Dual Interaction of the Malaria Circumsorozoite Protein with the Low Density Lipoprotein Receptor-related Protein (LRP) and Heparan Sulfate Proteoglycans

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Summary

Speed and selectivity of hepatocyte invasion by malaria sporozoites have suggested a receptor-mediated mechanism and the specific interaction of the circumsporozoite (CS) protein with liver-specific heparan sulfate proteoglycans (HSPGs) has been implicated in the targeting to the liver. Here we show that the CS protein interacts not only with cell surface heparan sulfate, but also with the low density lipoprotein receptor-related protein (LRP). Binding of 125I-CS protein to purified LRP occurs with a $K_d$ of 4.9 nM and can be inhibited by the receptor-associated protein (RAP). Blockage of LRP by RAP or anti-LRP antibodies on heparan sulfate-deficient CHO cells results in more than 90% inhibition of binding and endocytosis of recombinant CS protein. Conversely, blockage or enzymatic removal of the cell surface heparan sulfate from LRP-deficient embryonic mouse fibroblasts yields the same degree of inhibition. Heparinase-pretreatment of LRP-deficient fibroblasts or blockage of LRP on heparan sulfate-deficient CHO cells by RAP, lactoferrin, or anti-LRP antibodies reduces Plasmodium berghei invasion by 60-70%. Parasite development in heparinase-pretreated HepG2 cells is inhibited by 65% when RAP is present during sporozoite invasion. These findings suggest that malaria sporozoites utilize the interaction of the CS protein with HSPGs and LRP as the major mechanism for host cell invasion.

Malaria sporozoites invade hepatocytes where they subsequently develop into large numbers of erythrocyte-infected merozoites. The entire surface of these sporozoites is covered by the circumsporozoite (CS)$^1$ protein and it has therefore been proposed that this protein mediates the rapid and selective invasion of hepatocytes (reviewed in reference 1). The CS protein has been shown to interact specifically with liver heparan sulfate proteoglycans (HSPGs) (2–4) and CS protein is selectively cleared from the bloodstream by the liver (5, 6), most likely mediated by an interaction between clusters of basic amino acids in the CS protein and negatively charged sulfate groups in heparan sulfate (6). The selectivity of the CS protein for liver HSPGs may depend on their unusually high overall degree of sulfation compared to the heparan sulfate chains from other tissues, in particular the vascular endothelium (7). Therefore, the selective targeting of recombinant CS protein to the liver is likely to be mediated by recognition of heparin-like oligosaccharides in heparocyte HSPGs. The events of sporozoite invasion, however, appear to use additional receptor-ligand interactions, since Plasmodium berghei sporozoite invasion in vitro can also occur in the absence of cell surface heparan sulfate (8).

A variety of host substances, such as apolipoprotein E (apo E)-enriched chylomicron remnants, are also rapidly removed from the blood circulation by the liver and this process can be inhibited by lactoferrin (9–15). The intravenous administration of heparinase inhibits the clearance of lipoprotein remnants from the plasma and HSPGs have therefore been implicated in the initial trapping mechanism of the liver (13). The subsequent endocytic uptake of the remnants into hepatocytes (15) is mediated by the low density lipoprotein receptor (LDLR) in concert with the structurally related LDL receptor-related protein (LRP), both members of a family of endocytosis receptors (reviewed in reference 16, 17). The binding of all known ligands of LRP is inhibited by the 39-kD receptor-associated protein (RAP) (18, 19). Cell surface HSPGs play a synergistic role in the binding and internalization of various LRP ligands. For example, HSPGs are required for the hepatic lipase- or lipoprotein lipase-mediated enhanced binding and uptake.

$^1$Abbreviations used in this paper: CS, circumsporozoite; EEF, exoerythrocytic form; GST, glutathione-S-transferase; HSPGs, liver heparan sulfate proteoglycans; LDLR, low density lipoprotein receptor; LRP, LDL receptor-related protein; MEF, mouse embryonic fibroblast; RAP, receptor-associated protein; TRAP, thrombospondin-related adhesive protein.
of chylomicron remnants by LRP (20–24) and also facilitate the LRP-mediated endocytosis of lactoferrin (14) and apo E-enriched remnant lipoproteins (12, 25).

A recent report suggests the involvement of lipoprotein clearance pathways of the host in malaria sporozoite invasion of the liver. Apo E-enriched β-VLDL inhibit the development of P. berghei sporozoites in HepG2 cells in vitro and LDLR knock-out mice maintained on a high fat diet are less susceptible to infection by Plasmodium yoelii sporozoites (26). It appears, therefore, that remnant lipoproteins compete in vitro and in vivo for the same binding sites as the CS protein and malaria sporozoites. The similarities between the targeting of malaria sporozoites to the liver and the liver-mediated clearance of chylomicron remnants from the bloodstream prompted us to investigate the possibility that LRP or the LDLR play a role in sporozoite adhesion and invasion of hepatocytes. Our results suggest that the dual HSPG and LRP receptor system functions also as a mediator of sporozoite invasion.

