SYNTHETIC PEPTIDES FROM THE CIRCUMSPOROZOITE PROTEINS OF *PLASMODIUM FALCIPARUM* AND *PLASMODIUM KNOWLESI* RECOGNIZE THE HUMAN HEPATOMA CELL LINE HepG2-A16 IN VITRO

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Malaria sporozoites, upon injection by an infected anopheline mosquito, are rapidly and specifically localized in the hepatocytes of the mammalian host. Inhibition of invasion by mAbs or specific sera suggests that the circumsporozoite (CS)[1] protein plays a major role in this process (1–5), but the specific mechanisms involved are not understood. We have investigated (6) the possibility that the N1 conserved region of the CS proteins from *Plasmodium falciparum* and *Plasmodium knowlesi* is involved in sporozoite/hepatocyte recognition. Using synthetic peptides based on this region and the cloned human hepatoma cell line, HepG2-A16, as a target cell for invasion by *P. falciparum* sporozoites (3), we present evidence that *P. falciparum* sporozoites could use cell recognition of or by this portion of the CS protein as a part of the hepatocyte invasion process. Chemical crosslinking of radiolabeled N1 peptide to the HepG2-A16 cell line identified two hepatocyte proteins involved in peptide recognition.

Materials and Methods

Peptides were synthesized by the stepwise solid-phase method (7) on a multidetachable resin (8) (*p*-acyloxybenzhydrylamine-copolystyrene-1%-divinylbenzene). *t*-butyloxycarbonyl (Boc)–amino acids with benzyl alcohol–derived side chain–protecting groups were attached on *p*-acyloxybenzhydrylamine resin (0.4 mmol/g) and the peptides were assembled on a 990 M synthesizer (Beckman Instruments, Inc., Fullerton, CA). A double-coupling protocol via dicyclohexylacarbodiimide was used to give a coupling efficiency of 99.7% completion per step. Cleavage of peptides was effected by the degradative deprotection method (8). The imidazole-DNP protecting group was removed by 1 M thiophenol in dimethylformamide and the benzyl protecting groups by a mixture of reagent $CF_3SO_3H/CF_3CO_2H/CH_3SCH_3$/*p*-cresol (10:50:30:10, vol/vol) at 10°C for 4 h. The crude and unprotected peptide still attached to the resin was then released as peptide-*p*-hydroxybenzhydrylamine by methylaminolysis in CH_3NH_2/ethanol/tetrahydrofurane (10:45:45, vol/vol) at 20°C for 16 h.

The peptide-*p*-hydroxybenzhydrylamine was purified on a reverse-phase C-18 column (2.5 × 30 cm) using aqueous CF_3CO_2H (0.05%) and acetonitrile (80%) gradient. In some

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[1] *Abbreviations used in this paper:* CS, circumsporozoite; DSS, disuccinyl suberimidate; ISI, inhibition of sporozoite invasion; MHBI, methyl-*p*-hydroxybenzimidate; PfN1, *P. falciparum* N1 peptide.

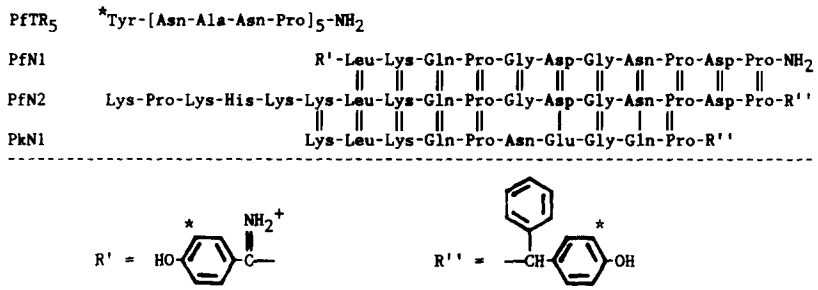

FIGURE 1. Description of peptides used for study. Sequences are aligned for maximal homology. ‖, conserved amino acids. |, conservative amino acid substitutions. Asterisk, iodination sites.

