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Plasmodium falciparum circumsporozoite (CS) protein peptides specifically bind to HepG2 cells

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Abstract

Hepatocyte invasion by malaria parasites is mediated by specific molecular interactions. Several lines of evidence suggest the importance of the surface plasmodial circumsporozoite (CS) protein in the sporozoite invasion of hepatocytes. Identification of the sequences involved in binding to hepatocytes is an important step towards understanding the structural basis for the sporozoite–hepatocyte interaction. In this study, binding assays between *Plasmodium falciparum* CS peptides and HepG2 cells were performed. Fifteen overlapping residue 20 mer long peptides, spanning the entire CS sequence, were tested in HepG2 cell binding assays. Five High Binding Activity Peptides (HBAPs) to HepG2 cells were identified: 4593, (NANPNANPNANP); 4383, (NSRSLGENDDGNNEDNEKLR); 4388, (GNGQGHNMPNDPNRNVDENA); 4389, (HNMPNDPNRNVDENANANSA) and 4390, (DPNRNVDENANANSAVKNNN). The HBAP HepG2 interaction is independent of charge and amino-acid composition, but sequence dependent. Four HBAPs (4383, 4388, 4389 and 4390) are bound with similar affinity to a 50 kDa molecule. These HBAPs define three Hepatocyte Binding Sequences (HBSs): HBS-1, located between residues 68 and 87 (HBAP 4383); HBS-11, the repeat NANP region (HBAP 4593), for which anti repeat antibodies are able to specifically inhibit sporozoite invasion of hepatocytes have been reported; and HBS-111, between residues 286 and 315 (HBAPs 4388, 4388 and 4390), respectively. Interestingly, HBS 111 carries two earlier-reported B-epitopes (underlined) in peptides 4388, 4389 and 4390 (GNGQGHNMPNDPNRNVD ENANANSAVKNN) in its sequence. The HBSs reported here show lesser interspecie-variability than the entire protein in species invading the same kind of hepatic cells. This data supports these HBSs' important role in CS-protein function; they could be used as ligand by the sporozoite to invade hepatic cells. © 2001 Published by Elsevier Science Ltd.

Keywords: CS; *Plasmodium falciparum*; Binding peptides

1. Introduction

Malaria is transmitted by *Plasmodium* sporozoites, which are inoculated by *Anopheles* mosquitoes into susceptible vertebrate hosts. A few minutes after injection, the sporozoites are clarified from the blood stream and found in the liver. They cross the Disse space, invade hepatocytes, develop into hepatic schizonts and undergo exoerythrocytic (EE) asexual multiplication [1]. The speed of invasion and target cell selectivity suggest that this process is mediated by specific, sporozoite–hepatocyte interactions.

The plasma membrane of sporozoites is entirely covered by the circumsporozoite (CS) protein, whose major structural features are highly conserved in all known rodent, monkey and human malaria species [2]. The CS protein is one of the most extensively characterized antigens and its role in protection against malaria infection is very well established [3]. It has been used as a target for antimalarial vaccines [4–6]. CS protein antibodies have been implicated in protection against in vivo [7–9] and in vitro [10–13] infection.

Furthermore, specific interaction between the CS protein and the hepatocyte plasma membrane basolat-

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eral domain [14–16], and the finding that intravenously injected CS protein is clarified from the blood circulation and selectively targeted to the liver [17,18], have suggested an important role for this sporozoite surface protein in sporozoites' homing to their hepatic multiplication site. The CS protein contains a signal sequence at its amino terminus, a central repeat region, two conserved amino acid motifs in region I and region II-plus and an anchor sequence at its carboxyl terminus [18–20]. The repeat domain is species-specific, immunodominant and constitutes about one half of the molecule [21].

In *P. falciparum*, this repeated sequences is conserved and composed of 37 NANP and four NVDP repeated sequences [20]. Indirect evidence suggests that the CS protein's repeat-containing central domain is involved in the initial steps of host cell invasion [22]. However, attempts to inhibit in vitro sporozoite invasion with peptides representing the repeats have not been successful [23] and clinical trials with recombinant and synthetic CS protein peptides, aimed at developing specific immune responses to the repeats, have proved disappointing [24,25]. The fact that the sporozoites from different malaria parasite species infect hepatocytes from the same mammalian host [26] suggests that the ligands used by parasites must be conserved.

