IDENTIFICATION OF PEPTIDES TARGETING THE SURFACE OF PLASMODIUM FALCIPARUM–INFECTED ERYTHROCYTES USING A PHAGE DISPLAY PEPTIDE LIBRARY

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Abstract. Drastic changes in the plasma membrane of Plasmodium falciparum–infected red blood cells (iRBCs) make the surface of iRBCs distinct from that of the uninfected erythrocyte. To identify small peptides that would specifically recognize the altered surface of iRBCs, we screened a phage display peptide library (PDL) on the surface of iRBCs. After the sixth panning of the PDL, eight phage clones of 18 sequenced clones had the same sequence, LVDAAAL (named P1) and specific binding of P1 to the surface of iRBCs was confirmed using phage expressing P1 peptides and synthetic P1 peptide. When P1 peptide was conjugated with a peptide having moderate hemolytic activity, the peptide conjugate inhibited the growth of intracellular parasites in a dose-dependent manner, whereas control peptides were without effect. Our results demonstrate that the P1 peptide may be a lead compound for the development of anti-malarial agents targeting the surface of iRBCs.

INTRODUCTION

Malaria, one of the most devastating infectious diseases in tropical countries, is caused by four species of Plasmodium, i.e., P. falciparum, P. vivax, P. malariae, and P. ovale. This disease infects 300–500 million people every year and results in 1–3 million deaths annually.1 Of the four species, P. falciparum is known to be the most widespread and life-threatening. Since emergence of chloroquine-resistant P. falciparum was reported in the late 1950s, parasites have acquired resistance to almost all the available anti-malarial drugs, except for artemisinin and its derivatives.2 The prevalence of drug-resistant malaria parasites has been an obstacle to malaria control efforts, and provokes the search for new anti-malarial drugs. After malaria parasites gain entry into the human blood stream by the bite of its vector, a female Anopheles mosquito, they infect liver cells and subsequently appear in the blood stream where they infect erythrocytes.3,4 The asexual blood stages of malaria remain within erythrocytes for a relatively long time, i.e., 48–72 hours, the biochemistry of these stages has been well investigated, and most of the presently available anti-malarial drugs target these stages, which are responsible for all of the pathology.5,6 It has been demonstrated by intensive biochemical and molecular biological analysis that malaria-infected red blood cells (iRBCs) undergo drastic membrane changes, including the appearance of cell surface protrusions, called knobs,7,8 changes in lipid composition,9 exposure of phosphatidylserine,10–12 insertion of parasite-derived proteins,13 and modifications of host erythrocyte proteins.14,15 The objective of this work was to use a phage display library (PDL) technique to identify peptides that would selectively recognize surface changes of iRBCs.

A PDL is a library of phage expressing random peptides or a diverse repertoire of antibodies on their surface and has been successfully used to identify ligands of receptors, substrates for enzymes or epitopes for antibodies.16 In these cases, highly purified molecules have preferentially been used as targets for PDL to avoid selection of peptides with large diversity. However, recent reports have, interestingly, shown that PDL could be used for the identification of peptides that specifically bind to the surface of cancer cells, endothelial cells, or microbes both in vitro and in vivo,17–20 and the selected peptides show significant homologies. One such peptide has been shown to selectively target adenovirus to human umbilical vein endothelial cells.20 In this paper, using a PDL, we have identified small peptides that selectively bind to the surface of iRBCs, and one of the identified peptides when conjugated to a hemolytic peptide had growth inhibitory activity against intra-erythrocytic parasites.

