injected sporozoites travel to the liver and invade hepatocytes where they develop into exoerythrocytic forms. The speed and specificity of sporozoite localization to hepatocytes suggest a receptor-mediated event. Previous studies have shown that the major sporozoite surface protein, the circumsporozoite protein (CS),<sup>1</sup> binds to heparan sulfate proteogly-

cans (HSPGs) on the hepatocyte surface and in the space of Disse (reviewed in Ref. 1). Despite the wealth of *in vitro* and *in vivo* data demonstrating CS binding to HSPGs, the function of this binding event in the life of the sporozoite remains unknown.

*In vivo* experiments with recombinant CS have shown that intravenously injected protein is rapidly cleared from the circulation by HSPGs of hepatocytes (2, 3). These results suggest that CS may mediate the rapid clearance of the sporozoites by hepatocytes. *In vivo* experiments with sporozoites that could prove this point, however, have been difficult to perform. To date, remnant lipoproteins (ligands for hepatic HSPGs) and sulfated glycoconjugates such as fucoidan and dextran sulfate have been shown to decrease sporozoite infectivity *in vivo* (3, 4). However, the inhibitory effect on sporozoite infectivity, while demonstrating that the CS-HSPG interaction is important, does not indicate if the glycan is required for sporozoite attachment, invasion, or subsequent development in hepatocytes.

*In vitro* assays (5, 6) have been used to determine whether the CS-HSPG interaction is critical for cell invasion. Frevert *et al.* (6) found that removal of the majority of cell surface HSPGs had a minimal inhibitory effect on sporozoite invasion of cells. One interpretation of these data is that the binding of CS to HSPGs does not function during sporozoite invasion. Another possibility, however, is that CS binding to HSPGs functions in the more dynamic conditions found in the blood circulation and leads to arrest of sporozoites in the liver sinusoids. In this paper we modify the standard sporozoite invasion assay and provide evidence that the interaction between CS and cell surface HSPGs functions during the initial attachment of sporozoites to cells under conditions that mimic flow. In addition, we show that the sulfate moieties of the HSPG glycosaminoglycan chains (GAGs) are important for attachment of sporozoites to cells.

## MATERIALS AND METHODS

**Sporozoites and Cell Lines**—*Plasmodium berghei* and *Plasmodium yoelii*, two species of rodent malaria, were maintained in the laboratory using *Anopheles stephensi* mosquitoes and mice (7). Sporozoites were obtained from salivary glands of infected mosquitoes on the day of the experiment. All invasion assays were performed with *P. berghei* that invades cells *in vitro* with higher efficiency than *P. yoelii*. Both *P. berghei* and *P. yoelii* were used in attachment assays as indicated in the figure legends. We used HepG2 cells (ATCC HB8065, American Type Culture Collection, Manassas, VA), a hepatoma cell line permissive for *P. berghei* sporozoite development *in vitro*, for all CS protein binding assays and for sporozoite invasion and attachment assays. Cells were maintained as described previously (3).

serum; TBS, Tris-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.

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<sup>1</sup> The abbreviations used are: CS, circumsporozoite protein; HSPGs, heparan sulfate proteoglycans; GAGs, glycosaminoglycan chains; TRAP, thrombospondin-related anonymous protein; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf

**Antibodies and Recombinant Protein**—Monoclonal antibodies (mAb) used were directed against the repetitive region of the respective CS protein as follows: mAb 3D11, *P. berghei* CS (8); mAb NYS1, *P. yoelii* CS ((9) kindly provided by Dr. Yupin Charoenvit, Naval Medical Research Center, Bethesda, MD); and mAb 2A10, *Plasmodium falciparum* CS (10). The *Escherichia coli*-derived CS27IVC represents the complete *P. falciparum* CS sequence from the T4 isolate, except that the hydrophobic NH<sub>2</sub>- and COOH-terminal amino acids 1–26 and 412–424 have been deleted, and 5 histidine residues have been added to the COOH terminus to facilitate purification (11). The recombinant protein used in these studies was kindly provided by Dr. Bela Takacs (Hoffmann-La Roche).

**Modified Heparins**—Intact and modified forms of hog mucosal heparin were obtained from Glycomed, Inc. (Alameda, CA). *N*-Desulfation of heparin was achieved by mild solvolysis (12, 13); 2-*O*,3-*O*-desulfated heparin was prepared according to the method of Jaseja *et al.* (14); and carboxy-reduced heparin was made by borohydride reduction in the presence of carbodiimide (15).

**Sporozoite Attachment Assay**—HepG2 cells ( $3 \times 10^5$  cells/ml) were plated (0.4 ml/well) in labtek chamber slides (model 177445, Nalgene Nunc Corp., Naperville, IL) and allowed to grow until subconfluent (36–48 h). On the day of the experiment, the medium was removed, and an equal number of sporozoites in Dulbecco's modified Eagle's medium with 10% fetal calf serum (DMEM/FCS) were added to each well. Because sporozoites are obtained from mosquito salivary glands and the efficiency of salivary gland infection varies on a weekly basis, different numbers of sporozoites were used for each experiment. The number of sporozoites added per well varied between 20,000 and 80,000, although within a given experiment identical numbers of sporozoites were added to each well. The slides were incubated at 37 °C under static or rotating conditions. Rotating conditions were created by taping the labtek chamber slide to a clinical rotator (Fisher) set at 200 rpm. After 1 h, unattached sporozoites were removed, and the slides were washed twice with Tris-buffered saline (TBS: 130 mM NaCl, 50 mM Tris, pH 7.4) and fixed with 4% paraformaldehyde. The cells were then permeabilized with cold methanol for 10 min, washed twice with TBS, and blocked for 1 h with TBS containing 1% bovine serum albumin (TBS/BSA). Sporozoites were visualized with the appropriate monoclonal antibody (10 µg/ml in TBS/BSA) and goat anti-mouse immunoglobulin conjugated to FITC (Amersham Pharmacia Biotech). Slides were mounted in Citifluor mounting medium (Ted Pella, Redding, CA) and counted on a fluorescence microscope using a 40× objective lens and 15× eyepieces. Each point was plated in triplicate wells, and 50–60 fields per well were counted. When heparin was used as an inhibitor, sporozoites were preincubated in DMEM/FCS ± heparin for 15 min on ice and then plated on cells in the presence of heparin. For experiments with chlorate-treated cells, HepG2 cells were plated in DMEM/FCS, and 48 h later the medium was changed to low sulfate medium (see below) with the indicated concentrations of chlorate. In wells with 20 mM chlorate and 20 mM chlorate + 20 mM magnesium sulfate, an appropriate amount of medium was replaced with water in order to maintain normal osmolarity. After 24 h, the chlorate-containing medium was removed; sporozoites in DMEM/FCS were added, and the assay was carried out as outlined above.