One of these conserved regions, region I (KLKQP), which is present in the CS protein of all malaria parasites infecting mammalian species, is not present in the *P. gallinaceum* CS protein. Earlier studies had shown that a synthetic peptide corresponding to region I binds to 30–50-kDa hepatocyte surface proteins, and that antibodies against this region inhibit *P. falciparum* sporozoite in vitro invasion of Hep-G2 cells [23]. Another report has recently shown that synthetic peptides representing region I inhibited the binding of the recombinant CS protein to Hep-G2 cells by 62%, in a concentration-dependent manner [27]. The region II-plus is an 18-amino acid motif, which is highly conserved in CS protein malaria parasites [28]. This region II amino-acid sequence has homology with other sporozoite surface proteins such as TRAP [29,30] and a variety of host proteins such as thrombospondin or properdin [31–33].

The amino-acid sequence for this region II includes two cysteines at the amino-terminal and a series of basic and hydrophobic amino acids at the COOH-terminal. In *P. falciparum* it is represented by EWSPCSVTCGNGIQVRIK. Several reports have shown that synthetic peptides representing this CS protein conserved region II-plus inhibited CS protein interaction with hepatocytes as well as sporozoite invasion of Hep-G2 cells [14,15,34–36].

Several studies have shown that the circumsporozoite (CS) protein's specific interaction with heparin sulfate proteoglycans (HSPGs) on the hepatocyte basolateral surface in the Disse space [14,15,17,18,37] is responsible for the rapid and specific homing of sporozoites to the liver. However, *P. berghei* sporozoites were able to invade the mutant CHO cells, which do not express any glycosaminoglycans on their surface [16].

Some other studies have revealed that the CS protein also interacts with low-density lipoprotein receptor-related protein (LRP) present on hepatocyte cell surface [38]. However, although the simultaneous elimination of LRP and HSPGs inhibits recombinant CS protein binding almost completely, this does not completely inhibit sporozoite invasion [16,38]. On the other hand, the identification of the exact residues involved in binding has yielded discrepant results [18,23,27,35,39]. Taking this data into account, an attempt was made to find hepatocytes binding sequences from *P. falciparum* CS protein (NF54 strain) [40], which might be implicated in sporozoite invasion of hepatocytes. Four high specific binding activity peptides were found from amongst 41 fifteen overlapping 20 mer peptides covering the entire protein length.

2. Materials and methods

2.1. Peptide synthesis

Fifteen overlapping residue 20 mer long peptides were synthesized by the solid phase method [41,42] with 100 mg *p*-methyl benzyhydrylamine resin (substitution 0.74 meq/g) Standard N t-Boc protected amino-acids were employed (Bachem). Peptides were cleaved by the Low-High HF technique [43], purified by RP-HPLC and freeze-dried. Amino acid analysis was performed on all peptides and amino acid sequences for those having high binding activity. The synthesized peptide sequences are shown in Fig. 1 in one-letter code. Peptides, which did not contain Tyr were added to the carboxiterminal end to allow radio labeling.

2.2. Human hepatoma cell

The cloned HepG2-A16 human hepatoma cell line [44,45] was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, ICN), penicillin (100 IU/ml, ICN), streptomycin (100 μg/ml, ICN) and amphotericin B (0.25 μg/ml, ICN), vitamins (ICN) and non-essential amino-acid solution (Gibco). The cells were grown as monolayers in 75–150 cm^2 culture flasks coated with a 2% collagen solution (Nunc and Falcon) at 37 °C in a 5% CO_2 atmosphere. Then 0.25% trypsin and 0.1% EDTA were added to the culture to detach them from the culture flask after 4 days of culture and expansion; cells were then kept in similar conditions to those described above. HepG2 cells were harvested by adding PBS-EDTA, followed by centrifugation. The