MATERIALS AND METHODS

Materials. Bacto tryptone and Bacto agar were obtained from Becton Dickinson and Co. (Sparks, MD). Yeast extract was obtained from Difco Laboratories (Detroit, MI) and a DNA preparation kit (QiAprep Spin M13 kit) was obtained from Qiagen, Inc. (Valencia, CA). Gentamicin and Albumax II were obtained from Gibco-BRL (Gaithersburg, MD). Biotinylated fluorescent beads (average diameter = 1 μm) were obtained from Spherotech, Inc. (Libertyville, IL) and diaphorase was obtained from the Worthington Biochemical Co. (Lakewood, NJ). Other chemicals were obtained from Sigma (St. Louis, MO). Synthetic peptides were obtained from the American Peptide Company (Sunnyvale, CA) and Genemed Synthesis, Inc. (San Francisco, CA). Peptides blocked by an amide group at the C-terminal end were purified by reversed-phase high performance liquid chromatography, and the molecular mass of the synthetic peptides was confirmed by mass spectroscopy.

Parasites. Three P. falciparum cloned lines, FCR-3 (knobby, CD36 preferring line), CS2 (knobby, chondroitin sulfate A–preferring line), and D3 (knobless line derived from FCR-3), were continuously cultured in O+ human erythrocytes as described by Trager and Jensen.21 Cultures of parasites were synchronized at the mature stage by gelatin floation22 and at ring stage by lysis of mature-stage iRBCs using 5% sorbitol solution.23

Screening of a PDL on iRBCs. The PDL and Escherichia coli strain (K91Kan) used in this work were kindly provided by Dr. Renata Pasqualini, (University of Texas, M. D. Anderson Cancer Center, Houston, TX). Details of library construction and protocols have been described previously.24,25 Phage in the library express 7-mer random peptides at the extracellular N terminal of coat proteins, pIII, and the random peptides are constrained by disulfide bonding of two cysteine residues at the flanking region of the peptides. The complex-
ity of the library was approximately $2 \times 10^6$. To remove phage that bound to surface of uninfected normal red blood cells (nRBCs), $1 \times 10^5$ transducing units (TU) of phage suspended in 100 μL of phosphate-buffered saline (PBS, 10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl) were mixed and incubated for two hours at 4°C with nRBCs (200 μL, 5% hematocrit) suspended in PBS-2% bovine serum albumin (BSA). Unbound phage were collected in the supernatant after centrifugation at 2,000 rpm for 30 seconds, mixed with 10 μL of packed erythrocytes infected with trophozoite and schizont-stage parasites (50–90% parasitemia), and incubated for two hours at 4°C. The suspension of iRBCs contained 2.5–4.5 × 10^9 iRBCs and 0.5–2.5 × 10^9 nRBCs. After incubation, iRBCs were washed by centrifugation twice with PBS-2% BSA and three times with PBS. To avoid collecting nonspecific phage that were bound to carrier proteins, Superblock (10% in PBS; Pierce, Rockford, IL) was used instead of a BSA solution in the second and fifth panning of phage against iRBCs. For the same reason, Blockaid (Molecular Probes, Eugene, OR) (10% in PBS) was used in the third and sixth panning. Phage bound to the surface of iRBCs were used to directly infect log-phase K91kan by co-incubation at room temperature for 20 minutes. Infected bacteria were selected by culturing in the presence of 100 μg/mL of kanamycin for one hour at 37°C. Titers of the infected E. coli suspension were checked by culturing the infected bacteria on tetracycline-containing agar plates. The remaining suspension of phage-infected bacteria was incubated overnight at 37°C in Luria-Bertani medium containing 20 μg/mL of tetracycline, and the amplified phage were isolated from the pellet after precipitation with polyethylene glycol. The titer of the phage was checked as described earlier, and 1 × 10^9 TU of phage were subjected to the following panning steps. After the sixth panning, phage clones were randomly selected, amplified, and used in assays to test for their binding specificity to iRBCs. The phage-iRBC binding assays were conducted using the same method as the panning procedure described earlier.

**DNA sequencing.** The DNA of selected phage was purified using QIAprep Spin M13 kit according to the manufacturer’s instructions. Partial sequencing of DNA to determine the sequence of the random peptide on the selected phage was carried out using the BigDyeR Terminator version 3.0 cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA) and a 5' primer (5'-CCCTCATAGTTAGCGTAACG-3'). The PCR products were analyzed on a 3730 DNA analyzer (Applied Biosystems, Inc.). All DNA sequencing was carried out at the University of California Riverside Genomics Institute.

