

***Plasmodium vivax* merozoite surface protein 1 paralog as a mediator of parasite adherence to reticulocytes**

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Abstract

Plasmodium vivax parasites preferentially invade reticulocytes in human beings. *P. vivax* merozoite surface protein 1 (PvMSP1) and PvMSP1 paralog (PvMSP1P) may have important functions in reticulocyte adherence during invasion. These proteins share similar structures, including the presence of two epidermal growth factor (EGF)-like and glycosylphosphatidylinositol (GPI)-anchored domains at the C-terminus. However, there have been no reports concerning the functional activity of PvMSP1P in reticulocyte adherence during *P. vivax* invasion. In this study, the ability of PvMSP1P-19 to bind to reticulocytes and normocytes was analysed. The reticulocyte binding activity of PvMSP1P-19 was 4.0-fold higher than its normocyte-binding activity. The binding of PvMSP1P-19 to reticulocytes and normocytes was inhibited in a dose-dependent manner by antibodies from immunized rabbits and by antibodies from vivax parasite-infected patients. Consistently, antibodies against PvMSP1P inhibited parasite invasion during short-term *in vitro* cultivation. Similar to PvDBPII binding activity, PvMSP1P-19 binding activity was reduced in chymotrypsin-treated reticulocytes. However, no significant difference was found in the binding of PvMSP1P-19 to Duffy-positive and Duffy-negative erythrocytes. The minimal binding motif of PvMSP1P-19 was characterized using synthetic peptides. The results showed that the residues at amino acid positions 1,791-1,808 may have an important function in mediating merozoite adherence to reticulocytes. The positively charged residues within the EGF-like domain were shown to constitute a key binding motif. This work presents strong evidence supporting the role of PvMSP1P in host target cell selection and invasion of Duffy independent pathway in *P. vivax*. Moreover, PvMSP1P-19-specific antibodies may confer protection against *P. vivax* re-invasion.

Key words: *Plasmodium vivax*, merozoite surface, Duffy independent, merozoite surface protein 1, merozoite surface protein 1 paralog, invasion inhibition

Introduction

Malaria remains a major problem, causing high morbidity and mortality in tropical and sub-tropical nations (1). An estimated 148-304 million cases of malaria occur annually, and this disease continues to be a serious health threat in developing countries (1). Of the six parasites that cause human malaria, *Plasmodium vivax* is the most prevalent outside sub-Saharan Africa. However, understanding of how *P. vivax* pathophysiology in the host during the intra-erythrocytic stage of the parasite is still limited, leading to difficulty in controlling vivax malaria. In particular, how and why *P. vivax* displays strict host tropism for immature erythrocytes (reticulocytes) must be addressed (2). To date, only the interaction between Duffy binding protein (PvDBP) and Duffy antigen receptor for chemokines (DARC) has been shown to be involved in the invasive mechanism of *P. vivax* (3). However, PvDBP interaction is insufficient to explain *P. vivax* host cell selection mechanisms because DARC expression decreases with erythrocyte maturation (4). In addition, *P. vivax* infection has been reported in Duffy-negative individuals in Madagascar (5). These studies suggest that *P. vivax* may use a variable and flexible invasion pathway and host cell selection (6); however, this important issue remains unresolved.

The transition from reticulocyte to normocyte involves dramatic changes in the cell surface as well as intracellular changes (7). A number of cell surface molecules function as adhesion molecules that interact with other blood cell components and with endothelial cells were found (8). The interaction of parasite ligands with erythrocyte surface molecules during *P. vivax* invasion is essential for successful invasion from initial contact to internalization (9, 10). In *P. vivax*, several adhesive ligands from the merozoite apical region that are important for specific interactions with reticulocytes, including PvRBP1a, PvRBP1b, PvRBP2a, PvRBP2b and PvEBP2, have been identified (11-14). All of these apically localized antigens may be

79 responsible for parasite internalization in reticulocytes (9). However, identification of the ligand
80 responsible for the initial contact has been neglected due to the lack of an *in vitro* cultivation
81 assay for *P. vivax*.