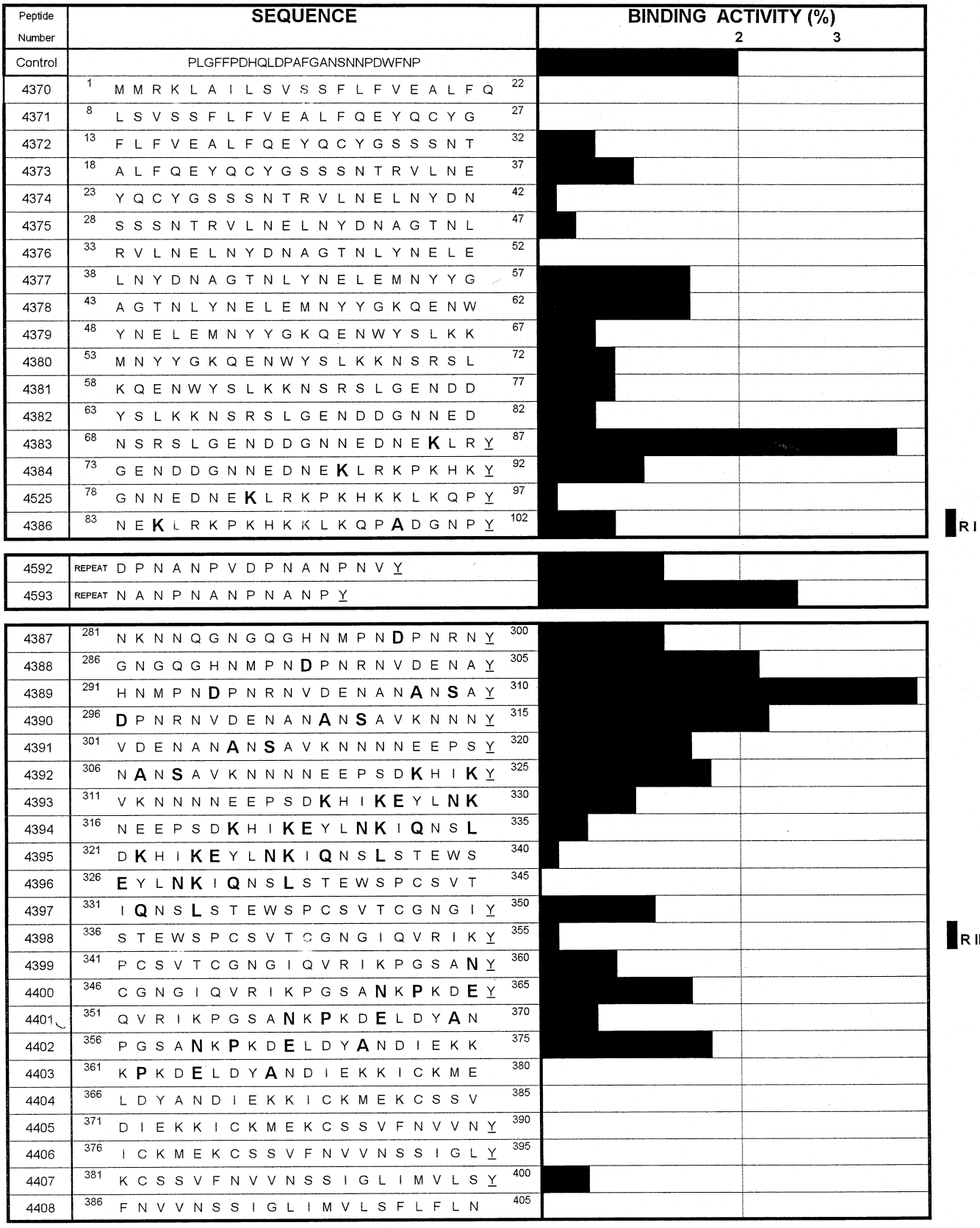

Fig. 1. *P. falciparum* CS protein peptide specific binding activity with HepG2 cells. The column on the left shows the amino acid sequences, indicating the position of each peptide in the protein. In those peptides that did not contain Tyr, this was added (underlined) at the carboxy-terminal. Amino-acids labeled in bold are variable. The column on the right shows each peptide's specific binding activity. This was constructed from specific binding slopes (specifically bound peptide/added peptide). The dotted line separates peptides with binding activity $\geq$ the positive control peptide, considered as having high specific binding activity to hepatocytes and called HBAPs.

cells were washed with PBS and counted in a Newbawer chamber with trypan blue and their viability assessed before using in binding assays.

2.3. Radio labeling

Peptides were labeled with ^{125}I [46]; 3.2 μl Na ^{125}I (17.2 mCi/μg) oxidized with 12.5 μl chloramine T (2.25 μg/μl) and added to 5 μl peptide (1 μg/μl) for 1 min at room temperature. The reaction was stopped by addition of 50 μl sodium bisulfate (2.75 μg/μl) and 50 μl isotonic PBS (pH 7.4).