Materials and Methods

Materials. BSA (fraction V), heparin (grade I), chloroquine, lactoferrin (from bovine colostrum) and RPMI 1640 were from Sigma (St. Louis, MO). Hepaminase and heparitinase were from Seikagaku (Rockville, MD), FCS from Hyclone (Logan, UT), IODO-GEN from Pierce (Rockford, IL), 125I-Iodine from Amersham. 125I-Iodine isomer from Isolab (Akron, OH), heparin-Sepharose from Pharmacia (Piscataway, NJ), 96-well (Removawell) tissue culture plates from Dynatech Laboratories, Inc. (Chantilly, VA), 12-well plates form Corning (Corning, NY), and 8-well Lab-Tek tissue culture slides from Nunc (Naperville, IL). The silver enhancement reagent was from Amersham.

Recombinant Proteins. Escherichia coli-derived Plasmodium falciparum CS proteins CS27IVC-His, (AA 27–123[NANPNVDP]3 [NANP]21 300–411) contains region 1 and II-plus as well as the complete repeat region, CSEZ(Cys)–His, (AA 27–123[NANP]300–411) also contains region I and region II-plus, but only one repeat were kindly provided by Dr. Bela Takacs (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and the yeast-derived Falc-1 (Chiton Corporation, Emeryville, CA), which terminates at amino acid 348 and therefore lacks region II-plus (2). LRP was a generous gift of Dr. Dudley K. Strickland (American Red Cross, Rockville, MD) (27). The recombinant Salmonella japonicum glutathione-S-transferase (GST)-RAP fusion protein was kindly provided by Dr. Joachim Herz (University of Texas Southwestern Medical School, Dallas, TX) (19).

Antibodies. The repeat region of the P. falciparum CS construct was detected by mAb 2A10 (28) and the repeat of the P. berghei CS protein by the mAb 3D11 (29). The mAb 2E6 is directed against a heat shock protein in P. berghei EEF (30). DAMP (Molecular Probes, Eugene, OR) as a marker for acidic organelles (31) was labeled with anti-DNP-KLH conjugated to Texas red (anti-DNP-TX; Molecular Probes). Affinity-purified polyclonal rabbit anti-LRP IgG antibodies (rab 777; designated here as anti-LRP), generated against purified human placental LRP, were a kind gift of Dr. Dudley K. Strickland (18). Goat anti-mouse IgG conjugated to FITC (GAM-FITC) or to peroxidase were from Boehringer (Indianapolis, IN) and goat anti-mouse IgG gold 10 nm (GAM 10) and protein A gold 10 nm (PAG 10) from Amersham.

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Sporozoite Invasion Assay. Intra- and extracellular sporozoites were distinguished in subconfluent CHO-K1, CHO pgsA or wild-type, LRP-deficient, LDLR-deficient, or LRP/LDLR-deficient MEF cells as described (8). Heparinase was added at a concentration of 10 mU/ml for 3 h before addition of 5 × 10⁴ P. berghei sporozoites per well (8). Anti-LRP antibodies (10 μg/ml) or GST-RAP (500 μg/ml) were added 30 min before and during co-cultivation with the sporozoites for 1 h.

EEF Development Assay. HepG2 cells were cultivated and EEF immunolabeled as described (2). Heparinase plus heparitinase were added at a concentration of 50 mU/ml each for 3 h before addition of 1 × 10⁵ P. berghei sporozoites per well. GST-RAP (500 μg/ml) was added 30 min before the parasites.

Immunoelectron Microscopy. Subconfluent HepG2 cells were incubated for 2 h at 4°C with 12.8 μg/ml CS27IVC in RPMI 1640 medium, incubated for up to 8 h at 37°C, embedded in Lowicryl K4M (Ted Pella Inc., Redding, CA) and sectioned with an RMC MT-7 ultramicrotome. Thin sections were sequentially labeled with 10 μg/ml mAb 2A10 and PAG10 and examined with a Zeiss EM 910 electron microscope.

Immunofluorescence. HepG2 cells were allowed to internalize 12.8 μg/ml CS27IVC for 4 h at 4°C, washed, and incubated for up to 8 h at 37°C. After fixation, the specimens were sequentially labeled with 10 μg/ml mAb 2A10 and GAM-FITC. In another set of experiments, 40 μg/ml DAMP were added for the last 2 h of the incubation time. The internalized DAMP was stained with anti-DNP-TX (31). The specimens were examined with an inverted Nikon Diaphot microscope equipped with a Superrastro 2000 laser confocal scanning system (Molecular Dynamics, Sunnyvale, CA).

 Autoradiography. Iodinated CS27IVC at a concentration of 10 nM was added to subconfluent HepG2 or CHO-K1 cells grown on Lab-Tek tissue culture slides (Nunc, Naperville, IL) for 1, 4, 12, or 24 h. The cells were washed, fixed with methanol, counterstained with 0.1% Evans blue and the slides coated with Hypcoat EM-1 photographic emulsion (Amersham). The silver grains in the photographic emulsion of autoradiography specimens were visualized in the reflection mode of the confocal microscope.

Results

The CS Protein Binds to both Cell Surface Heparan Sulfate Proteoglycans and LRP. To investigate the possibility of a dual interaction of recombinant CS protein with both HSPGs and LRP, we performed binding studies using three different cell types: (a) HepG2 cells, which possess HSPGs, LRP, and the LDLR on their surface (24, 38), (b) mutant CHO cell lines that express LRP and the LDLR, but are deficient in heparan sulfate biosynthesis and therefore express only the core proteins of proteoglycans (32, 38), and (c) several MEF cell lines which express HSPGs but are defective in either LRP, the LDLR, or both receptors (33).