**Flow Chamber Assay**—Sporozoite attachment under laminar flow was studied using a parallel plate flow chamber. HepG2 cells were cultured until confluent on tissue culture-treated Petri dishes, and a customized flow chamber was assembled on the well of the dish forming a 3.18-mm wide and 25-µm high flow channel on the cell monolayer. The wall shear stress was calculated as a function of flow rate. Medium (Hanks' balanced salt solution with 2% BSA and 10 mM Hepes, pH 7.4) ± sporozoites was drawn through the chamber at controlled flow rates with a syringe pump (model 22; Harvard Apparatus, Holliston, MA) attached to the outlet. The assay consisted of four steps: 1) 5-min prerinse with medium at 0.4 ml/min; 2) infusion of  $5 \times 10^5$  sporozoites (in 750 µl) into the inlet tubing (0.4 ml/min) followed by sporozoite perfusion of the cells at the indicated flow rate (the time of the perfusion was inversely proportional to the flow rate so that for each point an equal number of sporozoites was allowed to perfuse over the cell monolayer); 3) 6-min rinse with medium at 0.05 ml/min to remove nonadherent sporozoites; and 4) a variable period of stasis so that for each point the sporozoites had a total of 20 min of cell contact before fixation. After each assay the flow chamber was removed, and the cells were fixed with 4% paraformaldehyde, and sporozoites were stained as outlined above. The flow chamber, cells, and medium were kept on a slide warmer at 37 °C; and the room in which the assay was performed was

maintained at 30 °C since sporozoites do not attach to cells at temperatures below 25 °C.<sup>2</sup>

**Sporozoite Invasion Assay**—This assay is identical to the sporozoite attachment assay except that cells were stained with a double-staining procedure that allows differentiation of intracellular and extracellular sporozoites (5, 6). Briefly, cells were fixed with 4% paraformaldehyde and stained with mAb 3D11 (directed against the repeat region of *P. berghei* CS) followed by goat anti-mouse Ig conjugated to colloidal gold 10 nm (Amersham Pharmacia Biotech). Cells were then permeabilized with methanol and stained again with mAb 3D11 followed by goat anti-mouse Ig conjugated to FITC. The colloidal gold label was revealed with a silver enhancement kit (Amersham Pharmacia Biotech). Each field was counted under simultaneous fluorescence and low-light bright field microscopy. All sporozoites were FITC-positive, whereas only extracellular sporozoites appeared black. The percentage of sporozoites that invaded the cells was calculated using the following equation:

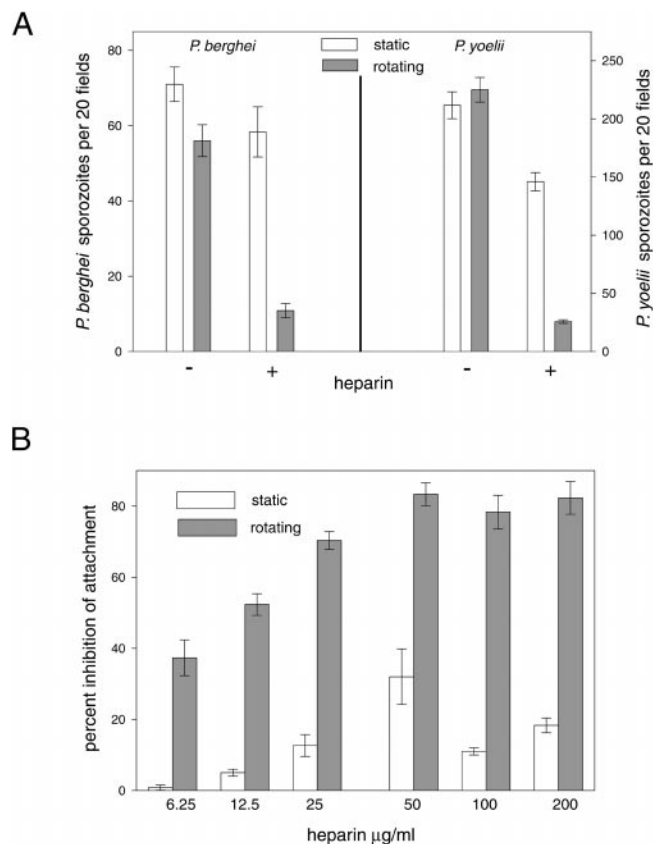
$$\frac{\text{total parasites} - \text{extracellular parasites}}{\text{total parasites}} \times 100 = \% \text{ invasion}$$

For the experiments with cytochalasin D (Sigma), the sporozoites were preincubated in DMEM/FCS ± 1 µM cytochalasin (± heparin) for 15 min at room temperature and then plated on cells in the presence of cytochalasin. For the cytochalasin recovery experiments, sporozoites were preincubated in DMEM/FCS with 1 µM cytochalasin and added to cells in the presence of cytochalasin for 30 min at 37 °C. Following this, the medium was removed and replaced with DMEM/FCS, without cytochalasin, containing different concentrations of heparin. Controls included sporozoites treated exactly as outlined above except that all incubations (preincubation, incubation with cells with initial medium, and incubation of cells with replacement medium) were performed in either (a) DMEM/FCS with no cytochalasin or heparin or (b) DMEM/FCS with cytochalasin but no heparin. After 1 h at 37 °C, all cells were fixed and stained with the double-staining procedure outlined above.

**Chlorate Treatment of HepG2 Cells**— $2 \times 10^5$  cells/well were plated into 6-well plates (Corning Glass) and grown for 12 h in DMEM/FCS with various concentrations of chlorate (sodium salt; Aldrich). In wells with 10 and 20 mM chlorate, an appropriate amount of medium was replaced with water in order to maintain normal osmolarity. After 12 h, cells were washed twice with phosphate-buffered saline and resuspended in 2 ml/well of low sulfate medium (Ham's F-12 (Life Technologies, Inc.), 1 mM glutamine, and 2% FCS that had been dialyzed extensively versus 150 mM NaCl, 10 mM Hepes, pH 7.3) with the indicated amount of chlorate and 100 µCi of carrier-free Na<sub>2</sub><sup>35</sup>SO<sub>4</sub> (Amersham Pharmacia Biotech). After 12 h, the cells were transferred to 4 °C, washed twice with cold phosphate-buffered saline, and then incubated with lysis buffer (130 mM NaCl, 50 mM Tris-HCl, pH 7.0, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml each of pepstatin and leupeptin) for 5 min. The lysate was transferred to Eppendorf tubes; the wells were washed once, and the wash was added to the lysate that was spun at 16,000 × *g* for 30 min at 4 °C. Both pellet and supernatant were counted in a Beckman LS 7500 scintillation counter. Over 95% of the counts/min were found in the supernatant. Protein concentrations were measured using the BCA protein assay (Pierce) using BSA as a standard. Lysates from chlorate-treated cells were loaded onto 5% polyacrylamide slab gels. Equivalent amounts of protein were loaded onto each lane. The gels were fixed with 10% glacial acetic acid and 30% methanol, impregnated for 30 min in 1 M salicylic acid, dried, and exposed to Kodak X-Omat AR film at –70 °C.