82 The surface-exposed antigens on merozoites are abundant and serve essential functions that
83 mediate initial contact with erythrocyte surface. This hypothesis was supported by previous
84 PvMSP1-19 and PfMSP1-19 functional studies (15, 16). Recently, PvMSP1P was reported as a
85 novel erythrocyte binding protein (17). PvMSP1P is expressed on the merozoite surface and
86 elicits a strong acquired immune response in *P. vivax* patients (17). PvMSP1P is similar to
87 PvMSP1 in terms of its amino acid sequence, with conservation of twelve cysteine residues in
88 its epidermal growth factor-like (EGF)-like domains in the C-terminal region. Moreover, these
89 cysteine residues were also shown to be highly conserved not only in *P. vivax* merozoite surface
90 antigens (PvMSP1, PvMSP8 and PvMSP10) but also in the surface proteins of diverse species
91 of human-invasive *Plasmodium* (18, 19). Although the human EGF domain was shown to be
92 related to dendritic cell maturation and T cell activation (20), the functions of the EGF-like
93 domain in *Plasmodium* spp. are unknown. PvMSP1P transcription levels increase at the
94 schizont stage, reflecting a major role for this protein in individual merozoites to involve egress
95 and invasion process. Accordingly, PvMSP1P-19 may play an important role in the initial
96 contact and the selection of host cells when merozoites invade new reticulocytes. Therefore,
97 evaluation of the reticulocyte selectivity of PvMSP1P and determination of its binding
98 mechanism is essential for understanding the complex process of *P. vivax* invasion involving
99 Duffy independent pathway.

100 In this study, the reticulocyte binding ability of the PvMSP1P EGF-like domain was
101 evaluated, and the inhibitory activity of an antibody against PvMSP1P on parasite re-invasion
102 were demonstrated.

Results

Structure and amino acid sequence homology of PvMSP1P-19

The N-terminal region of PvMSP1P consists of a signal peptide (SP, 1-28 amino acids (aa.)), a hepta-peptide tandem repeat domain (TR, 905-918 aa.) and a polymorphic Glu- and Gln-rich domain (PR, 1,157-1,172 aa.). PvMSP1P contains two highly conserved EGF-like domains (1,751-1,789 aa. and 1,792-1,834 aa.) with a GPI-anchored domain (1,834-1,854 aa.) at the C-terminal region (Fig. 1A). The two EGF-like domains are conserved in various human and non-human primate invasive *Plasmodium* species containing cysteine residues (Fig. 1B). The sequence similarity was shown in *P. knowlesi* (86.0%), *P. cynomolgi* (76.7%), *P. ovale* (54.7%), and *P. malariae* (57.0%) with PvMSP1P-19 (Fig. 1B).

Three-dimensional structure of PvMSP1P-19

The three-dimensional structure of the EGF-like domain was modelled using SWISS-MODEL. As a template for PvMSP1P-19, the EGF-like domain of PvMSP1-19 (PDB ID: 2npr) was found to display a highly fitted structure. For structural analysis of the template, the BL21(DE3) strain of *E. coli* was used for recombinant protein expression, and the solution nuclear magnetic resonance method was used for 3D structure identification (21). A Ramachandran plot revealed the favourable regions (72.6%) and disallowed regions (0.0%) in the model. The quality verified by ERRAT results was 75.4% of the *ab initio* model structure. After refinement, the 3D structure quality improved to a favourable region of 87.7% and a disallowed region of 1.4% in the Ramachandran plot and to 95.5% of the final allowed PvMSP1P-19 structure in ERRAT.

Three-dimensional structure prediction showed similarity to the PvMSP1-EGF domain, with a flat, disk-like shape with three disulphide bridges in each EGF-like domain (Fig. 1C). The surface of PvMSP1P-19 showed a distinct charge pattern; the negative charge was concentrated in the central concave portion, and the positive charge surrounded the concave portion (Fig.

1D).