**Peptide binding assay.** Binding of synthetic peptides to surface of iRBCs was tested using fluorescent beads. Biotinylated synthetic peptides dissolved in PBS (100 μg/mL) were incubated with 20 μL of streptavidin-conjugated fluorescent beads (average diameter = 0.85 μm; Spherotech) in the presence of 10% Blockaid blocking solution for one hour at room temperature. Unbound peptides were removed by washing the beads with PBS, and the peptide-coated beads were resuspended in PBS. The peptide-coated beads were then mixed with a suspension of iRBCs (50–90% parasitemia) in RPMI 1640 medium, HEPES, 10% Blockaid and incubated for one hour at 37°C with gentle shaking. The iRBCs and bound beads were fixed with 0.2% glutaraldehyde for 20 minutes at room temperature, and the cells were mounted on a glass slide. The number of beads bound to iRBCs was counted with a microscope using a phase contrast filter to distinguish iRBCs from nRBCs and fluorescence to visualize fluorescent beads.

**Growth inhibition assay.** Parasites were grown to the ring stage at a parasitemia of approximately 10% in RPMI 1640 medium supplemented with 0.5% Albumin II (Gibco-BRL). Synthetic peptides were added to cultures of ring-stage iRBCs (2% parasitemia, 1% hematocrit) and the iRBCs were then cultured for 24 hours or until parasites matured to the trophozoite or schizont stage. The growth of parasites was quantified by the ability of the malarial parasite to use the nicotinamide adenine dinucleotide analog, 3-acetylpyridine adenine dinucleotide, in a lactate dehydrogenase assay previously described.26

### RESULTS

**Panning of a PDL on iRBCs.** To isolate phage that would specifically bind to the surface of iRBCs, a library of phage expressing random peptides (7 mer peptides constrained by disulfide bonding of two cysteine residues) was first absorbed with nRBCs. Unbound phage were recovered in the supernatant and then mixed with a suspension of iRBCs at high parasitemia (50–90%). Since it has been shown that drastic membrane changes on the iRBC surface occur at the late stages of parasite development in erythrocytes,9–15 we used trophozoite and schizont stages of iRBCs for the panning of phage. After extensive washing, bound phage were used to directly infect host E. coli and amplified. The amplified phage were purified and subjected to panning on nRBCs and iRBCs. As shown in Table 1, the recovery of phage after each panning (the ratio of TU of bound phage to phage used for panning) was increased after the third and sixth panning. The binding specificity of phage for iRBCs (the ratio of TU of phage bound to iRBCs to that of phage bound to nRBCs) was also dramatically increased after the sixth panning (Table 1). After the sixth panning, the number of phage bound to iRBCs was 135 times greater than that bound to nRBCs, showing the accumulation of phage clones that bound specifically to the iRBC surface. To confirm the specificity of the panning, the fourth panning was conducted both on nRBCs and iRBCs and selected phage were used for the fifth panning. The phage isolated from iRBCs after the fourth panning bound 16.7-fold more to iRBCs than to nRBCs (fifth panning, Table 1),

<table>
<thead>
<tr>
<th>Round</th>
<th>Recovery of phage (× 10^9 %)</th>
<th>Ratio of phage bound to iRBCs/nRBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>6.0</td>
<td>10.0</td>
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<tr>
<td>4</td>
<td>5.7</td>
<td>3.7</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>16.7</td>
</tr>
<tr>
<td>6</td>
<td>27.6</td>
<td>135.0</td>
</tr>
</tbody>
</table>

*Panning of the phage display peptide library was conducted as described in the Materials and Methods section. Recovery of phage was calculated by dividing the transducing units (TU) of phage recovered from iRBCs by the total TU of phage used for panning and was expressed as a percentage. The ratio of bound phage to iRBCs/nRBCs was calculated by dividing the TU of phage bound to iRBCs by the TU of phage bound to nRBCs in each panning.*
Table 2