HepG2 cells were incubated with iodinated recombinant CS protein either in the presence of heparin to compete with the host receptor or after treatment of the cells with heparinase to remove cell surface heparan sulfate. Both heparin and heparinase treatment inhibited the binding of recombinant CS protein in a concentration-dependent fashion to a maximum of 85.8 and 71.4%, respectively (Table 1). When HepG2 cells were incubated with recombinant CS protein in the presence of a polyclonal anti-LRP IgG, a concentration-dependent inhibition of the CS protein binding was observed to a maximum of 64.2% (Fig. 1 A). When HepG2 cells were pretreated with heparinase and then incubated with recombinant CS protein in the presence of anti-LRP, the inhibition reached a maximum of 98.4%, whereas a control antibody had no effect (Fig. 1 A). We then used GST–RAP in combination with the heparinase treatment. RAP inhibits the LRP-mediated binding and endocytic uptake of all known ligands (19). Here, GST–RAP alone inhibited the binding of recombinant CS protein to HepG2 cells in a concentration-dependent manner, with a maximum of 82.6%, whereas in combination with heparinase treatment, GST–RAP yielded 93.4% inhibition (Table 1). These data indicate that interference with either heparan sulfate or LRP inhibits the binding of CS protein to HepG2 cells and almost complete inhibition can be obtained when both molecules are blocked.

To ensure the complete absence of cell surface heparan sulfate and to exclude possible nonspecific effects of the heparinase treatment on surface molecules other than heparan sulfate, we utilized CHO pgsA cells that are deficient in heparan sulfate (32). Controls were wild-type CHO-K1 cells that synthesize normal HSPGs. Both cell lines express LRP (39) and GST-RAP was used to block this receptor. CHO pgsA cells bind only 45.5% of the recombinant CS protein in comparison to CHO-K1 cells, presumably due to the lack of heparan sulfate. GST–RAP inhibited the binding of recombinant CS protein to CHO-K1 cells in a concentration-dependent manner with maximum inhibition being 77.3% (Fig. 1 B). However, with CHO pgsA cells, GST–RAP inhibited the binding of recombinant CS protein by 93.4% (Fig. 1 B). Similarly, anti-LRP inhibited CS binding to CHO-K1 cells to a maximum of 46.1%, but to CHO pgsA cells to a maximum of 93.7% (Table 1), whereas a control antibody was ineffective (not shown). Lactoferrin reduced the binding of recombinant CS protein to both CHO-K1 and pgsA cells to a similar degree (89.2 and 94.8%, respectively) (Table 1). All three competitors had the same strong inhibitory effect on the CS protein binding to heparan sulfate-deficient CHO pgsA cells, but lactoferrin, which interacts in a dual mode with both heparan sulfate and LRP (14, 34, 40–42), was a more potent inhibitor of the binding to wild-type CHO cells than anti-LRP. Moreover, it seems that RAP may have interacted weakly with CHO cell surface heparan sulfate and therefore exhibited an intermediate inhibitory effect.

Heparinase treatment of wild-type and LDLR-deficient MEF cells maximally reduced the binding by only 35 and 49%, respectively (data not shown). This contrasts with the results obtained by heparinase treatment of LRP-deficient MEF cells which inhibited the binding of recombinant CS protein in a concentration-dependent fashion by up to 84%. Thus the vast majority of the receptor interaction of CS protein on the cell surface is mediated by LRP and not the LDLR.
Table 1. Inhibition of Cell Surface Binding and Internalization of the CS Protein

| Cell type | Inhibitor | Concentration | Bound cpm | Percent inhibition | P Value |
|-----------|-----------|---------------|-----------|--------------------|---------|
|           |           | mean ± SD     |           |                    |         |
| Binding*  |           |               |           |                    |         |
| HepG2     | none      | 11056.8 ± 1126.4 | 0         | 0.007130           |         |
|           | heparin   | 1564.7 ± 173.2 | 85.8      | 0.007130           |         |
|           | Hase      | 11281.2 ± 872.9 | 0         | 0.006384           |         |
|           | RAP       | 3231.6 ± 207.8 | 71.4      | 0.029425           |         |
|           | Hase + RAP| 7182.4 ± 1444.5 | 82.6      | 0.000921           |         |
|           | none      | 1253.1 ± 275.4 | 0         | 0.000921           |         |
|           | CHO-K1    | 8339.9 ± 65.1 | 93.4      | 0.000921           |         |
|           | lactoferrin| 7513.5 ± 924.8 | 0         | 0.001638           |         |
|           | CHO pgA   | 810.4 ± 807.2 | 89.2      | 0.006120           |         |
|           | lactoferrin| 3862.6 ± 969.2 | 48.6      | 0.009948           |         |
|           | CHO-K1    | 6768.2 ± 230.4 | 0         | 0.008760           |         |
|           | anti-1LRP | 3653.4 ± 345.2 | 46.1      | 0.008132           |         |
|           | none      | 4418.0 ± 194.6 | 34.7      | 0.00675            |         |
|           | anti-1LRP | 429.1 ± 34.7 | 93.7      | 0.00675            |         |
| Internalization† | | | | | |
| HepG2     | none      | 6463.9 ± 332.1 | 0         | 0.012860           |         |
|           | Chloroquine| 8949.6 ± 227.4 | -38.5     | 0.012860           |         |
|           | RAP       | 6732.3 ± 1293.1 | 0         | 0.009898           |         |
|           | Hase + RAP| 1810.9 ± 678.3 | 73.1      | 0.009898           |         |
|           | 7327.2 ± 348.5 | 0         | 0.007850           |         |
| CHO-K1    | none      | 847.9 ± 304.7 | 88.4      | 0.007850           |         |
|           | anti-1LRP | 5576.9 ± 204.2 | 0         | 0.001722           |         |
| CHO pgA   | none      | 761.2 ± 195.9 | 86.4      | 0.001722           |         |
|           | anti-1LRP | 4060.9 ± 210.1 | 27.2      | 0.001722           |         |
| CHO-K1    | none      | 528.4 ± 80.6 | 90.5      | 0.00944            |         |
|           | lactoferrin| 7310.5 ± 1692.6 | 0         | 0.026501           |         |
| CHO pgA   | none      | 103.4 ± 39.9 | 98.6      | 0.026501           |         |
|           | lactoferrin| 3368.2 ± 468.7 | 53.9      | 0.086548           |         |
|           |           | 435.1 ± 209.8 | 94.1      | 0.029417           |         |