cases the *p*-hydroxybenzhydrylamine handles of the peptides were removed by acid hydrolysis in CF_3SO_3H/CF_3CO_2H/*p*-cresol (2:88:10, vol/vol) for 1 h, and the peptides were further purified on a reverse-phase C-18 column as described above. The purified material gave a single symmetrical peak on analytical HPLC, and the correct molar amino acid rations upon 6 N HCl hydrolysis. Designations and amino acid sequences for each of the peptides are given in Fig. 1.

To provide a phenolic site for radio-iodination, the peptide PfN1 was derivatized at the amino terminus with methyl-*p*-hydroxybenzimidate (MHBI; Pierce Chemical Company, Rockford, IL) as described by Tolan et al. (9). Peptides PkN1 and PfN2 were purified with removable *p*-hydroxybenzhydrylamine handles that were suitable for radio-iodination. Peptide $PfTR_5$ contained a tyrosine residue to permit radio-iodination.

Each peptide was radiolabeled using $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) and Iodogen (Pierce Chemical Co.) as previously described (10). Uncoupled iodide was removed by passing the radiolabeled peptides through Dowex 1 (Sigma Chemical Co., St. Louis, MO). Typical specific activities of radiolabeled peptides were: *P. falciparum*-N1 peptide (PfN1), 3.8 μCi/μg; PfN2, 7.2 μCi/μg; *P. knowlesi* N1 peptide (PkN1), 5.2 μCi/μg; and $PfTR_5$, 8.8 μCi/μg.

Purified IgG from rabbit serum raised against the PfN1 was generously provided by Dr. Victor Nussenzweig, Department of Pathology, New York University Medical Center.

Human hepatoma cloned cell line HepG2-A16 was cultured in MEM (Earl's) supplemented with 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin in 5% CO_2 in air at 37°C (11).

The binding of peptides to hepatoma cells was assayed by incubating peptides with cells grown on Thermolux coverslips (LUX; Miles Laboratories Inc., Naperville, IL) in 0.45 ml complete medium and incubated in 5% CO_2 at 37°C for 1 h. Coverslips were then washed five times with Dulbecco's PBS (Gibco Laboratories, Grand Island, NY) and bound peptide was determined by counting the coverslips directly in an LKB CliniGamma counter (LKB Wallac, Turku, Finland).

P. falciparum sporozoites were isolated from the salivary glands of *Anopheles stephensi* mosquitoes fed 14 d previously on in vitro cultures of gametocytes of strain NF-54. Inhibition of invasion assay (ISI) was performed by the addition of 30,000 sporozoites in the medium containing control or immune serum to a confluent monolayer of hepatoma cells on a 1-cm glass coverslip, incubating for 2.5 h, and counting invaded sporozoites as previously described (3).

For crosslinking of peptide to associated hepatoma cell proteins, radiolabeled peptide was incubated with cells in Earl's balanced salt solution for 1 h at 37°C in 5% CO_2 in air. Cells were washed twice and incubated in Earl's salts containing 200 μg/ml of disuccinyl suberimidate (DSS; Pierce Chemical Co.) for 20 min at 24°C. Reaction was stopped by the addition of excess glycine for an additional 20 min at 24°C. The medium was removed and the adherent cells were solubilized in 0.0625M Tris-HCl, pH 6.8, containing 10% glycerol, 5% SDS, and 5% 2-ME. Solubilized proteins were separated by SDS-PAGE

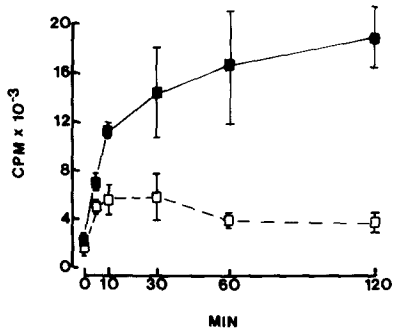

FIGURE 2. Specific uptake of PkN1 by HepG2-A16 cells. ^{125}I-PkN1 was incubated with hepatoma cells as described in text in the presence or absence of unlabeled, homologous peptide. Cell-associated label was plotted as a function of time. (■) ^{125}I-PkN1, (□) ^{125}I-PkN1 plus 10 μg PkN1 per ml.