Peptide sequences of phage collected after the sixth panning*

<table>
<thead>
<tr>
<th>Clone</th>
<th>Sequence</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LVDAAAAL</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>PIALGLK</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>GPLKGL</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>INLGLTM</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>FSLGLK</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>PAYKLYS</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>GSRKGT</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>NVGGRS</td>
<td>1</td>
</tr>
</tbody>
</table>

*Eighteen phage clones bound to infected red blood cells were randomly selected after the sixth panning, and the sequences of random peptides expressed on phage were determined. The peptides were cyclized by disulfide bonding of two cysteine residues at the flanking region and expressed at the N-terminus of a coat protein of phage, called pIII. The common motif LGL found in different clones is underlined.

**Sequences of peptides on selected phage.** Eighteen clones from the phage mixture obtained after the sixth panning were randomly selected, and the sequences of random peptides expressed on the phage were determined. As shown in Table 2, the sequences of clones 1, 2, and 3 were found in 8, 3, and 2 clones of 18 sequenced clones, respectively, and the peptide motif LGL was found in clones 2, 4, and 5, constituting 28% of the sequenced clones. This suggested that during the panning process specific selection of peptides occurred. The homology of the identified peptidic sequences with known proteins was obtained using the BLAST program (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD); however, no proteins reported to bind to the surface of iRBCs were found in the list of proteins having homology with the peptides in Table 2.

**Binding specificity of selected phage and synthetic peptides against iRBCs.** To confirm the binding specificity of isolated clones, phage of clones 1, 2, and 3 were amplified and tested for binding to nRBCs and iRBCs. Phage of clones 1, 2, and 3 specifically bound to the surface of iRBCs as shown in Figure 1, whereas phage in the original PDL showed no such specificity. Since the objective of this work was to identify small peptides that bind to the surface of iRBCs to selectively deliver anti-malarial materials, identified peptides on phage were chemically synthesized and tested for their binding specificity against iRBCs. In the following experiments, we have focused only on the peptide of clone 1 (P1), which was the most frequent (44.4% of the total clones) of those sequenced. Since the plasma membrane of the iRBC has been reported to be permeable to small molecules such as oligopeptides, we used fluorescent beads coated with peptides to test for the binding activity of the peptides against the outer surface of the iRBC. Fluorescent beads coated with P1 peptides were incubated with a suspension of iRBCs and beads bound to the surface of iRBCs were observed by light microscopy. Beads coated with P1 peptides bound to the surface of iRBCs as shown in Figure 2A. The number of peptide-coated beads bound per iRBC was determined; the binding of P1-coated beads to the iRBC surface was more than four-fold higher than that of uncoated beads (Figure 2B). Furthermore, the binding of beads coated with a control peptide was lower than that with the P1 peptide (Figure 2B), demonstrating that P1 peptide binding to the surface of the iRBC was specific.

**Effect of P1 peptide conjugated with a membrane-active peptide on the growth of malaria parasites.** Various amphipathic peptides have been reported to exert bactericidal and/or hemolytic activity through their activity to destabilize the cell membrane.27 More recently, it was reported that a synthetic amphipathic peptide having sequence of (KLAKLAK)2, called L peptide in this work, showed hemolytic activity at a concentration of 750 μM (1,200 μg/mL).28 We prepared a peptide having the sequence of P1 peptide at the N-terminal and L peptide at the C-terminal (designated P1L, Table 3), and tested for its activity to inhibit the growth of intraerythrocytic parasites. The growth of parasites in the presence of peptide was quantified by measuring a parasite-specific enzyme activity as described in the Materials and Methods. It was confirmed that the P1L and P1LS peptides were without effect on this enzyme reaction. The P1L peptide inhibited parasite growth in a dose-dependent manner and inhibition reached 90% at a concentration of approximately 100 μg/mL (40 μM) with all parasite strains used (FCR-3, CS2, and D3), whereas P1 and L by themselves showed no significant effect even at the highest concentration of 200 μg/mL (180 μM for P1 peptide and 120 μM for L peptide) (Figure 3). This demonstrated that the conjugation of P1 peptide conferred growth inhibitory activity against intraerythrocytic malaria parasites to the L peptide.