**HepG2 Cell Binding Assay**— $10^5$  cells per well were plated in 96-well plates (Removawell tissue culture plates, Dynatech Laboratories, Chantilly, VA) and allowed to grow for 18 h. In the experiments investigating CS binding to chlorate-treated cells, the cells were grown in low sulfate medium with the indicated concentrations of chlorate. In the experiments with the substituted heparins, the cells were grown in DMEM/FCS. The cells were then fixed with 4% paraformaldehyde for 10 min, rinsed three times with TBS, and blocked with TBS/BSA for 1 h at 37 °C. In the binding experiments with chlorate-treated cells, varying concentrations of CS in TBS/BSA were added to the cells for 1 h at 37 °C. After washing, cells were incubated with mAb 2A10 (10 µg/ml) followed by anti-mouse Ig conjugated to horseradish peroxidase (1:5000, Amersham Pharmacia Biotech). Bound enzyme was revealed by the addition of substrate (2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonate, Kirkegaard & Perry Laboratories, Gaithersburg, MD) following the manufacturer's instructions. After 1 h, absorbance at 405 nm was read

<sup>2</sup> P. Sinnis, unpublished data.

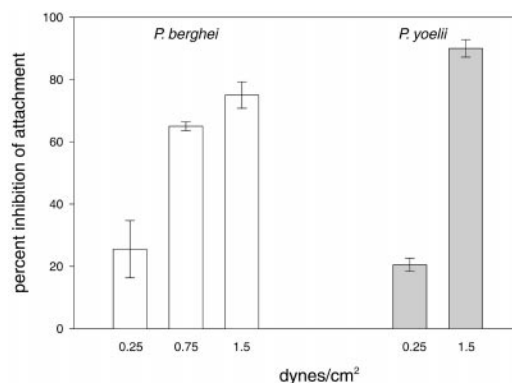


**FIG. 1. Heparin is a better inhibitor of sporozoite attachment under conditions that mimic flow.** Sporozoites were preincubated  $\pm$  25  $\mu\text{g/ml}$  of heparin (A) or the indicated concentration of heparin (B) for 15 min on ice and then plated on cells in the continued presence of heparin. After 1 h at 37 °C under static or rotating conditions, unattached sporozoites were removed by washing, and the attached sporozoites were visualized with the appropriate monoclonal antibody (mAb 3D11 for *P. berghei* and mAb NYS1 for *P. yoelii*) and goat anti-mouse Ig conjugated to FITC. Each point was plated in triplicate and shown are the means with standard deviations. A, attachment of *P. berghei* and *P. yoelii* sporozoites to cells under static and rotating conditions. B, dose-dependent inhibition of *P. berghei* sporozoite attachment. Percent inhibition was calculated using the mean number of sporozoites attached in the absence of heparin under static or rotating conditions.

in an enzyme-linked immunosorbent assay plate reader. In the experiments using the substituted heparins as inhibitors of CS binding, 5  $\mu\text{g/ml}$  CS was preincubated with the indicated concentrations of heparin for 30 min at 37 °C. These solutions were then added to the cells for 1 h at 37 °C; the cells were washed, and 200,000 cpm/well of iodinated mAb 2A10 was added for 30 min at 37 °C. The plates were washed 3 times, and wells were counted in a gamma counter.

## RESULTS

**Heparin Is a Better Inhibitor of Sporozoite Attachment to Cells Under Conditions That Mimic Flow**—Previous studies have failed to demonstrate a significant role for HSPGs during sporozoite invasion of cells (6). Since sporozoites are in the blood circulation when they contact HSPGs in the liver, we reasoned that heparin might be a better inhibitor of sporozoite invasion of cells under conditions that mimic flow. We created shear force between sporozoites in liquid medium and immobilized target cells by placing the experimental chamber on a rotator. We then compared sporozoite attachment to cells in the presence and absence of heparin, under static and rotating conditions. As shown in Fig. 1A, heparin is a much more potent inhibitor of sporozoite attachment under rotating conditions compared with static conditions. Inhibition of attachment under rotating conditions is dose-dependent and reaches a maximum of 85% with 25–50  $\mu\text{g/ml}$  heparin (Fig. 1B). Under static



**FIG. 2. Heparin inhibition of sporozoite attachment to HepG2 cells in a parallel plate flow chamber.** Sporozoites were preincubated  $\pm$  25  $\mu\text{g/ml}$  of heparin for 15 min on ice, infused into the inlet tubing of the flow chamber, and then perfused over the cells at controlled flow rates. The wall shear stress was calculated as a function of flow rate. The perfusion time was inversely proportional to the flow rate so that for each point an equal number of sporozoites were allowed to perfuse over the cell monolayer. Nonadherent sporozoites were washed off with medium infused at 0.05 ml/min (resulting in a shear force of 0.25 dynes/cm<sup>2</sup>) for 6 min. The cells were fixed, and sporozoites were stained as outlined previously. For each point, the entire area covered by the flow chamber was divided into 2, and  $\geq$ 100 fields per region were counted. Percent inhibition was calculated using the mean number of sporozoites attached in the absence of heparin with the same flow rate.

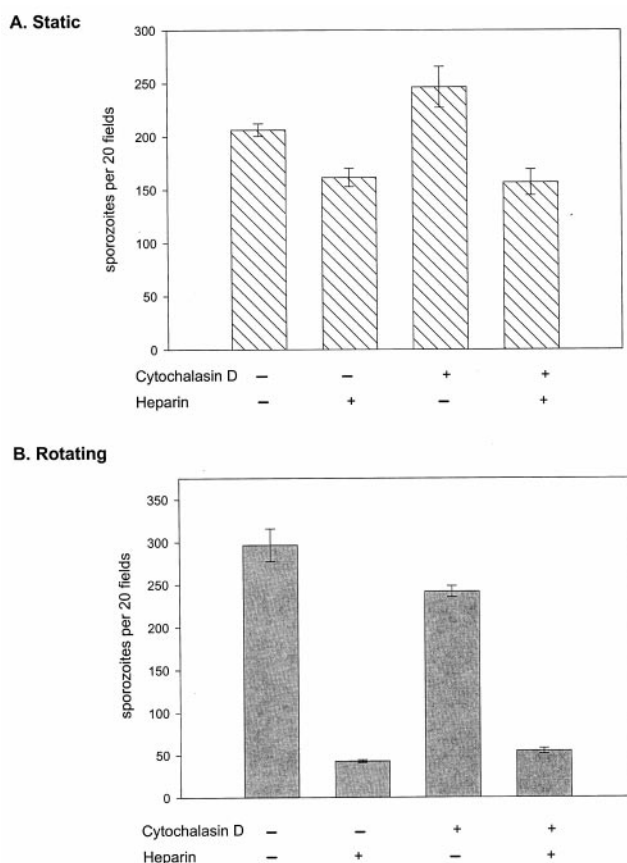
conditions, increasing the concentration of heparin does not increase its inhibitory activity beyond 15–25% (Fig. 1B). Importantly, sporozoite attachment to cells is not significantly altered under rotating conditions in the absence of inhibitor (Fig. 1A).