*Subconfluent HepG2 cells were preincubated for 30 min at 4°C with or without 0–150 µg/ml GST-RAP or Hase plus GST-RAP (10 µg/ml + 0–150 µg/ml). The heparinase treatment (0–10 mU/ml) was performed for 3 h at 37°C. CHO cells were treated with 0–10 µg/ml anti-1LRP or 0–400 µg/ml lactoferrin. 125I-CS protein was then added at a concentration of 4 nM and the incubation continued at 4°C for another 2 h. Cell surface binding was determined by liquid scintillation counting.

†125I-CS protein was added to subconfluent HepG2 cells at 4 nM after a 30-min preincubation period with or without 0–150 µg/ml GST-RAP, Hase plus GST-RAP (10 µg/ml + 0–150 µg/ml) and to CHO cells after preincubation with anti-1LRP or lactoferrin. The incubation was then continued for 3 h at 37°C before addition of other inhibitors or iodinated CS protein. Chloroquine was added to some of the wells during the internalization period to prevent lysosomal degradation. Cell surface-bound ligand was then proteolytically removed and internalized cpm measured by liquid scintillation counting. Values represent the mean of duplicate determinations ± SD. P values were calculated using an independent equal variance t test. Shown are the results of one representative of duplicate experiments performed independently.

Taken together, these binding studies indicate that blockage of either the HSPGs or LRP singly results in partial inhibition of the binding of recombinant CS protein, whereas simultaneous blockage of both HSPGs and LRP abolishes the interaction nearly completely. These binding studies suggest that: (a) the CS protein interacts with both cell surface heparan sulfate and LRP and (b) no additional receptors appear to be required for binding.
Figure 1. Heparan sulfate proteoglycans and LRP mediate the cell surface binding of iodinated recombinant CS protein. (A) Anti-LRP IgG (α-LRP) inhibits the CS protein binding to HepG2 cells by 64%, whereas anti-LRP in combination with heparinase pretreatment (10 mU/ml) results in 98% inhibition. A control IgG is ineffective. Subconfluent cell cultures were pretreated for 3 h at 37°C with increasing concentrations of heparinase (Hase) and/or preincubated for 30 min at 4°C with increasing amounts of inhibitor. Iodinated CS271VC at a final concentration of 4 nM was then added and the incubation continued for another 2 h at 4°C. Cell-associated radioactivity was determined by liquid scintillation counting. The indicated values represent the mean of duplicates ± SD. (B) GST-RAP inhibits the binding of iodinated CS protein to wild-type CHO-K1 cells by 77% and to heparan sulfate-deficient CHO pgsA cells 93%. Shown is one of two independent experiments for each experimental condition.

**LRP Mediates the Endocytic Uptake of Recombinant CS Protein.** HepG2 cells were incubated with 125I-CS protein and the amount of internalized CS protein determined after proteolytic removal of cell surface-associated ligand. The accumulation of intracellular CS protein reached its maximum after 3–5 h and decreased subsequently (Fig. 2 A). This decrease was completely prevented in the presence of 0.1 mM chloroquine (Table 1), which interferes with intracellular protein degradation by blocking lysosomal acidification (35). When binding of CS protein to cell surface HSPGs was selectively inhibited either by heparinase digestion or by competition with heparin, the uptake of recombinant CS protein was inhibited by 70.4 and 92.7%, respectively (Fig. 2 A). Blockage of the interaction of CS protein and LRP on the HepG2 cell surface by increasing amounts of anti-LRP inhibited the uptake of recombinant CS protein in a concentration-dependent manner to a maximum of 71.5%, while a control antibody was inactive (Fig. 2 B). If the cells were pretreated with heparinase, anti-LRP antibody blocked endocytosis of CS protein by up to 98.6% (Fig. 2 B). Taken together, these results suggest that in HepG2 cells, both HSPGs and LRP are important for the endocytosis of recombinant CS protein. The complete inhibition of CS protein uptake after elimination of the two molecules also suggests that no other receptors (3, 43) are required.