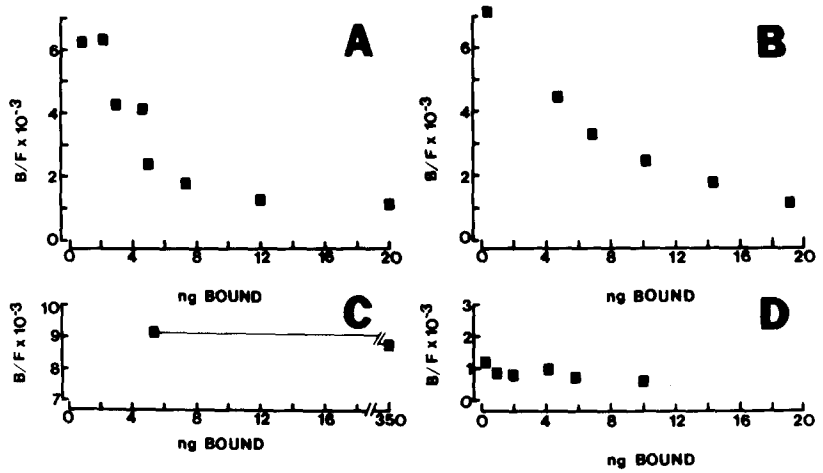

FIGURE 3. Scatchard analysis, equating cell accumulation with binding, of various peptides derived from either *P. falciparum* or *P. knowlesi* CS protein. Various concentrations of ^{125}I-peptides were incubated with hepatoma cells as described in text and cell associated and free concentration determined directly. (*A*) PfN1 peptide; (*B*) PkN1 peptide; (*C*) *P. falciparum* repeat region, Tyr-(NANP)$_5$; (*D*) PfN2 region.

according to the method of Laemmli and Favre (12), and proteins crosslinked to radiolabeled peptide were visualized by autoradiography on XAR-5 film (Eastman Kodak Co., Rochester, NY) at −70°C using a Cronex Lightning plus enhancing screen (DuPont Co., Wilmington, DE).

Results

Peptide Binding. Human hepatoma cells, incubated with ^{125}I-labeled PfN1 or PkN1, rapidly accumulated radiolabel (Fig. 2). This accumulation was largely inhibited by the simultaneous addition of 10 μg/ml of unlabeled peptide, indicating that the process was specific and not due to random endocytic uptake. When these data were plotted as for a Scatchard analysis, equating binding and accumulation, similar specific binding curves were seen for N1 peptides from either species (Fig. 3, *A* and *B*). Saturation of available receptors occurred at a

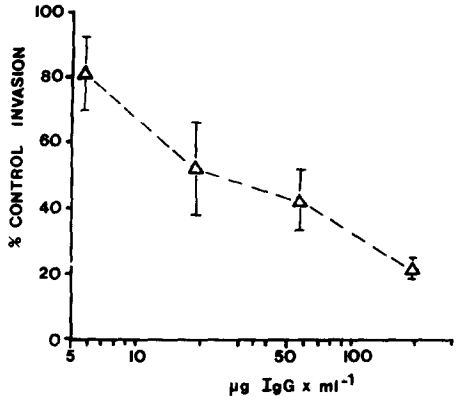

FIGURE 4. ISI by IgG against the PfN1 peptide. *P. falciparum* sporozoites were incubated in the presence of Ig and the resulting invasion rate was calculated as a percentage of invasion in the presence of normal rabbit Ig. ISI_{50}, 20 μg IgG per ml.

concentration of ~10 μg/ml of peptide. Binding of the N1 peptides was inhibited equally well by unlabeled peptide from either species of malaria. In combination, the inhibition was additive, but not synergistic (data not shown).