The radio labeled peptide was separated from reaction products by elution with PBS in a Sephadex G-10 column (Pharmacia, Upsala, Sweden) at a 300 μl/min flow rate.

2.4. Binding assay

Increasing concentrations of radio labeled peptide (2, 4, 8, 12 nM) were incubated with a hepatocyte suspension (10^4 cells/μl), in the absence (total binding) or presence (non-specific binding) of unlabelled peptide (1.25 μM) and incubated for 1 h at room temperature, according to the methodology earlier described for red blood cells (RBC) [47]. After 1 h, the unbound peptide was separated from cells by spinning through a mixture of dibutylphthalate–dioctylphthalate cushion ($d =$ 1.015 g/ml) [48] and centrifugation at $5000 \times g$ for 3 min. Cell-bound peptide was measured in an automatic gamma counter (4/200 plus ICN Biomedicals, INC.) The assay was carried out in triplicate under identical conditions for each radio labeled peptide concentration.

Specific binding was calculated as being the difference between total and non-specific binding. For each peptide, the specific binding curve slope (specific bound CPM/ total added CPM) was determined. The methodology was standardized by using the 27 mer 4980 peptide (PLGFFPDHQLDPAFGANSNNPDWDFNP), a hepatitis B virus peptide which specifically binds to HepG2 cells [49]. This peptide's binding activity to HepG2 cells is about 2% and it was used as control peptide. Based on earlier criteria [46,47], peptides with binding activity greater than or equal to the positive control peptide, were considered as having high specific binding activity to hepatocytes and called high binding activity peptides (HBAPs). High binding peptides' saturation curves were performed with 10^4 cells/μl, ^{125}I-Iabelled peptide at concentrations between 5 and 400 nM, in absence or presence of 40 μM unlabelled peptide.

2.5. Competition binding assay

In order to identify the residues involved in peptide binding to hepatocytes, a competition binding assay was carried out with the HBAPs and their glycine analogue peptides [50,51]. HepG2-A16 (10^4 cells/μl) were incubated with 20 nM ^{125}I-labelled HBAP and with 0, 10,100 and 800 nM of each non-radio labeled original or analogue peptide for 1 h at room temperature. The unbound peptide was separated from cells by spinning through a mixture of dibutylphthalate–dioctylphthalate cushion ($d = 1.015$ g/ml) [48], at $5000 \times g$ for 3 min. The cell-bound peptide was measured in an automatic gamma counter (4/200 plus ICN Biomedicals, INC). The assay was done in triplicate. Critical amino-acids were defined as being those residues, which, if changed for glycine, then the analogue peptide would not be able to inhibit ^{125}I-labelled high binding peptide binding by at least 50%.

2.6. HepG2 cell membrane preparation and purification

Membranes were obtained by earlier reported methodology [52]; 2.5 million cells were suspended in 6 ml buffer A (Sucrose 0.25 M, Tris–HCl 5 mM pH 8.0, $MgCl_2$ 1 mM) and passed through 27 gauge syringe ten times. The mixture was centrifuged at $250 \times g$ for 5 min at 4 °C; the supernatant was withdrawn and stored at 4 °C. The pellet was suspended in buffer A and this step was repeated twice. The supernatants were centrifuged at $1500 \times g$ for 10 min at 4 °C and the pellets were suspended in buffer A, pooled and centrifuged for 30 min at $16000 \times g$, at 4 °C. The supernatant was discarded and buffer B (Sucrose 1,42 M, Tris–HCl 5 mM pH 8.0, $MgCl_2$ 1 mM) was added to the pellet. This solution was poured into the bottom of the tube and, afterwards, 2 ml buffer B and 1 ml buffer A were added on top. The sucrose gradient was centrifuged at $80000 \times g$ for 60 min at 4 °C. The ring observed in the interface was suspended in PBS and centrifuged for 60 min at $16000 \times g$. The pellet composed of plasmatic membranes was suspended in 1 ml PBS and stored at −20 °C