Reticulocyte enrichment and reticulocyte preference of PvMSP1P-19

A total of twenty-two cord blood samples were collected for reticulocyte binding assay, reticulocyte binding inhibition assay, and competition assays in this study. Approximately 15 mL of each cord blood sample was used for reticulocyte enrichment. The fresh cord blood contained various concentrations of reticulocytes ranging from 1.6% to 6.9% (mean: 2.7%, 95% confidence interval: 2.2%-3.2%). The enriched reticulocytes were obtained at concentrations ranging from 71% to 88% (mean: 76.6%, 95% confidence interval: 74.3-78.7%) (Fig. 2A, upper panel).

The enriched reticulocytes were diluted in suspensions of erythrocytes (peripheral blood), ranging from 80.0% to 0.5% reticulocytes in normal peripheral blood, and confirmed by methylene blue staining (Fig. 2A, upper panel). The reticulocyte concentration dependent manner of binding ability was confirmed by COS-7 cells binding assay and rosette formations were counted under the light microscopy (Fig. 2A, middle and bottom panel). The rosette formation of erythrocyte (reticulocyte count > 0.5%) and reticulocyte (reticulocyte count > 80%) fraction was specific to PvMSP1P-19 and PvDBPII expressed COS-7 cells (Fig. S1A-C). The transfection efficiency was shown as 85% for PvMSP1P-19 and 42% for PvDBPII in COS-7 cells in three independent experiments (Fig. S1A and B). PvMSP1P-19 and PvDBPII binding activities were normalized to the number of rosettes that would have been obtained at 100% transfection efficiency (number of rosettes \times 100/transfection efficiency). The number of rosettes was normalized by setting PvDBPII binding to peripheral blood (reticulocyte count > 0.5%) as 100% relative binding for comparison with binding activity under other conditions (Fig. 2B). Both PvMSP1P-19 binding and PvDBPII binding increased with reticulocyte concentration in a dose-dependent manner. PvMSP1P-19 demonstrated half of the binding

ability of PvDBPII; however, its binding increased dramatically (3.2-fold) as the reticulocyte concentration was increased from 0.5% to 80.0% (Fig. 2B). PvDBPII showed 4.0-fold higher binding to 80.0% reticulocytes than to peripheral erythrocytes. In particular, the binding of both proteins increased significantly at a concentration of 10.0% reticulocytes compared to peripheral blood conditions (Fig. 2B).

Inhibition of the binding and invasion of *P. vivax* by antibodies against PvMSP1P-19

An *in vitro* binding inhibition assay was used to determine whether anti-PvMSP1P-19 antibodies in mouse and vivax-infected patient sera had inhibitory effects on the binding of the protein to erythrocytes and reticulocytes. Antibodies against PvDBPII and PvMSP1P-19 recognized target antigens on the COS-7 cell surface (Fig. 3A and B, upper panel). Pre-immune mouse sera (PI) and mouse sera against Pvs25 at a dilution of 1:10 were used as negative controls. In this study, PvDBPII and PvMSP1P-19 binding inhibition assays were performed simultaneously (Fig. 3A). The inhibitory effects were measured by normalizing the number of rosettes observed in the presence of test antibody dilutions ranging from 1:1,000 to 1:50. The mouse antibodies against PvDBPII and PvMSP1P-19 exerted significant concentration-dependent inhibition of PvDBPII and PvMSP1P-19 binding to erythrocytes and reticulocytes, respectively (Fig. 3A and B). For PvMSP1P-19, the percentage of binding inhibition at a dilution of 1:50 was approximately 75% for both erythrocytes and reticulocytes (Fig. 3B). Furthermore, to investigate whether sera from vivax patients also contain blocking antibodies against PvMSP1P-19, we tested the effects of patient sera on erythrocyte and reticulocyte binding. Vivax-infected patient sera (from the Republic of Korea, ROK+) inhibited the binding of PvMSP1P-19 to human erythrocytes ($78.1 \pm 6.1\%$ relative binding, mean \pm standard deviation (S.D.)) and reticulocytes ($71.8 \pm 8.0\%$) at a dilution of 1:20 and dose-dependently reduced the binding ability (Fig. 3C). In contrast, control sera from healthy individuals (normal

sera, ROK-) showed no ability to block PvMSP1P-19 binding to erythrocytes ($0.0 \pm 9.8\%$) or reticulocytes ($0.0 \pm 9.6\%$), even at the highest serum concentration tested (1:10) (Fig. 3C).