The 50% inhibitory concentration (IC50) of P1L for growth inhibition of the FCR-3, CS2, and D3 strains was in the range of 15–29 μM (Table 4). The specificity of activity of P1L was confirmed by the fact that the IC50 of P1LS (a peptide in which alanine residues in P1 portion of P1L were substituted with serine residues, see Table 3) was 2.3–2.9-fold higher than that of P1L (Table 4). The inhibitory effect of the P1L peptide...
tide, showed anti-malarial activity. Although the IC50 of P1L tide, a conjugate of P1 peptide with a membrane active peptide bound to the surface of iRBCs. Furthermore, P1L more than 40% of the sequenced phage clones, and specifically bound to the surface of the iRBC. We have identified some membrane-targeting peptides using such a peptide to selectively deliver a cytotoxic agent to the parasites. These reports are consistent with our results in that specific clones, such as P1, and the LGL motif were accumulated when a PDL was panned and selected on the surface of intact cells. Though lysis of nRBCs was caused by P1L peptide, the lysis was less than 10% at the IC50 of P1L growth inhibition of FCR-3 (40 μg/mL).

**DISCUSSION**

Specific structures in the membrane of cancer cells and microbes have been regarded as good candidates for the development of probes for the selective delivery of drugs. Despite the reports of various dramatic changes in the membranes of iRBCs in the last few decades, few malarial drugs have been developed through the targeting of such changes. We used a PDL technique to identify a small peptidic probe to target the surface of the iRBC with the goal of using such a peptide to selectively deliver a cytotoxic agent to the iRBC. We have identified some membrane-targeting peptides using a PDL and one of the peptides (P1), constituted more than 40% of the sequenced phage clones, and specifically bound to the surface of iRBCs. Furthermore, P1L peptide, a conjugate of P1 peptide with a membrane active peptide, showed anti-malarial activity. Although the IC50 of P1L (15 μM) was not low enough for P1L to be considered as a basis for the development of a practical drug, modifications of P1L, i.e., a conjugate of P1 with other membrane-active peptides, could be a lead compound for a potent membrane-targeting anti-malarial drug.

The PDL has mainly been used to identify peptides with the capacity to bind to a purified target molecule, such as a monoclonal antibody or an enzyme. Theoretically, it could be expected that specific peptides would not be selected when a PDL was panned on a surface with multiple components. However, interestingly, recent attempts by some investigators to isolate phage that would bind selectively to the surface of cells have been successful both *in vitro* and *in vivo.* For example, Samoylova and others screened a PDL with random 7-mer peptides for microglial cell-targeting peptides and identified several peptides with a common motif, S/T-F-T/X-Y-W. Phage and synthetic peptides with the motif were shown to specifically bind to the surface of a microglial cell line. Similarily, PDLs with different types of random peptides were used to identify peptides that would specifically interact with the surface of *Eimeria acervulina* sporozoites. Interestingly, a peptidic motif, called the WW motif, was found in all libraries used. Furthermore, such peptides showed activity of membrane destabilization and inhibited the invasion and growth of *E. acervulina* sporozoites. These reports are consistent with our results in that specific clones, such as P1, and the LGL motif were accumulated when a PDL was panned and selected on the surface of intact cells. Although the LGL motif in clones 2, 4, and 5 was not found in the most frequent clone i.e., P1 (Table 2), all of these clones share a similarity in that all peptides contain a sequence of 3–4 amino acids with aliphatic side chains and lack polar groups, i.e., L, A, I, and G. It is possible that such hydrophobic sequences represent the minimal requirement for the specific binding of the peptides to the surface of the iRBC and indeed the addition of three hydroxyl groups to such hydrophobic sequence, i.e., substitution of three alanine residues in P1L with serine residues, decreased the anti-malarial activity of P1L peptide (Figure 3).