Mutant CHO cell lines were used to exclude possible nonspecific effects on the HepG2 cells. In comparison to the wild type CHO-K1 cells, endocytosis of CS protein by heparan sulfate-deficient CHO pgsA cells was reduced by 46.4% (Fig. 2 C). When LRP was blocked on CHO-K1 cells by GST-RAP (Fig. 2 C) or anti-LRP (Table 1), the internalization of recombinant CS protein was inhibited by 85.3% and 86.4%, respectively. When CHO pgsA cells were exposed to GST-RAP or anti-LRP, endocytosis was inhibited by more than 90% in both cases. Thus, compared to HepG2 cells, GST-RAP and anti-LRP exhibited a somewhat stronger inhibitory effect on the CS protein uptake by CHO-K1 cells indicating that CHO cells rely more on LRP for the binding of CS protein than on heparan sulfate compared to HepG2 cells. Lactoferrin, which binds to both LRP and cell surface heparan sulfate, blocked endocytosis by more than 94% independently of the CHO cell type (Table 1).

The relative roles of LRP and LDLR were analysed in MEF cell lines. Wild-type MEF cells expressing both receptors exhibit an endocytosis curve with a maximum intracellular accumulation of CS protein at 5 h (not shown). Compared to wild-type cells, LRP-deficient and LRP/LDLR-deficient double mutant MEF cells both showed no significant uptake of CS protein (92.9 and 94.8% inhibition, respectively; data not shown) emphasizing the crucial role of LRP in this process. However, uptake of recombinant CS protein by LDLR-deficient cells was reduced by 35.1% suggesting that, although the LDLR receptor is not obligatory for the internalization of CS protein, it may support the action of LRP. Degradation of cell surface heparan sulfate of wild type MEF cells by heparinase caused a 53.9% inhibition of uptake at the highest enzyme concentration (data not shown) suggesting that the heparan sulfate of MEF cells does facilitate endocytotic uptake of CS protein to a similar degree as for CHO cells where heparinase treatment inhibits by ~50%. This effect is lower, however, than in HepG2 cells where the same treatment inhibits uptake by ~70%. When HSPGs were eliminated by heparinase pretreatment of MEF mutant cell lines lacking either LRP or LRP/LDLR, we found 93 and 95% inhibition, respec-
The CS Protein Binds with High Affinity and Specificity to LRP. Iodinated CS protein was allowed to react with purified human LRP in a solid phase assay in the presence of increasing concentrations of competitors: either unlabeled CS protein (Fig. 3A) or GST-RAP (Fig. 3B). The resulting curve represents the best fit to a single class of sites and the apparent $K_d$ of 4.9 nM suggests a specific ligand-receptor interaction. GST-RAP inhibited the interaction of the iodinated CS protein with purified LRP with a resulting $K_i$ of 9.5 nM. No binding of iodinated CS protein occurred in control wells coated with BSA (Fig. 3, A and B). These results suggest a specific receptor-ligand interaction between recombinant CS protein and purified LRP. The binding of iodinated CS protein to the surface of HepG2 cells was inhibited by unlabeled CS protein or GST-RAP in a concentration-dependent and saturable fashion (Fig. 3 C). The iodinated CS protein was displaced by cold ligand with an apparent $K_d$ of 0.4 nM (Fig. 3C) and by GST-RAP with an apparent $K_i$ of 0.8 nM (Fig. 3D), which is in the same order of magnitude as the RAP-mediated displacement of tissue-type plasminogen activator from the surface of rat hepatoma cells (40).

Internalized CS Protein Enters the Endocytotic Pathway and Is Degraded in Lysosomes. When HepG2 cells were incubated with recombinant CS protein at 4°C, the CS protein was found to be restricted to the cell surface microvilli (not shown). When the cells were incubated at 37°C for up to 8 h, the CS protein gradually disappeared from the cell surface and entered cytoplasmic vesicles (Fig. 4A). DAMP was then used as a marker for acidic organelles (31) to determine the nature of the CS protein-containing vesicles. Double labeling studies in combination with confocal colocalization revealed that the CS protein had entered lysosomes (Fig. 4, B and C). Microautoradiography was used to determine the intracellular, long-term fate of internalized $^{125}$I-labeled CS protein (data not shown). Thus, similarly to the above results with HepG2 and CHO cells, the elimination of both HSPGs and LRP as CS protein binding sites completely abolished the endocytotic uptake of the CS protein. The LDLR appears to play only a minor role in these events.

Figure 2. Heparan sulfate proteoglycans and LRP mediate the endocytotic uptake of iodinated recombinant CS protein. Subconfluent cell cultures were incubated at 37°C with iodinated CS-P27IVC at a final concentration of 4 nM in the presence various inhibitors. Internalized CS protein was determined after proteolytic treatment of the cells to dissociate surface-bound CS protein. (A) Time course of the internalization of iodinated CS protein (CSP) into HepG2 cells. The intracellular accumulation of CS protein reaches a peak at 3-6 h and is inhibited by pretreatment of the cells with 10 nU/ml heparinase or in the presence of 10 nM/ml heparin. (B) At 3 h, the endocytotic uptake of recombinant CS protein into HepG2 cells is inhibited by anti-LRP (α-LRP) by a maximum of 72%. After pretreatment of the cells with 10 nU/ml heparinase, anti-LRP inhibits the uptake by a maximum of 99%. (C) After a 3-h incubation period, GST-RAP inhibits the uptake of recombinant CS protein into wild-type CHO-K1 cells by a maximum of 85% and into heparan sulfate-deficient CHO pgsA cells by 95%. Note that in the absence of inhibitors, the endocytotic uptake of CS protein into CHO pgsA cells is only ~30% of the CHO-K1 cells.
tein. This approach was necessary since available antibodies recognize only the repeat region and neither the COOH or NH2 terminus of the CS protein would have been detected by immunolabeling. After 24 h of incubation, the iodine label had redistributed from an initially uniform staining of the peripheral cytoplasm (corresponding to endocytosis vesicles) to a juxtanuclear localization (Fig. 4 D) suggesting that the CS protein was confined to the lysosomal compartment of the cell. Taken together with the effect of chloroquine, these data show that recombinant CS protein remains confined to the endosomal compartment of the cell and is lysosomally degraded.