When the cells with the sporozoites are rotated, the medium moves with respect to the cells that are adhering to the bottom of the wells, and a shear force is generated. In a rotating system, however, the shear force is not readily measurable. To circumvent this problem, we performed a sporozoite attachment assay in a parallel plate flow chamber. As shown in Fig. 2, at physiologic shear forces (0.75–2 dynes/cm<sup>2</sup>) heparin is a better inhibitor of sporozoite attachment compared with a very low shear force of 0.25 dynes/cm<sup>2</sup>.<sup>3</sup> Thus, similar results were obtained in both systems.

In the experiments shown in Figs. 1 and 2, we did not distinguish between intracellular and extracellular parasites because the cells were permeabilized before the sporozoites were stained. Since sporozoites must attach to cells before entry, we reasoned that attachment occurs by the same mechanism regardless of whether sporozoites have entered the cells. In order to confirm this, we performed the assay in the presence of cytochalasin D, an inhibitor of sporozoite invasion but not attachment (16, 17). By using a double-staining procedure that enables us to distinguish intracellular from extracellular sporozoites, we found no intracellular sporozoites in the presence of cytochalasin, whereas without cytochalasin the invasion rate was  $\sim$ 40% (data not shown). As shown in Fig. 3, cytochalasin-treated sporozoites attached with approximately the same frequency as untreated sporozoites. In addition, the inhibitory effect of heparin on sporozoite attachment was more dramatic under rotating conditions, regardless of whether cytochalasin was present in the medium. It appears, therefore, that sporozoite attachment to cells is a separate, distinguishable phase of cell invasion and that heparin acts on the attachment phase of sporozoite entry into cells.

<sup>3</sup> We used a very low shear force instead of static conditions because it was technically not feasible to perform a static assay in our flow chamber.





**FIG. 3. Sporozoite attachment to HepG2 cells in the presence of heparin and cytochalasin D.** *P. berghei* sporozoites were preincubated with or without 1  $\mu$ M cytochalasin D  $\pm$  25  $\mu$ g/ml heparin and then plated on cells (in the continued presence of these compounds). After 1 h at 37  $^{\circ}$ C under static (A) or rotating (B) conditions, unattached sporozoites were removed, and the cells were stained with a double-staining procedure that allows the differentiation of intracellular from extracellular sporozoites. Each point was plated in triplicate and shown are the means with standard deviations of the total number of sporozoites attached to the cells. Invasion data for this experiment is not shown; however, in the presence of cytochalasin D there was no invasion, and in the absence of cytochalasin D,  $\sim$ 40% of attached sporozoites were found intracellularly regardless of whether heparin was present or not.

**The CS-HSPG Interaction Functions during Sporozoite Attachment to Cells**—We then went on to determine the effect of heparin on sporozoite invasion of HepG2 cells under both rotating and static conditions. Results from three experiments indicate that heparin does not have a significant effect on sporozoite invasion of cells (Fig. 4A). These experiments were performed using a double-staining procedure that distinguishes between intracellular and extracellular sporozoites, and invasion efficiency is expressed as a percentage of total sporozoites bound. Since, as one would expect, the total number of sporozoites bound to cells in the presence of heparin under rotating conditions is low, the absolute number of intracellular sporozoites in these wells is also low. However, the percentage of total sporozoites that are found intracellularly is the same as in the other groups, suggesting that once the sporozoite has attached to the cell, heparin does not affect its ability to enter.

In order to confirm our findings that heparin does not inhibit sporozoite invasion of cells, we used cytochalasin D to separate attachment from invasion of cells. Sporozoites were allowed to attach to cells in the presence of cytochalasin and then the drug was removed. We have found that sporozoites incubated with cytochalasin can, upon its removal, recover from its effects and invade cells (Fig. 4C). Recovery appears to be a stochastic process that begins immediately upon cytochalasin removal

and reaches a maximum after  $\sim$ 30 min. However, only 30–50% of the sporozoites recover and are able to invade cells (Fig. 4C). This is likely because incubation of sporozoites with cytochalasin is performed at 37  $^{\circ}$ C, and it has been shown that sporozoites lose between 60 and 100% of their infectivity after 1–2 h at 37  $^{\circ}$ C (18).