2.7. Binding-assays and SDS-PAGE

Washed HepG2-A16 cells (10^4 cells/μl) were incubated with 100 nM ^{125}I-labelled peptide in the absence or presence of unlabelled peptide (1 μM) for 2 h at 4 °C. The cells were centrifuged at $10000 \times g$, for 5 min at 4 °C. Supernatant was discarded and the pellet was washed with PBS. The pellet was incubated with 20 μM Bis (sulfosuccinimidyl suberate) (Bs3, Pierce), for 20 min at 4 °C and the reaction was stopped with Tris–HCl 0.1 M, pH 6.8. The cells were washed with PBS, then the cell-membranes were obtained and purified as described above. These membranes were treated with Laemmli buffer, heated for 2 min at 90 °C. Solubilized proteins were separated by 12% SDS-PAGE [53], exposed for 24 h at −70 °C and developed autoradio-

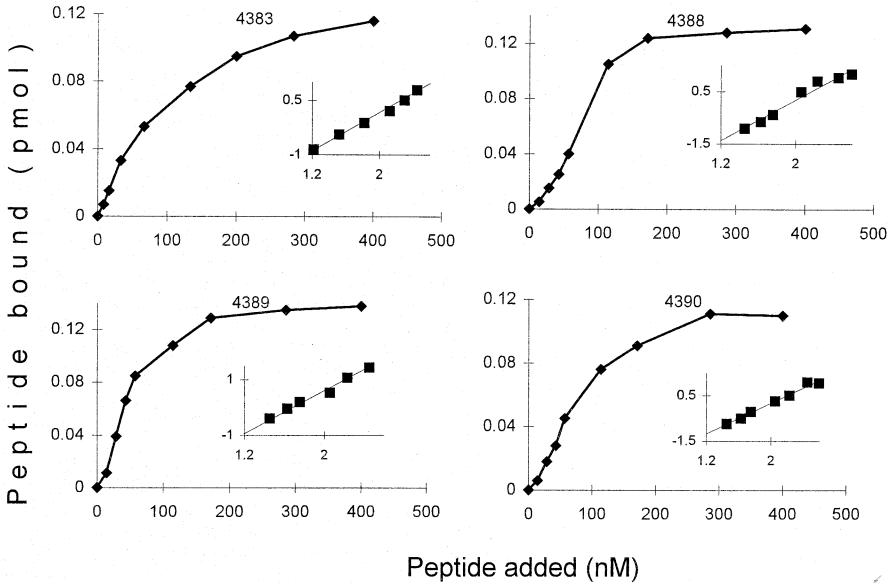

Fig. 2. Saturation curves for peptides 4383, 4388, 4389 and 4390 with HepG2 cells. The large graphs correspond to saturation curves and the smaller are of the Hill graphs for each peptide. The X axis is: $\log(F)$, the Y axis is $\log(B/\mathrm{BmX}-B)$. F is free peptide concentration (nM). B represents specifically bound peptide picomoles. Bmx represents maximum specifically bound peptide picomoles. Each point was done in triplicate.

graphically on Kodak film (X_OMAT). The labeled bands' molecular weight was determined by comparison with the apparent standard weight markers low range (BIO-RAD).

3. Results

The binding assay to HepG2 cells was standardized with a peptide from hepatitis-B virus that has been reported as having a high binding activity to HepG2 cells [49] (this peptide was chosen as the reference peptide). This binding assay was carried out at concentrations of radio labeled peptide between 2 and 12 nM. Forty-one 20 mer peptides, having 15 overlaps, were tested in this assay. Five of them showed higher binding than reference peptide (Fig. 1). HBAP 4383 (NSRSLGENDDGNNEDNEKLR) is located close to region I. Only 4593 (NANPNANPNANP) from the repeat region was considered to be HBAP. A hepatocyte binding region was found next to the repeat region (residues 286–315) formed by HBAPs 4388 (GNGQGHNMPNDPNRNVDENA), 4389 (HNMPNDPNRNVDENANANSA) and 4390 (DPNRNVDENANANSAVKNNN).

The affinity constants, the number of binding sites per cell and HABP (4383, 4388, 4389 and 4390) Hill coefficients were determined by saturation binding assays to HepG2 cells (Fig. 2). Affinity constants were between 50 and 80 nM (Table 1), indicating these sequences' high affinity for hepatocytes. The number of binding sites was around 130000 per cell. The Hill coefficients were 1.0 for peptide 4383 and 2.0 for the other HBAPs, suggesting a simple interaction for HBAP 4383 and positive cooperativity for the other HBAPs.