Both HSPGs and LRP Are Required for P. berghei Sporozoite Invasion. Next, we investigated the role of both LRP and LDLR in the invasion of host cells by P. berghei sporozoites using mutant MEF cells deficient in either LRP or in both LRP and LDLR. Invaded sporozoites were distinguished from attached parasites by double labeling (8). Heparinase treatment of wild-type MEF cells had some effect, i.e., 18.3–23.9% inhibition, on parasite entry (Table 2). Sporozoite invasion of LRP-deficient MEF cells was inhibited by 31.1% in comparison to the wild-type MEF cells. Prior heparinase treatment of these LRP-deficient MEF cells lead to a marked 60.5% inhibition of sporozoite invasion. Likewise, invasion of LRP/LDLR-deficient cells was inhibited by 45.9% compared to wild type cells and heparinase treatment increased the inhibition to 72.4% (Table 2). These data suggest that sporozoite invasion is mediated predominantly by LRP in concert with HSPGs and perhaps supported to a minor extent by the LDLR.

P. berghei sporozoite invasion of CHO-K1 and heparan sulfate-deficient CHO pgsA cells was inhibited by lactoferrin by more than 60% (Fig. 5 A). Notably, lactoferrin blocks LRP and interferes also with the HSPG-mediated CS protein binding on the CHO-K1 cells (14) thus explaining the equally strong effect on invasion of both the wild-type and the heparan sulfate-deficient cells. GST-RAP, which blocks predominantly LRP and appears to interact less strongly with CHO cell surface HSPGs than lactoferrin, inhibited sporozoite invasion of CHO-K1 cells by...
Figure 4. Recombinant CS protein enters the lysosomal compartment. (A) Subconfluent HepG2 cells were incubated for 2 h at 4°C with CS27IVC, washed, incubated for 10 min at 37°C, fixed, and embedded in Lowicryl K4M. CS protein is found associated with microvilli (MV) on the cell surface and internalized in vesicles (arrow) as detected by sequential immunolabeling with mAb 2A10 and PAG10. M, mitochondria; N, nucleus. (B and C) After a 4-h incubation period, HepG2 cells contain CS protein-positive vesicles (C, arrows). By confocal microscopy, most of the vesicles that contain endocytosed CS27IVC colocalize with DAMP-positive acidic organelles (B, arrows) suggesting that the CS protein had entered lysosomes. CS27IVC in cryosections of HepG2 cells was labeled with mAb 2A10 and GAM-FITC, DAMP was detected with anti-DNP-TX. N, nucleus. (D) HepG2 cells were incubated for 24 h with iodinated CS protein and prepared for microautoradiography. Evans blue-stained specimens were scanned by confocal microscopy and the localization of the silver grains visualized in relation to the cellular compartments. The distribution of the silver grains (arrows) suggests the targeting of the internalized CS27IVC to a juxtanuclear compartment. N, nucleus. Bars: (A) 0.2 μm; (C and D) 10 μm.
Table 2. Inhibition of Plasmodium berghei Sporozoite Invasion In Vitro

| Cell type             | Inhibitor | Invaded Sporozoites | Percent Inhibition | P Value   |
|-----------------------|-----------|---------------------|--------------------|-----------|
|                       |           | mean ± SD           |                    |           |
| MEF/wt                | none      | 253.5 ± 25.0        | 0                  |           |
|                       | Hase      | 193.0 ± 12.7        | 23.9               | 0.009642  |
| MEF/LRP-def.          | none      | 174.7 ± 15.0        | 31.1               | 0.008084  |
|                       | Hase      | 100.0 ± 30.7        | 60.5               | 0.000338  |
| MEF/wt                | none      | 185.0 ± 67.2        | 0                  |           |
|                       | Hase      | 151.0 ± 25.9        | 18.3               | 0.444994  |
| MEF/LRP/LDLR-def.     | none      | 100.0 ± 14.5        | 45.9               | 0.074696  |
|                       | Hase      | 51.0 ± 13.8         | 72.4               | 0.014472  |
| CHO-K1                | none      | 483.8 ± 53.3        | 0                  |           |
|                       | anti-LRP  | 325.3 ± 48.3        | 32.8               | 0.008812  |
| CHO pgsA              | none      | 290.5 ± 30.0        | 39.9               | 0.001553  |
|                       | anti-LRP  | 204.5 ± 40.9        | 57.7               | 0.000363  |
| CHO-K1                | none      | 345.5 ± 28.8        | 0                  |           |
|                       | RAP       | 229.8 ± 22.5        | 33.5               | 0.001539  |
| CHO pgsA              | none      | 177.5 ± 20.0        | 48.6               | 0.000166  |
|                       | RAP       | 85.8 ± 7.0          | 75.2               | 0.000005  |