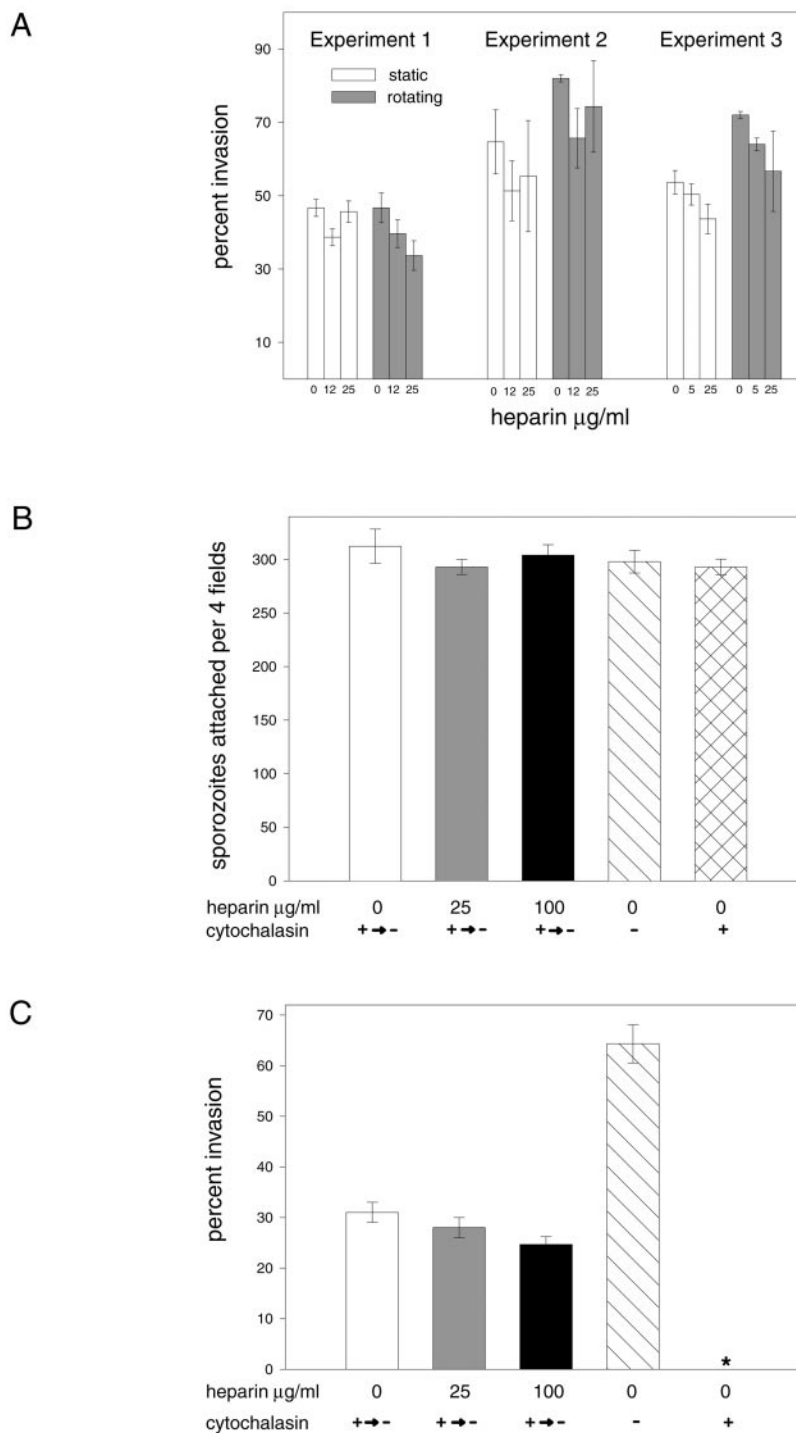
We performed the cytochalasin recovery experiment under static conditions because our previous data indicated that rotation had no effect on invasion efficiency (Fig. 4A). As shown in Fig. 4C, when heparin was added to the medium after sporozoite attachment to cells, it did not significantly inhibit sporozoite invasion. As expected, sporozoite attachment was the same in all groups because cytochalasin does not inhibit sporozoites from attaching to cells, and heparin was added after sporozoite attachment had occurred (Fig. 4B). When sporozoites were added to the cells in medium without cytochalasin, the invasion rate was 2–3-fold higher compared with sporozoites that were initially incubated with the cells in the presence of cytochalasin (Fig. 4C). As stated above, this is likely due to a loss of sporozoite infectivity during the time in which the sporozoites were allowed to attach to but not invade the cells.

**The Sulfate Moieties of HSPGs Are Critical for CS Binding to HSPGs**—Previous studies have shown that CS binds to the glycosaminoglycan chains (GAGs) and not the protein core of HSPGs (19). In addition, CS binds to regions of the GAGs that are more highly sulfated (20, 21). In order to determine more precisely if CS binds to the sulfated domains of the GAGs, we performed CS binding assays with HepG2 cells in which the sulfation of the HSPGs was decreased by treating cells with sodium chlorate. Chlorate inhibits ATP-sulfurylase, the first enzyme in the synthesis of the high energy donor of sulfate, phosphoadenosine phosphosulfate (PAPs). Previous studies have shown that chlorate inhibits sulfation of cellular proteins and carbohydrates without affecting cell growth or protein synthesis (22–24). Here we show that the effect of chlorate on HepG2 cells is similar to what has been reported previously for other cell lines. As shown in Fig. 5, chlorate causes a dose-dependent decrease in [ $^{35}$ S]sulfate incorporation into cellular macromolecules of HepG2 cells. Although many proteins and carbohydrates can be sulfated, previous studies have shown that most free sulfate is incorporated into proteoglycans (25). This is likely also true for HepG2 cells since we found that the sulfate-labeled material ran as a high molecular weight smear on an SDS-polyacrylamide gel (*inset*, Fig. 5), consistent with its being predominantly composed of proteoglycans. We found no differences in cell growth or protein synthesis in cells incubated in chlorate (data not shown).

We investigated CS binding to chlorate-treated cells and found a dose-dependent decrease in CS binding (Fig. 6A). When cells were incubated in medium containing chlorate and an equimolar amount of sulfate, there was no effect on CS binding (*inset*, Fig. 6A), suggesting that chlorate is not toxic to the cells, and its effect on CS binding is due to its inhibition of sulfation. These studies, taken together with previous results showing that CS binds to HSPGs on the surface of HepG2 cells (6, 19), suggest that CS binding is correlated with the degree of sulfation of the HSPG GAGs.

In order to determine whether specific sulfate moieties were important for CS binding, we used modified heparins as inhibitors of CS binding to HepG2 cells. These heparins are selectively desulfated in either the *N*- or *O*-positions. As shown in Fig. 6B, both 2-*O*,3-*O*-desulfated heparin and *N*-desulfated heparin inhibited CS binding to HepG2 cells less well than the parent compound. In addition, the more fully desulfated compound (2-*O*,3-*O*-desulfated and *N*-desulfated heparin) had very

**FIG. 4. Heparin does not inhibit sporozoite invasion of HepG2 cells.** *A*, sporozoite invasion in the presence of heparin under static and rotating conditions. *P. berghei* sporozoites were preincubated in medium  $\pm$  heparin (25  $\mu\text{g/ml}$ ) and then plated on cells in the presence of heparin. After 1 h at 37 °C under static or rotating conditions, unattached sporozoites were removed, and the cells were stained with a double-staining procedure that allows the differentiation of intracellular from extracellular sporozoites. The percentage of sporozoites that invaded the cells was calculated using the following equation:  $((\text{total parasites} - \text{extracellular parasites})/\text{total parasites}) \times 100$ . Each point was plated in triplicate and shown are the means with standard deviations. Because of the variation in invasion efficiency among different batches of sporozoites, we show results from three separate experiments. *B* and *C*, sporozoite invasion after recovery from cytochalasin treatment. *P. berghei* sporozoites were preincubated in medium with 1  $\mu\text{M}$  cytochalasin, added to cells, and allowed to adhere under static conditions in the continued presence of cytochalasin. After 30 min, the cytochalasin-containing medium and any unattached sporozoites were removed; medium containing the indicated concentrations of heparin was added, and sporozoites were allowed to invade cells in the presence of heparin. *White bars*, no heparin; *gray bars*, 25  $\mu\text{g/ml}$  heparin; *black bars*, 100  $\mu\text{g/ml}$  heparin. Controls included sporozoites preincubated and added to cells in medium without cytochalasin or heparin (*slanted line bars*) and sporozoites preincubated and maintained in medium with cytochalasin for the entire experiment (*cross-hatched bar*). Sporozoites were allowed to invade cells for 1 h and then the cells were double-stained so that intracellular and extracellular sporozoites could be distinguished. The total number of sporozoites attached for each experimental condition (*B*) and the percentage of attached sporozoites that were found intracellularly (*C*) are shown. None of the sporozoites that were in the continuous presence of cytochalasin were found intracellularly (the *asterisk* in the graph indicates that the data were collected but the error bar cannot be seen since it is 0). Each point was plated in triplicate and shown are the means with standard deviations.