A synthetic peptide containing five copies of the repeat sequence of the *P. falciparum* CS protein, $PfTR_5$ (Fig. 1), showed no specific (saturable) accumulation by cells, although consistently high nonspecific association was seen even at extreme concentrations of peptide (Fig. 3*C*). PfN2, a peptide that contains the entire PfN1 sequence plus an additional five amino acids, including three lysine residues, from the *P. falciparum* CS protein sequence, did not show any significant cell association, either specific or nonspecific (Fig. 3 *D*).

ISI Assay of Anti-PfN1. Purified rabbit IgG against PfN1 was tested for inhibition of sporozoite invasion in the ISI assay. These antibodies were strongly inhibitory, with an ISI_{50} at a concentration of about 20 μg/ml (Fig. 4).

Crosslinking Studies. To identify possible hepatic cell receptor proteins, HepG2-A16 cells previously incubated with radiolabeled N1 peptide were treated with the bifunctional reagent, DSS, to crosslink the N1 peptide with the putative hepatocyte receptor. These cells were solubilized, and total proteins were separated by SDS-PAGE. In the presence of crosslinker, radioactive ligand was found to be covalently linked to two polypeptides with M_r of 55,000 and 35,000 (Fig. 5*A*). A substantial portion of the radiolabel, presumably the peptide itself, was found near the dye front of the gel in all samples regardless of whether or not crosslinking reagent was added (Fig. 5*B*). Inclusion of 10 μg/ml unlabeled peptide in the initial incubation with PkN1 greatly reduced all cell-associated label.

Discussion

Because of the ability of antibodies against the repeat region of the CS protein to inhibit malaria sporozoite invasion of hepatic cells, the CS protein is thought to have an important role in the recognition or invasion process. However, previous work in this laboratory (Aley, S., personal communication) using either synthetic peptides or recombinant proteins containing the tandem repeat region

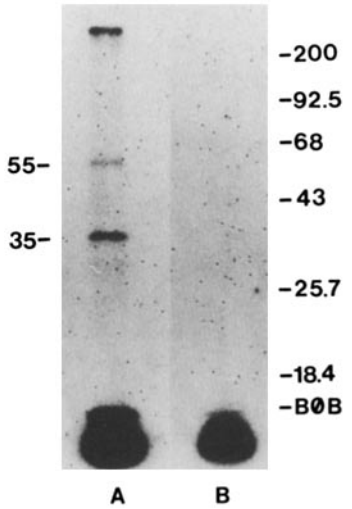

FIGURE 5. Autoradiogram of human liver cell proteins specifically crosslinked to radiolabeled N1 peptide, PkN1. HepG2-A16 cells were incubated with radiolabeled PkN1, washed, and then treated with 200 μg/ml DSS. Unreacted DSS was blocked by addition of glycine. Proteins were solubilized in SDS sample buffer and separated by SDS-PAGE on 10% acrylamide gels. Proteins covalently linked to the radiolabeled N1 peptide visualized by autoradiography. (*A*) ^{125}I-PkN1, crosslinked by DSS to hepatoma cell proteins. (*B*) ^{125}I-PkN1, without subsequent crosslinking by DSS. Molecular weight standards are: myosin (200,000), phosphorylase B (92,000), BSA (68,000), OVA (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and bromphenol blue (BΦB).

of the *P. falciparum* CS protein failed to demonstrate any specific affinity for liver cells.