The inhibitory effects of the polyclonal sera on parasite invasion were evaluated FACS using field vivax parasite isolates from Mae Sot, Thailand (The FACS gating described in Fig. S2). Three antibodies (anti-PvMSP1-19, anti-PvMSP1P-19 and anti-PvDBPII) were used in the test. An anti-Fy6 (α -2C3) monoclonal antibody was used as a positive control (22, 23). Consistently, anti-Fy6 ($95.4 \pm 2.9\%$ relative binding, mean \pm S.D.) significantly reduced the invasion of the vivax parasite. Although the PI sera showed an invasion inhibition effect ($27.3 \pm 15.5\%$), re-invasion by the vivax parasite was significantly inhibited by antibodies against PvMSP1-19 ($61.3 \pm 19.4\%$) and by antibodies against PvMSP1P-19 ($53.6 \pm 13.2\%$). However, antibodies against PvDBPII did not significantly reduce vivax invasion ($45.2 \pm 15.3\%$) (Fig. 3D).

Minimal binding motif of PvMSP1P-19

As a control, PvDBPII-expressing COS-7 cells were incubated with synthesized peptides in PvMSP1P-19 competition assays to detect possible nonspecific masking of receptors on the reticulocyte surface. Peptides other than Ser1764 and Asn1809 showed less nonspecific masking. Peptide N1791 displayed the greatest inhibition of PvMSP1P-19 binding, with a mean \pm S.D. of $57.4 \pm 11.5\%$ in erythrocytes and $52.2 \pm 10.0\%$ in reticulocytes in three independent experiments (Fig. 4A and B). The peptides S1782 (erythrocytes, $30.3 \pm 8.4\%$; reticulocytes, $31.7 \pm 10.6\%$) and N1800 (erythrocytes, $32.4 \pm 6.0\%$; reticulocytes, $32.7 \pm 6.4\%$) also showed inhibitory activity, as did peptide N1791 in both erythrocytes and reticulocytes (Fig. 4A and B). To confirm this result, the highest inhibitory peptide, N1791, and the least inhibitory peptide, S1764, were serially diluted to concentrations ranging from 100 μ M to 0.1 μ M. The inhibitory activity of N1791 increased in a concentration-dependent manner. However, S1764 showed a slightly increase in reticulocyte binding ability with PvMSP1P-19 (Fig. 4C).

Point mutants of the PvMSP1P-19 protein were used to analyse binding properties as well as to identify critical binding residues. The mutant PvMSP1P-19 fragments were confirmed by SDS-PAGE (Fig. S3). The mutant 1 and mutant 4 fragments showed decreased reticulocyte binding ($66.2 \pm 2.2\%$ and $75.9 \pm 11.2\%$ relative binding, respectively). However, mutants 2 and 3, which affect residues that are located within the concave portion of the mutated fragment, did not interfere with binding (Fig. 4D). Thus, three important amino acid residues (Ser1782, Glu1792, and Glu1810) and one critical amino acid residue (Glu1786) were shown to directly influence reticulocyte binding (Fig. 4E and F).

DARC independence of PvMSP1P-19-specific receptors

The presence of a specific receptor for PvMSP1P-19 was confirmed using enzyme-treated erythrocyte and reticulocyte binding assays (Fig. S4). PvDBPII, a well-known receptor that interacts strongly with erythrocytes via chymotrypsin-sensitive receptors such as DARC, was used as a positive control (Fig. 5A). Consistently, neuraminidase (Nm) and trypsin (T) did not affect the binding ability of PvDBPII. However, chymotrypsin (Ct)-treated erythrocytes and reticulocytes failed to bind PvDBPII (Fig. 5A). PvMSP1P-19 binding showed neuraminidase and trypsin resistance but was chymotrypsin-sensitive (Fig. 5B). Both erythrocytes and reticulocytes displayed dramatically decreased binding activity when host cell receptors were cleaved by chymotrypsin, similar to PvDBPII (Fig. 5B).