Critical residues for HABP binding to HepG2 cells were determined by competition binding assays between ^{125}I-labelled HABPs and their glycine analogues. The change of one of these residues by glycine in a HBAP, diminishing ^{125}I-Iabelled HABP-binding to HepG2 cell inhibition capability by more than 50%, led to it being considered as a critical residue (Fig. 3). These critical residues, located along the sequence, are 47% polar, 18% non polar and 35% charged. The most frequently found critical residue was asparagine. Critical residues for peptides 4383 (NSRSLGENDDGNNEDNEKLR); 4388 (GNGQGHNMPNDPNRNVDENA); 4389 (HNMPNDPNRNVDENANANSA) and 4390 (DPNRNVDENANANSAVKNNN) are underlined.

To identify the putative hepatic receptor(s), HepG2 cells membrane, earlier incubated with each radio labeled HBAP, were cross linked and membrane proteins separated by SDS-PAGE. The 4383, 4388, 4389 and 4390 HBAPs from CS protein bind to molecules located on HepG2 cell membrane surface (Fig. 4). The

Table 1
Binding constants of CS peptides to HepG2 cells

Peptide	Kd (nM)	n	Binding sites per cell
4383	80 ± 8	1.0 0.1	$120\,000 \pm 11\,500$
4388	80 ± 11	2.0 0.2	$140\,000 \pm 12\,000$
4389	50 ± 6	2.0 0.2	$140\,000 \pm 12\,000$
4390	80 ± 9	2.0 0.2	$120\,000 \pm 10\,000$