Wild-type, LRP-deficient or LRP/LDLR-deficient MEF cells or CHO-K1 or pgsA cells were incubated for 2 d to subconfluency. The MEF cells were pretreated with or without 10 mU/ml heparinase (Hase) and cocultivated with *P. berghei* sporozoites for 1 h. The CHO cells were cocultivated with *P. berghei* sporozoites for 1 h in the presence or absence of 10 µg/ml anti-LRP IgG or 500 mg/ml GST-RAP. The cultures were then fixed and double labeled to distinguish between extracellular and intracellular sporozoites. The indicated values represent the mean number of sporozoites counted in entire 4 wells ± SD. P values were calculated using an independent equal variance t test. Shown are the results of one representative of duplicate experiments performed independently.

33.5%, but invasion of CHO pgsA cells by a maximum of 75.2% (Table 2). When anti-LRP was used to selectively block LRP, sporozoite invasion of CHO pgsA cells was inhibited by 57.7%, whereas under the same conditions, the wild-type cells exhibited only 32.8% inhibition (Table 2).

HepG2 cells support the intracellular differentiation of malaria parasites from the sporozoite to the mature exoerythrocytic form (EEF) stage, whereas in all other cells, including MEF and CHO cells, parasite development is arrested shortly after sporozoite invasion. Development of *P. berghei* EEF in HepG2 cells that had been pretreated with heparinase plus heparitinase was inhibited only by 17% (Fig. 5 B), corroborating our previous data (8). Likewise, GST-RAP inhibited EEF development only by 21%. However, GST-RAP in combination with removal of HSPG exhibited a strong 65% inhibitory effect. These data demonstrate clearly the relevance of the dual CS protein binding sites during the interaction of sporozoites with the host cell.

### Discussion

The major implication of this work is that malaria sporozoites depend on the dual binding to the low density LRP and cell surface HSPGs for host cell invasion. This conclusion is based on the finding that, firstly, recombinant *P. falciparum* CS protein binds with high affinity and specificity to purified human LRP, an interaction that can be inhibited by a protein known to block the receptor function of LRP, i.e., RAP. Secondly, binding and internalization of recombinant CS protein into HepG2, CHO, or MEF cells was virtually eliminated by the combined inhibition of heparan sulfate and LRP. Thirdly, the same result was obtained whether competitors of the interaction or mutant cells deficient in the participating molecules were used or if the cells were enzymatically pretreated. Moreover, elimination of only heparan sulfate or only LRP consistently resulted only in partial inhibition of binding or endocytosis of recombinant CS protein and had only a minor effect on sporozoite invasion. Finally, the strong inhibitory effect of the combined blockage of HSPGs and LRP on *P. berghei* sporozoite invasion in vitro suggests that this dual interaction of the CS protein represents the major molecular mechanism for host cell entry used by this apicomplexan parasite.

The cell surface binding properties of the CS protein have a striking similarity to those of apo E-enriched β-VLDL, which bind initially in an HSPG-mediated process to the liver and to HepG2 cells (12–14). Subsequently, these β-VLDL are thought to be transferred to LRP and inter-
The basic amino acid motifs in the conserved regions I and II-plus of the CS protein may bind to highly sulfated heparin-like oligosaccharides in heparan sulfate (unpublished data) and also to the negatively charged SDE (serine-aspartate-glutamate) motifs in the ligand binding domains of LRP and the LDLR (44). The unique heparin-like nature of the liver-specific heparan sulfate (7) may mediate the initial arrest of the CS protein in this organ and our observation of the larger contribution to the CS protein binding of HepG2 cell heparan sulfate (~70%) compared to that from CHO or MEF cells (~45 or ~35%, respectively) supports this notion. HepG2 cells are transformed hepatocytes and in contrast to heparan sulfate from human skin fibroblasts and bovine kidney tissue, the distal two-thirds of rat liver heparan sulfate chains are clearly heparin-like (7). Basic oligopeptide motifs have also been implicated in the binding of lactoferrin and apo E to LRP (11, 47, 48). Significantly, these motifs resemble the clusters of basic amino acids NH₂-terminal from the conserved region I and within region II-plus of the CS protein both of which have been implicated in host cell binding and sporozoite invasion (2, 49, unpublished data). These considerations and our finding that lactoferrin inhibits the binding of the CS protein in the absence of cell surface heparan sulfate to the same degree as the universal LRP-inhibitor RAP (34) suggest the possibility that the CS protein binds to the same site on LRP as apo E and lactoferrin.