To determine whether PvMSP1P is a Duffy phenotype-dependent binding protein, Duffy-negative erythrocytes were used. The binding ability of PvDBP was lost in Duffy-negative erythrocytes ($13.6 \pm 4.8\%$, relative binding mean \pm S.D.) (Fig. 5C). In contrast, PvMSP1P bound both Duffy-positive ($41.6 \pm 8.0\%$) and Duffy-negative erythrocytes ($25.4 \pm 18.7\%$), indicating that PvMSP1P is a ligand for erythrocyte binding that is not related to the Duffy blood group antigen and revealing the presence of a potential receptor in addition to DARC on

the erythrocyte surface (Fig. 5C).

Discussion

Typically, two human EGFs form a complex and interact with two EGF receptor complexes in human cells, thereby triggering numerous downstream signalling pathways (24-26). Interestingly, the merozoite surface antigens of various *Plasmodium* species also contain EGF-like domains. Among them, the MSP1 EGF-like domain has been considered a *P. vivax* and *P. falciparum* blood stage vaccine candidate. MSP1 is the most abundant antigen on the merozoite surface and has an important role in initial attachment, as shown by experiments with processed fragments of the 19-kDa EGF-like domain using proteoglycans with heparin-like side chains (27). Recent study demonstrated that PfMSP1 is important for merozoite egress (28). The presence of antibodies against PfMSP1 is associated with a reduced risk of clinical *P. falciparum* infection; however, vaccine trials have shown low efficacy due to allelic diversity (29, 30). Consistent with PfMSP1 allelic specificity, weak immune recognition of the variable domains of PvMSP1 has been detected (31). The limited immune recognition observed with *P. vivax* vaccine development is considered at least partly attributable to the presence of species-preserved and allele-conserved antigens such as MSP1P (32).

P. vivax invasion is strictly restricted to reticulocytes by unknown host cell selection mechanisms. It is likely that the invasion mechanisms of both *P. vivax* and *P. falciparum* are adapted to their surroundings via complex and multiple protein interactions with their host cells (33, 34). During the maturation of erythrocytes from reticulocytes to normocytes, some receptors and channels such as CD71, CD47, ICAM4, NHE1 and GLUT4 show decreased expression (35). Those receptors are strong candidates for involvement in reticulocyte selection by *P. vivax*, as is the interaction of CD71 with PvRBP2b (14).

PvMSP1P interacts with a specific receptor on the erythrocyte surface that is dominant in

reticulocytes and is cleaved by chymotrypsin. The specific features of this receptor are similar to DARC; however, PvMSP1P binding is not related to the Duffy phenotype of erythrocytes, but Duffy independent. Therefore, PvMSP1P is a novel molecule involved in merozoite contact, and its binding properties provide strong evidence for infection of Duffy-negative populations by the vivax parasite. Based on the sequence of events that occurs during invasion by merozoites, our results show that PvMSP1P mediates initial contact with reticulocytes and normocytes. Following the general concept of *Plasmodium* species invasion process, after initial contact with an unknown receptor, actin-myosin motor activity-dependent reorientation and invasion occur sequentially (10). During this step, secretion of the microneme and rhoptry proteins occurs following successful calcium-mediated invasion (10, 36-38). In Duffy-positive hosts, PvDBP strongly interacts with DARC on erythrocytes and reticulocytes (39, 40). At the same time, proteins of the PvRBP family and PvEBP2 support the selection of reticulocytes as target cells for successful merozoite invasion via multiple and complex mechanisms (11-14, 41). Another invasion pathway mediated by PvGAMA may occur during infection of the Duffy-negative population (42).