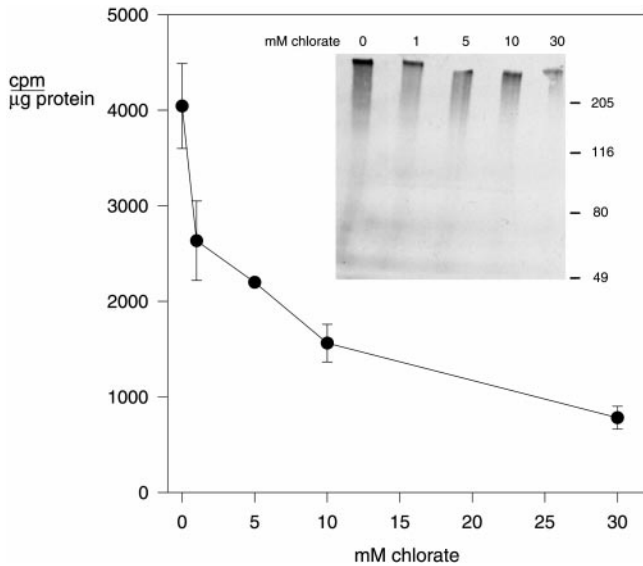


little inhibitory activity. These results suggest that both *N*- and *O*-sulfate moieties participate in CS binding. Results using carboxy-reduced heparin as an inhibitor were somewhat surprising. As shown in Fig. 6*B*, this was a weak inhibitor of CS binding to HSPGs. At physiologic pH the carboxyl groups of the GAGs are negatively charged, and our results suggest that they participate, along with the sulfate moieties, in binding to CS.

**The Sulfate Moieties of HSPGs Are Critical for Sporozoite Attachment to Cells**—To extend these findings and determine whether the requirements for CS binding to cells parallel the requirements for sporozoite attachment, we tested the ability of sporozoites to attach to chlorate-treated HepG2 cells under static and rotating conditions. As shown in Fig. 7, chlorate treatment of HepG2 cells results in a dose-dependent decrease

in sporozoite attachment, and as expected, the effect is much more dramatic under rotating conditions compared with static conditions. Cells incubated with chlorate and an equimolar amount of sulfate showed no inhibition of sporozoite attachment under static or rotating conditions (*inset*, Fig. 7).

We then used the modified heparins as inhibitors of sporozoite attachment to HepG2 cells under rotating conditions. As shown in Fig. 8, heparin that is selectively desulfated in the 2-*O* and 3-*O* positions inhibited sporozoite attachment by only 40%, whereas the parent compound inhibited sporozoite attachment by 80%. *N*-Desulfated heparin had similar inhibitory activity to the 2-*O*,3-*O*-desulfated compound. When we used heparin that was both *N*-desulfated and 2-*O*,3-*O*-desulfated, the effect was additive, and sporozoite adhesion was inhibited



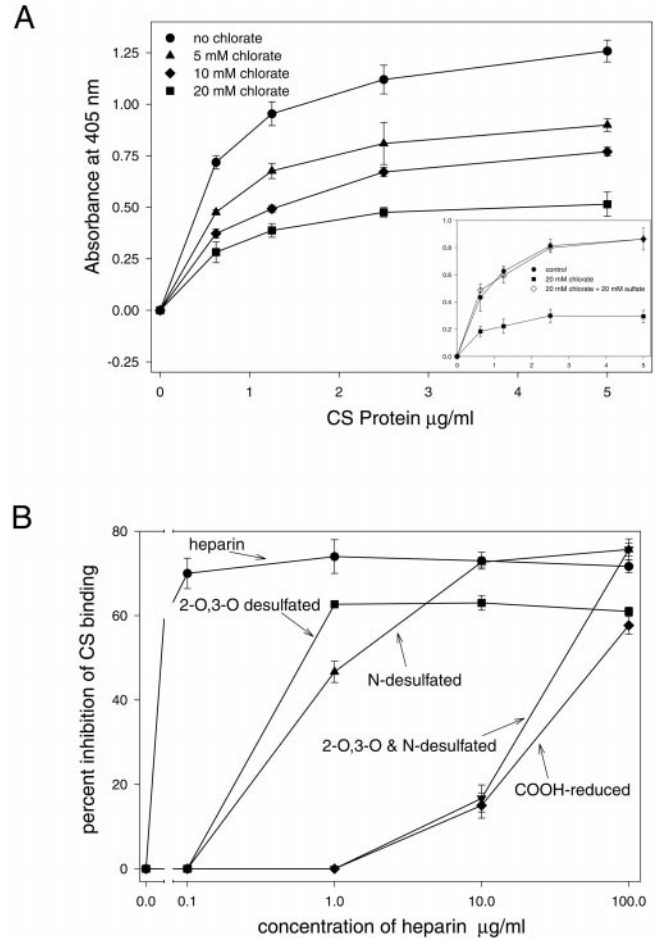
**FIG. 5. Chlorate inhibits sulfate incorporation into proteoglycans of HepG2 cells.** HepG2 cells were plated in 6-well plates in medium with the indicated concentration of chlorate. After 12 h, the medium was changed to low sulfate medium with the indicated concentrations of chlorate and [ $^{35}$ S]sodium sulfate. 12 h later the cells were washed, lysed, and total cell-associated counts/min were measured in a  $\beta$ -counter. Each point was performed in triplicate and shown as the means with standard deviations. *Inset*, lysates from chlorate-treated cells were loaded onto a 5% SDS-polyacrylamide gel that was then subjected to autoradiography. Equivalent amounts of protein were loaded onto each lane. Most of the labeled material migrated as a broad high molecular weight smear, typical of proteoglycans.

by only 20%. The lack of inhibitory activity of the more fully desulfated heparin shows that, similar to CS, sporozoites utilize both types of sulfate groups to bind to HSPGs. Interestingly, the carboxy-reduced heparin inhibited sporozoite attachment by over 50%. This is in contrast to its very low inhibitory activity in the CS protein binding assay. One possible explanation for this discrepancy is that recombinant CS may have sites not exposed on the native protein, and these sites may bind to the negatively charged carboxyl groups of the uronic acids.