In this study we present a sequence of about 10 amino acids (the N1 region), immediately adjacent to the repeat of the CS protein, as a candidate for the sporozoite ligand in hepatocyte invasion. Synthetic peptides of the amino acid sequence from this region of either the *P. falciparum* or *P. knowlesi* CS protein were rapidly and specifically accumulated by human liver target cell line under conditions identical to those used for sporozoite invasion. Accumulation did not occur with cell lines that are not readily invaded by *P. falciparum* (data not shown). This accumulation was progressively inhibited by inclusion of increasing amounts of unlabeled ligand, giving a typical Scatchard plot and implying a saturable, specific association. The accumulation was not associated with the presence of the iodinatable handles on the peptides, as the peptide PfN2 also contained a similar handle but demonstrated no binding activity toward the hepatoma cells. The nonbinding PfN2 also contained more positively charged amino acids, eliminating ionic interaction with the negatively charged cell surface as a major factor. A synthetic peptide consisting of five copies of the tetrapeptide repeat sequence from the *P. falciparum* CS protein likewise did not demonstrate any specific, saturable cell association; however, this peptide did show a relatively high nonspecific association over a broad range of concentrations.

The lack of binding by the extended *P. falciparum* N1 region, PfN2, has interesting implications for the identification of the amino terminus of native CS protein. A common feature of plasmodial sporozoites is that the malaria CS

protein is processed into at least three different forms (1, 2), but the nature of the process is as yet unknown. It is interesting to speculate that the multiple lysine residues immediately preceding the *P. falciparum* N1 region might act as a target for proteolytic enzymes from either the mosquito salivary glands, the sporozoite, or the host, activating the protein for binding, and that the CS protein might not be functional in cell recognition until it is properly processed.

Inhibition of invasion by antibodies against the N1 region of the CS protein is consistent with a major role of this region in sporozoite invasion. The concentrations used for 50% inhibition are only 5–10-fold higher than those required with highly immunogenic repeat region peptides (5). Antibodies against several synthetic peptides of CS protein sequences outside of the repeat and N1 regions were not inhibitory in similar assays (13). Inhibition of invasion by sera against either repeat or N1 regions may be explained in one of two ways. Because the N1 and repeat regions are immediately adjacent there is the possibility of antibodies against one region having steric or conformational effects on the adjacent sequence. A more interesting possibility is that both regions are involved, the repeat region providing a nonspecific affinity for cell surfaces and the N1 region providing specific interactions, stimulating or assisting in invasion. In this second case the specific interactions of the N1 region would impose stringent requirements on sequence and structure, with the result that its sequence is highly conserved through a wide range of species (14). The general functions of the repeat region would, however, permit the substantial variation in sequence seen among and within (15) species as long as the general character was retained.

A specific ligand/receptor interaction has long been postulated to explain the selectivity of the sporozoite for hepatocytes (16). The activities of the peptides studied here, including specific cell interaction and immune reactivity, fulfill a number of the criteria for that ligand. We are currently working to determine where this protein sequence belongs in the complex scheme of hepatocyte invasion and whether knowledge of its role would be useful to refine the sporozoite vaccine formulations now under development.

Summary

Several lines of evidence have emphasized the importance of the malaria circumsporozoite (CS) protein as a factor in sporozoite invasion of the hepatocyte; however, the specific mechanism of cell recognition and invasion has not been explained. In this study we present evidence that a highly conserved region of the CS protein immediately adjacent to the repeat region, the N1 region, specifically recognizes receptors on the human hepatoma cell line HepG2-A16 under conditions where invasion by sporozoites can occur. Peptides consisting of sequences from the repeat region or of the more extensive N2 region showed no such specific association. Antibody against the N1 peptide could inhibit sporozoite invasion in vitro. Covalent coupling of radiolabeled N1 peptide to HepG2-A16 cells identified two hepatic cell proteins to be closely associated with the peptide. We suggest that these proteins could act as receptors or mediators, via the N1 region of the CS protein, for the *P. falciparum* sporozoite in the process of invasion of the hepatocyte.

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