The vivax patient elicited high antibody response in acute phase against PvMSP1P-19 and these antibodies were stable up to nine months (17, 43). The longevity of naturally acquired antibody responses of PvMSP1-19 was showed two months stable after treatment (44). Binding inhibition of PvMSP1P-19 to erythrocyte and reticulocyte was abrogated by immune sera against PvMSP1P-19 as shown in positive control of PvDBPII. However, the reticulocyte binding inhibition by antibodies was slightly higher rather than erythrocyte binding inhibition. It suggested that binding activity of PvMSP1P-19 was specific to reticulocyte, even though there is no statistical different. Additionally, the higher inhibition activity of PvMSP1P-19 compared with PvDBPII was observed at 1:200 dilution. Taken together, this result supported that PvMSP1P-19 is effective target antigen to interrupt the adherence. In recent study, ex vivo

invasion assay showed that antibodies against PvMSP1P-19 inhibited the invasion as well as anti-PvDBPII antibody (22). Moreover, the invasion inhibition rate obtained with an anti-PvMSP1P antibody was higher than that obtained with an anti-PvDBPII antibody. Taken together, these results indicate that PvMSP1P is a new promising vaccine candidate that may offer protection against *P. vivax* invasion.

Reticulocyte-dominant receptor binding by PvMSP1P-19 is mediated by the region of the protein comprising Ser1782 to Asn1817. The binding motif screening's result suggests that targeting this small motif is important for protection against invasion by the vivax parasite. The minimal binding motif in the EGF-like domains of various *Plasmodium* proteins such as MSP1, MSP8 and MSP10 contains conserved cysteines (19). However, the role of the EGF-like domain in erythrocyte adherence and invasion by *Plasmodium* species remains unclear.

Our current work provides new insight into the complicated invasion mechanisms of *P. vivax*. Furthermore, the inhibitory effects of its antibody makes PvMSP1P-19 as one of potential vaccine developments. In the future, determining how PvMSP1P interacts with its receptor will aid in our understanding of how initial contact with the host cell influences the selection by *P. vivax*.

Materials and Methods

Ethics statement

The sample collection, informed consent and the clinical protocol were approved by the Ethics Committee of Kangwon National University Hospital (IRB No. 2014-08-008-002). Written informed consent was obtained from all subjects. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University, Republic of Korea. The experiments were conducted according to relevant national and international guidelines, and the Ethical Guidelines for Animal Experiments of Kangwon

National University (KIACUC-13-0001).

Structure prediction, determination and refinement

A three-dimensional model of the structure of PvMSP1P was generated by homology-based modelling. Suitable structural templates were searched and modelled using SWISS-MODEL (45). The three-dimensional model quality and potential errors were evaluated by Ramachandran plots (46) and ERRAT (47). To improve accuracy, the generated model was refined using a combination of two programs, Mode Refiner (48) and Galaxy Refine (49). The structure was visualized using UCSF CHIMERA software (50).

Reticulocyte enrichment from cord blood

Cord blood and peripheral blood samples were collected in heparin tubes (Becton-Dickinson, Franklin Lakes, NJ). Reticulocytes were enriched by gradient centrifugation using Nycodenz (Axis-Shield, Dundee, UK). Briefly, the leukocytes were removed from cord blood using NWF filters (Zhixing Bio, Bengbu, China). The packed cells were collected, resuspended in high KCl buffer, and incubated at 4°C for 3 hours. The erythrocyte-high KCl buffer mixture was transferred to the surface of a 19% Nycodenz solution. The layered solution was centrifuged at $3,000 \times g$ for 30 minutes, and the interface layer was collected. The reticulocyte concentration was calculated by observing more than 2,000 RBCs under light microscopy in thin blood smears prepared after staining the cell suspension with new methylene blue (Sigma, St Louis, MO).

Production of recombinant proteins and immune sera

The production of recombinant proteins and animal immune sera has been described previously (17). BALB/c mice and Japanese white rabbits were immunized with PvMSP1P-19, PvDBPII, Pvs25 (ookinete surface protein, not related to erythrocyte binding) and PvMSP1-19.

Mice and rabbits were injected intraperitoneally with 20 µg and 250 µg, respectively, of the recombinant proteins mixed with Freund's complete adjuvant (Sigma). Booster containing Freund's incomplete adjuvant (Sigma) were given after a 3-week interval.