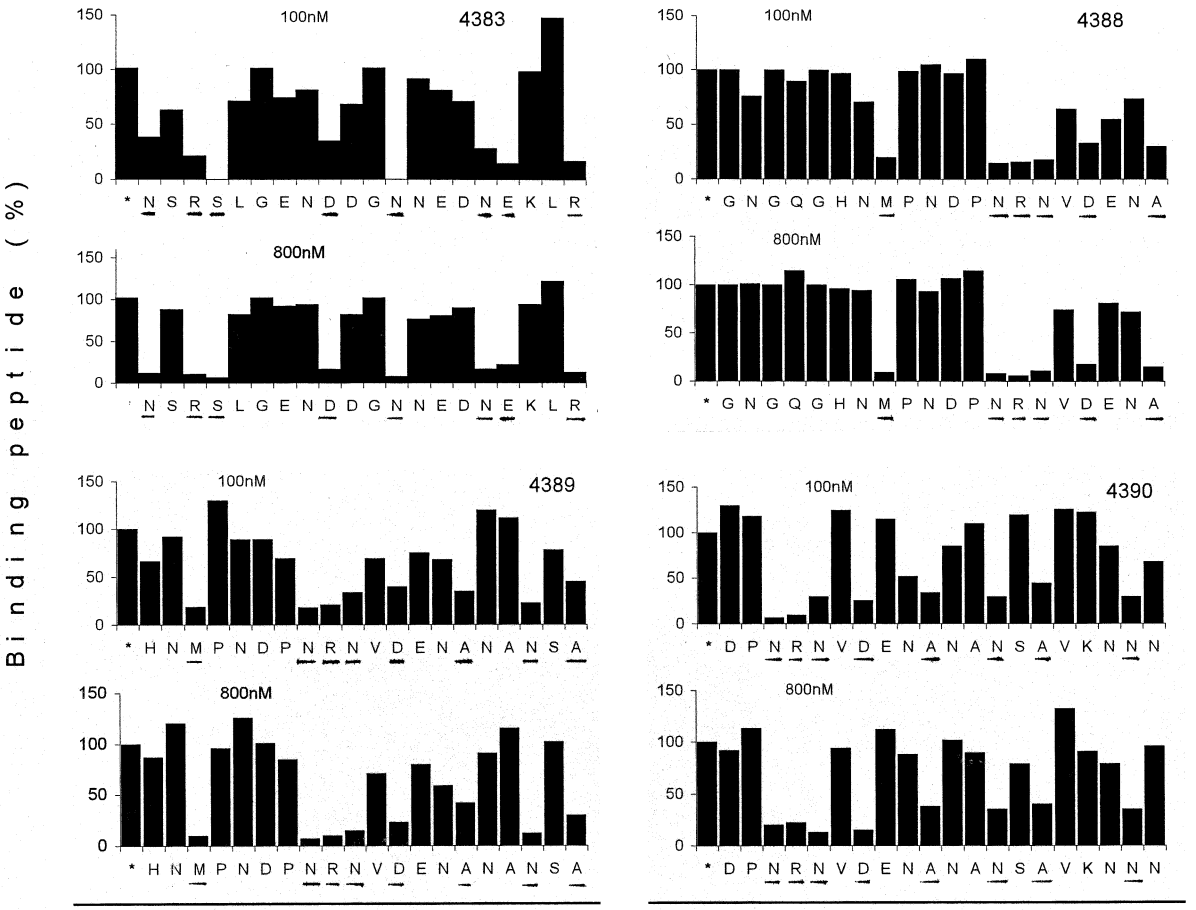

Fig. 3. Specific binding of HBAPs to HepG2 cells in competition binding assays with glycine analogue peptides The height of each bar indicates bound ^{125}I-peptides 4383, 4388, 4389 and 4390 inhibited by original (*) or by their respective analogues. Each assay was done in triplicate and used three different unlabeled peptide concentrations: 10, 100 and 800 nM. Only two concentrations (100 and 800 nM) are shown.

autoradiography for 4383 and 4389 peptides showed a strong band of about 50 kDa. This band disappeared when the binding assay was performed in presence of an excess unlabelled peptide.

4. Discussion

CS-protein may be playing an important role during sporozoite invasion of hepatocytes. It has earlier been demonstrated that CS protein binds specifically to hepatocyte membrane zones exposed to the blood stream in the Disse space [14]. The CS protein does not bind to endothelial or Kupffer cells in the liver, nor to cells from other organs.

This study reports three Hepatocyte Binding Sequences (HBSs) which specifically bind to HepG2 cells. These sequences are located between residues 68 and 87 (HBS-1), the repeat region NANP (HBS-11) and residues 286 and 315 (HBS-111). In HBS-1, the residues between 68–72 (NSRSLGENDDGNNEDNEKLR) and 83–87 (NSRSLGENDDGNNEDNEKLR) seem to be very important for HepG2 binding, since peptides upstream of 4382 and downstream of 4384, not containing these residues, did not show any high binding activity to hepatocytes. Furthermore, the critical residues (N_{68}, R_{70}, S_{71}, N_{83}, E_{84} and R_{87}) for binding to HepG2 cells are located in these implicated sequences. For HBS-111 (residues 286–315), the residues between 297 and 305 (HNMPNDPNRNVDENANANSA) seem to be very important for HepG2 binding, since peptides 4387 (residues 281–300) and 4391 (residues 301–320) did not show any high binding activity to hepatocytes. Also, the critical residues N_{298}, R_{299}, N_{300}, D_{302} and A_{305} are located in this zone.

Peptides 4383, 4388, 4389 and 4390 specificity for human hepatocytes is also demonstrated by the fact that these peptides do not bind to human erythrocytes nor to the C32 epithelial cell line (data not shown). The specific binding of these four HBAPs is saturable (Fig. 2). The dissociation constants (Table 1) for these HBAPs are below 100 nM, showing a high affinity for human hepatocytes. The Hill coefficients are 1.0 for HBAP 4383, indicating a simple interaction, and 2.0 for HABPs 4388, 4389 and 4390, showing a cooperative interaction. There may be positive cooperativity for

these HBAPs, indicating an increase of affinity with progressive binding. The number of binding sites per cell (120000–140000) is sufficiently high to be relevant to hepatocyte invasion.

It is likely that critical amino-acids could be implicated in maintaining the peptide structure or directly implicated in the binding to HepG2 cells. The interaction with HepG2 cells seems to be mediated by polar and charged residues since these residues represent around 80% of the critical residues. The most implicated residue in this interaction is asparagine. It can be clearly seen in this study that HBAP HepG2 binding is independent of charge and peptide amino-acid composition, but is sequence dependent. For example, in HABP 4383 the replacement of the same residue in different positions ($\underline{D}_{76}$ and D_{77} or $\underline{N}_{79}$ and N_{80}: critical residues are underlined) shows very different effects on binding activity, regardless of their proximity in the sequence.