We show here that malaria sporozoites utilize the interaction between the CS protein and a dual receptor system for host cell binding. Similarly, P. falciparum-infected erythrocytes with the parasite-derived variant antigen PfEMP-1 bind to CD36, thrombospondin and ICAM-1 (50, 51). Thus, like many other microorganisms (see references in 8), Plasmodium has developed multiple recognition strategies to guarantee its survival in the mammalian host. After the initial binding event, however, malaria sporozoites appear not to depend on the endocytic machinery of the mammalian cell for invasion, since shortly after entry, sporozoites lie free in the cell and release considerable amounts of CS protein directly into the cytosol (52). Thus, in contrast to solu-

![Diagram](image-url)

Figure 5. P. berghei sporozoites depend on the dual binding to heparan sulfate and LRP during host cell invasion. (A) Wild-type CHO-K1 or heparan sulfate-deficient CHO pgsA cells were co-cultivated with P. berghei sporozoites for 1 h at 37°C in the absence or presence of increasing concentrations of lactoferrin (L, concentrations in μg/ml) or transferrin (T). Lactoferrin inhibits sporozoite invasion into both CHO-K1 and pgsA cells in a concentration-dependent fashion to a maximum of 71 and 60%, respectively. Transferrin has no effect. Intra- and extracellular parasites were distinguished by double labeling. Each bar represents the mean number of invaded sporozoites counted in four wells ± SD. Note that whereas the enzyme treatment alone or the blockage by RAP of LRP alone resulted only in 19 and 21% inhibition, respectively. However, elimination of both HSPGs and LRP as CS protein binding sites inhibits sporozoite invasion by 65%.

obtained for the interaction of the CS protein with the surface of HepG2 cells did not fit a one or two-site model using the program LIGAND (35) as might be expected since we used an aggregated preparation of recombinant CS protein for these studies. A more complex interaction may therefore have occurred on the cell surface involving multiple CS protein molecules, LRP and/or heparan sulfate chains, much like that which would occur between a sporozoite and a host cell. Nevertheless, an estimate of the apparent dissociation constants suggests that the overall affinity of the CS protein to the surface of HepG2 cells is ~10 times higher than to purified LRP. This is in agreement with the ~10-fold increased affinity between bFGF and the growth factor receptor 1 in the presence of heparin (44, 45). Malaria sporozoites are covered by a dense surface coat of CS protein and it is probable that the host cell affinity of these relatively large microorganisms exceeds greatly the affinity values presented here.

The basic amino acid motifs in the conserved regions I and II-plus of the CS protein may bind to highly sulfated heparin-like oligosaccharides in heparan sulfate (unpublished data) and also to the negatively charged SDE (serine-aspartate-glutamate) motifs in the ligand binding domains of LRP and the LDLR (44). The unique heparin-like nature of the liver-specific heparan sulfate (7) may mediate the initial arrest of the CS protein in this organ and our observation of the larger contribution to the CS protein binding of HepG2 cell heparan sulfate (~70%) compared to that from CHO or MEF cells (~45 or ~35%, respectively) supports this notion. HepG2 cells are transformed hepatocytes and in contrast to heparan sulfate from human skin fibroblasts and bovine kidney tissue, the distal two-thirds of rat liver heparan sulfate chains are clearly heparin-like (7). Basic oligopeptide motifs have also been implicated in the binding of lactoferrin and apo E to LRP (11, 47, 48). Significantly, these motifs resemble the clusters of basic amino acids NH₂-terminal from the conserved region I and within region II-plus of the CS protein both of which have been implicated in host cell binding and sporozoite invasion (2, 49, unpublished data). These considerations and our finding that lactoferrin inhibits the binding of the CS protein in the absence of cell surface heparan sulfate to the same degree as the universal LRP-inhibitor RAP (34) suggest the possibility that the CS protein binds to the same site on LRP as apo E and lactoferrin.

We show here that malaria sporozoites utilize the interaction between the CS protein and a dual receptor system for host cell binding. Similarly, P. falciparum-infected erythrocytes with the parasite-derived variant antigen PfEMP-1 bind to CD36, thrombospondin and ICAM-1 (50, 51). Thus, like many other microorganisms (see references in 8), Plasmodium has developed multiple recognition strategies to guarantee its survival in the mammalian host. After the initial binding event, however, malaria sporozoites appear not to depend on the endocytic machinery of the mammalian cell for invasion, since shortly after entry, sporozoites lie free in the cell and release considerable amounts of CS protein directly into the cytosol (52). Thus, in contrast to solu-
ble recombinant CS constructs, which remain restricted to the endocytic compartment, malaria sporozoites possess mechanisms to cross mammalian membranes and thereby enable the CS protein to fulfill possible intracellular functions after its association with ribosomes of the host cell (52).

Although elimination of both LRP and HSPGs was sufficient to completely abolish cell surface binding of recombinant CS protein, additional ligand receptor pairs may be involved in sporozoite invasion. Blockage or absence of LRP and HSPGs caused only a 60–80% inhibition of sporozoite invasion. One explanation is that the multimeric high-avidity sporozoite / host cell interaction may not effectively be inhibited with monomeric competitors. Alternatively, native CS protein expressed on the surface of sporozoites and/or other parasite surface molecules such as the thrombospondin-related adhesive protein (TRAP) may contribute to the host cell recognition by binding to the same and possibly also to additional receptors. Support for this comes from the finding that highly sulfated polymers such as fucoiand or dextran sulfate consistently yield an inhibitory effect on sporozoite invasion in the range of 80–90% (3, unpublished data). It is possible that these sulfated polymers block the CS protein and TRAP simultaneously, since both molecules contain region II plus–like heparin-binding motifs (4, 53). Moreover, TRAP contains sequences homologous to the β2-integrin A-type domain superfamily (54) and it is conceivable that malaria sporozoites use this molecule for the binding to additional host cell receptors. Nevertheless, the molecule that is expressed most abundantly on the sporozoite surface is the CS protein and our data suggest that the binding to LRP and HSPGs represents the predominant interaction during parasite invasion.

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