#### DISCUSSION

Previous studies have shown that CS binds to HSPGs (reviewed in Ref. 1). Although investigators have speculated that this binding event functions in sporozoite attachment to target cells, there is little experimental data to support this hypothesis. Previous *in vitro* studies failed to demonstrate a significant decrease in sporozoite attachment or invasion using cell lines deficient in HSPGs or cells in which surface HSPGs were removed with heparinase (6). In the present study, using an inhibitor of the CS-HSPG interaction (heparin), or modifying cell surface HSPGs using chlorate, we significantly inhibit sporozoite attachment to cells under conditions that mimic flow.

Why do the more dynamic conditions of our rotating assay (or the assay performed with a flow chamber) result in a more dramatic inhibition of sporozoite attachment in the presence of heparin compared with the same assay under static conditions? We know from previous studies (26, 27) that only multimers of CS bind with high affinity to HSPGs. Since CS forms a coat on the surface of the sporozoite, we can consider the sporozoite to be a very large CS multimer. When heparin is added to the sporozoites, it will bind to many of these CS molecules. It is likely that under static conditions, a low affinity interaction between the sporozoite and the cell is sufficient for parasite attachment so that even in the presence of heparin enough CS

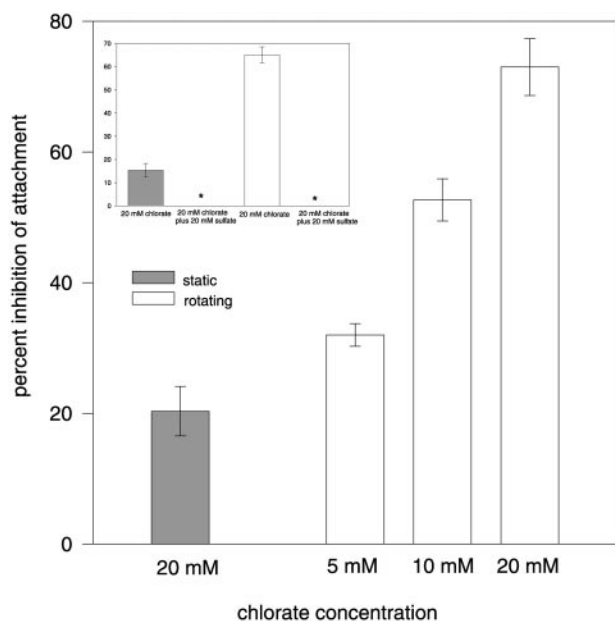


**FIG. 6. A, CS binding to chlorate-treated HepG2 cells.** Cells were plated in 96-well plates and grown in low sulfate medium with the indicated concentrations of chlorate. After 18–24 h, they were fixed, blocked, and incubated with increasing amounts of CS protein. CS binding was revealed with mAb 2A10 specific for the CS repeats, followed by anti-mouse Ig conjugated to horseradish peroxidase and peroxidase substrate. The CS binding curves for cells grown in 20 mM chlorate (squares), 10 mM chlorate (diamonds), 5 mM chlorate (triangles), and no added chlorate (circles) are shown. Each point was assayed in triplicate, and the means with standard deviations are shown. *Inset* shows CS binding curves for a control experiment where cells were grown in low sulfate medium with 20 mM chlorate (squares), 20 mM chlorate plus 20 mM magnesium sulfate (open diamonds), or no chlorate (circles). **B, inhibition of CS binding to HepG2 cells with modified heparins.** 5  $\mu$ g/ml CS was preincubated with the indicated concentrations of each heparin for 30 min at 37  $^{\circ}$ C. These solutions were then added to paraformaldehyde-fixed HepG2 cells for 1 h at 37  $^{\circ}$ C; the cells were washed, and bound CS was determined using iodinated mAb 2A10, specific for the CS repeats. Shown is the percent inhibition of binding of CS to HepG2 cells in the presence of inhibitor compared with results obtained in the absence of inhibitor. Inhibitors were heparin (circles), 2-O,3-O-desulfated heparin (squares), N-desulfated heparin (triangles), 2-O,3-O- and N-desulfated heparin (inverted triangles), and carboxy-reduced heparin (diamonds). Each inhibitor concentration was assayed in triplicate, and the means with standard deviations are shown.

will be unoccupied to enable the parasite to attach to the cell. However, the number of unoccupied CS molecules on the surface of the sporozoite in the presence of heparin may not be sufficient for the sporozoite to attach to the cell under more dynamic conditions. These results suggest that the multimeric binding between sporozoite CS and hepatic HSPGs may function to arrest the sporozoite in the liver under conditions of flow.

One important consideration, however, is that neither the rotating assay nor the flow chamber is likely to mimic precisely blood flow in the liver. Given the architecture of the liver

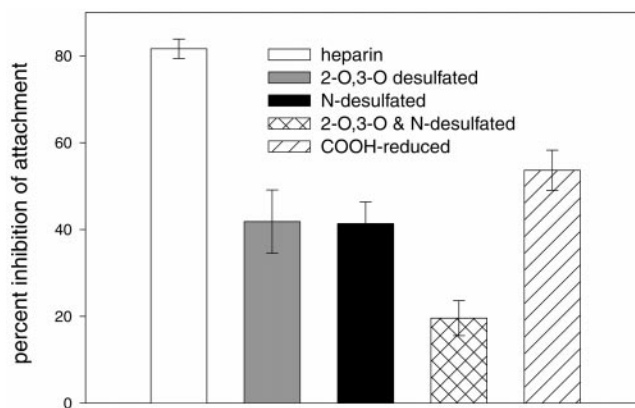




**FIG. 7. Inhibition of sporozoite attachment to chlorate-treated HepG2 cells.** HepG2 cells were plated in labtek chamber slides, and 2 days later the medium was replaced with low sulfate medium containing the indicated concentrations of chlorate. After 24 h, the chlorate-containing medium was removed, and *P. berghei* sporozoites in DMEM/FCS were added to each well. After 1 h at 37 °C under static (gray bars) or rotating conditions (white bars), unattached sporozoites were removed; the cells were fixed, and the attached sporozoites were stained. Shown is the percent inhibition of sporozoite attachment to cells in the presence of chlorate compared with the mean number of sporozoites attached in the absence of chlorate (under static or rotating conditions). Each point was plated in triplicate and shown are the means with standard deviations. *Inset* shows sporozoite attachment, under static and rotating conditions, to cells that had been incubated (as above) in medium with no chlorate, 20 mM chlorate, and 20 mM chlorate plus 20 mM magnesium sulfate. There was no inhibition of sporozoite attachment to cells in 20 mM chlorate plus 20 mM magnesium sulfate under both static and rotating conditions (asterisks indicate that the data were collected, but the error bar cannot be seen since it is 0). Each point was plated in triplicate and shown are the means with standard deviations.

sinusoids, this is a challenge for any experimental set up. However, in both assays, a shear force is created between the medium containing the sporozoites and the immobilized cells. Our experiments therefore allow us to conclude that when shear forces are present, heparin is a more potent inhibitor of sporozoite attachment. The difference between our rotating assay and the parallel plate flow chamber is that in the latter case the flow is more uniform and can be measured. The conclusions we draw from both assays, however, are the same. Although we cannot measure the shear forces in our rotation assay, we think they are in the physiologic range since sporozoites attach well to the cells in this assay, and they do not attach to cells when subjected to high shear forces in the flow chamber (data not shown).