The nature of HepG2 cell receptors for HABPs is unknown; however, the HBAPs are bound with similar affinity to a 50 kDa molecule. The radio labeled HABPs interaction with this 50 kDa protein is specific and inhibited by the same non-radio labeled peptide. This band shows similar apparent molecular weight to that earlier reported for HepG2 peptide binding protein and to a protein that inhibited *P. falciparum* sporozoite protein binding and in vitro sporozoite invasion [23].

Our data also shows that these three HBSs bind to HepG2 cells with higher affinity than other earlier reported sequences such as the region II-plus conserved sequence (EWSPCSVTCGNGIQVRIK) [14,18,39] entirely contained in our 4398 low activity binding peptide. Similarly, region I conserved sequence (KLKQP) [23,27] is also contained in our 4525 and 4586 low activity binding peptides. These sequences have been proposed as being the CS ligands used in hepatocyte invasion. However one has to stress that peptides containing these reported 4386, (NEKLRKPKHKKLKQPADGNP); 4397, (IQNSLSTEWSPCSVTCGNGI) and 4400, (CGNGIQVRIKPGSANKPKDE) HepG2 binding sequences also show some degree of HepG2 binding activity, but with low affinity. The differences could be due to the highly stringent and specific conditions of our methodology used for detecting HABPs.

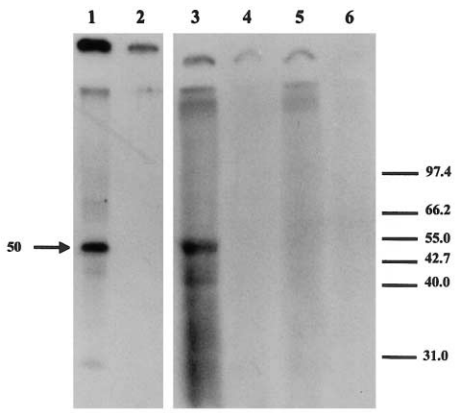

Fig. 4. Binding of peptides 4383 and 4389 to human hepatocytes. HepG2 cells were incubated with ^{125}I-labelled peptide 4383 (100 nM) in the absence (lane 1) or presence (lane 2) of unlabelled peptide 4383 (1 μM) and with ^{125}I-labelled peptide 4389 (100 nM) in the absence (lane 3) or presence of unlabelled peptide 4388 (lane 4), 4389 (lane 5) and 4390 (lane 6) (1 μM). After the binding, cells were washed, BS3-crosslinked, membranes were obtained and the samples were electrophoresed and ^{125}I-labelled bands were visualized by autoradiography.

HBAP 4383 also showed similarity with HBAP 4388 and these two HBAPs present one critical residue in common (R_{87} and R_{299}). Interestingly, HBS-111 also showed 43.3 and 41.4% identity with the NVDPNANP and NANPNANP repeats in which HBAP 4593 is located. However, it has been reported that a peptide with the same 4593 peptide sequence located in the repeated HBS-11 was not able to inhibit CS recombinant protein binding to HepG2 cells. It has also been reported that antibodies against the repeat region are able to specifically inhibit sporozoite invasion of hepatocytes and that there is indirect evidence that the repeat region may be involved in the initial steps of hepatic cell invasion [22]. It was also shown that repeat sequence peptides from different *P. falciparum* CS protein strains specifically bind to HepG2 cells (data not shown). Interestingly, HBS-111 carries two earlier reported B-epitopes in its sequence (GNGQGHNMPNDPNRNVDENANANSAVKNN) [54].

HBS-1 and HBS-111 peptides bind to a protein on hepatic cell surface (HepG2) having similar molecular weight. Moreover, affinity constants and number of binding sites per cell are similar; suggesting that the HepG2 receptor for these HBSs could be on the same protein, since different sequences are probably binding to the same region. This might be an advantage for sporozoites evading the immune system. This data could also explain controversial results for the repeat region, since hepatocyte binding sequence cross-reactive antibodies could efficiently inhibit CS protein binding to hepatic cells, but non-cross-reactive antibodies could not. However, it remains to be determined if antibodies against these hepatocyte binding regions show cross-reactivity; which could increase antibodies' capability to inhibit CS binding to HepG2 cells. The results shown here strongly support an important role for these HBSs in CS-protein function, maybe as ligand used by the sporozoite to invade hepatic cells.

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