**TABLE 3**

Sequences and molecular weight of the synthetic peptides used in this work*

<table>
<thead>
<tr>
<th>Designation</th>
<th>Molecular weight</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1,102</td>
<td>X-C<em>LVDAAALC</em></td>
</tr>
<tr>
<td>Control</td>
<td>1,338</td>
<td>X-FFSATLGN</td>
</tr>
<tr>
<td>L</td>
<td>1,652</td>
<td>GA (KLAKLAK)</td>
</tr>
<tr>
<td>PIL</td>
<td>2,639</td>
<td>GAC<em>LVDAAALC</em>GA (KLAKLAK)</td>
</tr>
<tr>
<td>PILS</td>
<td>2,687</td>
<td>GAC<em>LVDSSLGC</em>GA (KLAKLAK)</td>
</tr>
</tbody>
</table>

* X represents a biotin residue conjugated to the N-terminal of the peptide. P1, PIL, and PILS peptides were cyclized at the asterisked cysteine residues.
LVDAAAL did not show any homologies of P1 with proteins reported to bind to the surface of iRBCs, such as CD36, intercellular adhesion molecule 1, and thrombospondin. Therefore, the results of this homology search were unable to predict whether the P1 peptide is mimicking a known iRBC-binding protein. Since the aim of this work was to find a probe that would selectively deliver a drug to the iRBC, we did not conduct additional experiments to identify such proteins.

As a candidate of materials to be delivered to the surface of iRBCs, we elected to conjugate an amphipathic peptide with P1 because such a conjugate could easily be obtained by chemical peptide synthesis and because amphipathic peptides have been shown to destabilize the erythrocyte membrane. For the following reasons, we used a peptide having a sequence (KLAKLAK)_2, designated L peptide in this work, as a model amphipathic peptide: 1) L peptide has a simple amino acid sequence designed to be perfectly amphipathic, and 2) the peptide was shown to have moderate activity to lyse erythrocytes so that enhancement of anti-malarial activity by conjugation of the L peptide with P1 would be clearly observed. Indeed, P1L peptide inhibited the growth of intraerythrocytic parasite with an IC$_{50}$ of 15–29 μM, whereas L peptide did not show such activity even at a concentration of 120 μM (Figure 3). We tested three strains of $P$. falciparum with different surface properties to confirm that the parasiticidal activity of P1L peptide is not specific to FCR-3 strain that had been used for the selection of the peptide. The IC$_{50}$ of the peptide was 0.5 μM. These investigators also tested a variety of derivatives of dermaseptin S4 and found that the potency of the peptide was further increased when two amino acid residues (methionine or asparagine) in the peptide were substituted with lysine residues. The IC$_{50}$ of the derivatives could achieve a value as low as 0.2 μM, which is much lower than the IC$_{50}$ obtained in this work. Therefore, based on our present findings and those of Krugliak and others, substitutions of the L peptide in P1L with other amphipathic peptides having anti-malarial activity would be a rational approach to finding a more potent anti-malarial peptide, and such a peptide could serve as a lead compound for the design of a potent anti-malaria agent.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>FCR-3 (μM)</th>
<th>CS2 (μM)</th>
<th>D3 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>180&lt;</td>
<td>180&lt;</td>
<td>180&lt;</td>
</tr>
<tr>
<td>L</td>
<td>120&lt;</td>
<td>120&lt;</td>
<td>120&lt;</td>
</tr>
<tr>
<td>P1L</td>
<td>15</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>P1LS</td>
<td>34</td>
<td>49</td>
<td>68</td>
</tr>
</tbody>
</table>

* IC$_{50}$ = 50% inhibitory concentration.

IC$_{50}$ values were obtained from the results in Figure 3 and were expressed in μM. 180< and 120< indicates the IC$_{50}$ values were higher than 180 and 120 μM, respectively.
REFERENCES