HSPGs are ubiquitous molecules found on the surface of most mammalian cells. How then do we account for the specificity of sporozoites for hepatic HSPGs? Previous studies (2, 3) showing that intravenously injected CS is cleared by hepatic HSPGs suggest that CS binds to either a unique GAG chain structure or to a subset of GAGs found only in the liver. HSPG GAG chains are based on repeating disaccharide units of *N*-acetyl-glucosamine (GlcNAc) and glucuronic acid which undergo a series of modification reactions. Glc-NAc residues can be *N*-deacetylated and *N*-sulfated as well as 3-*O*- and 6-*O*-sulfated; and glucuronic acid can undergo epimerization to iduronic acid and 2-*O*-sulfation. These modification reactions



**FIG. 8. Sporozoite attachment to HepG2 cells under rotating conditions in the presence of modified heparins.** *P. berghei* sporozoites were preincubated for 15 min on ice in medium alone or with 25 µg/ml heparin (white bar), 2-*O*,3-*O*-desulfated heparin (gray bar), *N*-desulfated heparin (black bar), 2-*O*,3-*O*- and *N*-desulfated heparin (cross-hatched bar), and carboxy-reduced heparin (diagonally lined bar) and then added to HepG2 cells in labtek chamber slides. Sporozoite incubation with cells was under rotating conditions and in the continued presence of the inhibitor. After 1 h, unattached sporozoites were removed; the cells were fixed, and the attached sporozoites were stained and counted. Each point was plated in triplicate wells. Percent inhibition of sporozoite attachment was calculated using the mean number of sporozoites attached in the absence of heparin. This experiment was performed three times and shown are the pooled results from all three experiments.

are not evenly distributed throughout the chain but tend to occur in blocks, giving rise to highly modified, sulfated stretches of saccharides. In addition, within the modified blocks, these reactions do not occur uniformly so that heparan sulfate GAGs contain a large amount of structural heterogeneity. These modifications can provide specific binding sites for a variety of proteins (reviewed in Ref. 28). In the case of CS, previous work by Ying *et al.* (20) found that CS binds preferentially to more highly sulfated regions of HSPG GAGs. We have extended these studies and found that both CS binding and sporozoite attachment to cells decreases as the degree of GAG chain sulfation decreases. The role of sulfation in CS and sporozoite adhesion to cells is also supported by our experiments using modified heparins as inhibitors. These studies show that both *N*- and *O*-sulfation are important for CS and sporozoite adhesion to cells and that binding is not dependent on a single class of sulfate moieties. Although these results do not rule out the possibility that a specific sequence of sulfated sugar residues mediates sporozoite adhesion, they suggest that the interaction between sporozoite CS and hepatic HSPGs may be based largely on the anionic properties of HSPG GAGs. Could this account for the selective binding of CS and sporozoites to hepatic HSPGs?

*In vivo* the HSPGs on most cells are not exposed to the circulation since most organs are behind an endothelial cell barrier that does not permit direct contact with the blood circulation. The sinusoidal lining of the liver, however, is highly fenestrated, and these fenestrations are permanently open, allowing for direct contact between the blood circulation and the underlying hepatocytes and space of Disse (the loose basement membrane between hepatocytes and endothelia). However, endothelial cells themselves express HSPGs on their surface, and these could directly compete with hepatic HSPGs for sporozoite binding. Previous work (29) on the structure of rat liver heparan sulfate has shown that, compared with heparan sulfate of other organs, it is more extensively modified and highly sulfated. This is in contrast to endothelial cell heparan sulfate, which is among the most undersulfated heparan sulfate in the body (30). These findings together with the work

presented here suggest that the degree of GAG chain sulfation and the architecture of the liver sinusoids may account for the selective targeting of CS and sporozoites to the liver.

We also present data suggesting that the binding of CS to HSPGs is involved in sporozoite attachment to but not invasion of cells. It is generally recognized that attachment and invasion of cells by intracellular pathogens are separate steps requiring different molecular interactions. Parasites in the phylum Apicomplexa, of which *Plasmodium* is a member, are no exception. Previous studies have shown that another sporozoite protein, thrombospondin-related anonymous protein (TRAP (31, 32)), is required for invasion (33). Although a small amount of TRAP is found on the sporozoite surface, it is found most abundantly in secretory vesicles called micronemes (34). Upon contact with cells, sporozoites release TRAP onto the apical end of the parasite (35). The cell surface receptors for TRAP have still not been determined; however, several reports (36, 37) have shown that recombinant TRAP binds to heparin and HSPGs. Our finding that heparin has a minimal effect on sporozoite invasion of cells contradicts these data since one would expect that if TRAP is also binding to HSPGs, soluble heparin would have also inhibited this interaction and therefore inhibited invasion. One possible explanation for the lack of inhibitory activity of heparin on sporozoite invasion could be due to the timing and location of microneme release. If microneme contents are released only after sporozoite attachment to cells, then the high local concentration of TRAP in close proximity to its binding sites may make it difficult for heparin to compete with the binding of TRAP to its receptor. In fact it has been shown recently (35) that antibodies to TRAP do not inhibit *Plasmodium* sporozoite infectivity either *in vivo* or *in vitro*, and the likely reason is that these antibodies do not have access to TRAP.

In summary, the work we present here is the first demonstration that the binding of CS on the sporozoite surface to HSPGs functions during initial attachment of the sporozoites to their target cell. Our hypothesis is that the multimeric interaction between sporozoite CS and hepatic HSPGs functions to arrest circulating sporozoites in the liver. Our demonstration that sulfation of HSPG GAGs is required for sporozoite attachment to cells provides a theoretical basis for the selectivity of sporozoites for hepatic HSPGs *in vivo*. In addition, the data also indicate that initial attachment of sporozoites to hepatic HSPGs is a distinct step in target cell invasion and is likely followed by other molecular interactions that then lead to invasion. The precise nature of these other molecular interactions awaits further investigation.

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**The Binding of the Circumsporozoite Protein to Cell Surface Heparan Sulfate  
Proteoglycans Is Required for *Plasmodium* Sporozoite Attachment to Target Cells**  
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