Peptide synthesis and RBC masking

The sequence of PvMSP1P-19 (1,764-1,835 amino acids) from the Sal-1 strain was used for peptide synthesis. Seven sequential 18-mer peptides were chemically synthesized on 9 offset by the Solid Phase Multiple Peptide Synthesis Technique. The purity of the synthesized peptides was analysed by analytical reversed-phase high-performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption ionization-time of flight mass spectrometry. All peptides were of greater than 90% purity and were solubilized in dimethyl sulphoxide. Erythrocytes from peripheral blood and purified reticulocytes were pre-incubated for 1 hour at 37°C with individual peptides at 100 µM in incomplete DMEM. The packed cells were prepared as 10% hematocrit (*Hct.*) for competition binding assays.

Enzymatic treatment of RBCs

Each erythrocyte and reticulocyte sample was prepared at 50% *Hct.* The erythrocytes and reticulocytes were incubated with neuraminidase (Sigma), trypsin (Sigma) and chymotrypsin (Sigma) at 37°C for 1 hour. To terminate the biological activity of trypsin and chymotrypsin, trypsin inhibitor (Sigma) was added and incubated at 37°C for 10 minutes. The samples were washed with incomplete RPMI 1640 and resuspended at 10% *Hct.* for binding assays.

COS-7 cell-based RBC binding, inhibition and competition assays

The design and construction of the primers used to amplify PvMSP1P-19 and PvDBPII were described previously (17). Briefly, the pEGFP-HSVgD-N1 vector (a kind gift from John H.

Adams, USF Health, USA) was used to construct COS-7 cell surface expression vectors encoding PvMSP1P-19 and PvDBPII. The PCR products were ligated into the linearized vector using an In-Fusion[®] HD Cloning Kit (Clontech, Mountain View, CA). The construct was purified using an UltraClean[®] Endotoxin-Free Mini Plasmid Prep Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

COS-7 cells were cultured in 8-well culture slide chambers and transfected with PvMSP1P-19 or PvDBPII using Lipofectamine[®] 2000 (Invitrogen, Carlsbad, CA). At 42 hours post-transfection, COS-7 cells were incubated with 10% *Hct.* of serial dilutions of reticulocytes (0.5%-80.0%) to analyse PvMSP1P-19 binding preference. In the same way, the difference of Duffy status erythrocytes (positive or negative) and enzyme-treated erythrocytes were done for receptor identification. The peptide-treated erythrocytes were used for binding motif screening. The number of rosettes in 30 fields was counted under the light microscope using a 200x objectives lens. Positive rosettes were defined as adherent erythrocytes covering more than 50% of the COS-7 cell surface.

A binding inhibition assay was performed using mouse sera against PvMSP1P-19 and PvDBPII. COS-7 cells were pre-incubated with mouse sera that was serially diluted 1:1,000 to 1:50. Pre-immune mouse sera and sera from Pvs25-immunized animals were used as negative controls. The sera were washed out with PBS, and binding assays were then performed as described above. To investigate the acquired antibodies in vivax-infected patients, sera from ten patients were mixed in incomplete DMEM at dilutions ranging from 1:640 to 1:20. The transfection efficiency was determined by monitoring the expression of green fluorescent protein (GFP), and antibodies against the target protein were specified using Alexa 546-conjugated anti-mouse secondary antibodies (Invitrogen). GFP signals and antibodies were detected and scored in unfixed cells using a Fluoview FV1000 Laser Scanning Confocal Imaging System (Olympus, Tokyo, Japan) at a magnification of 200 \times .

379

380 **Mutant PvMSP1P recombinant protein expression**

381 Four mutant fragments containing 258 bp of the PvMSP1P-19 gene were generated by DNA
382 synthesis (Bioneer, Daejeon, Korea). The first mutant fragment involved mutation of the nine
383 negative charges (Asp and Glu) in the full EGF-like domain to positive charges (Lys). The
384 second mutation also changed three negatively charged residues (Glu1773, Glu1774 and
385 Asp1804) in place of the positively charged Lys in the pocket domain. The third and fourth
386 mutant fragments replaced a sequence in PkMSP1p with an orthologue sequence of PvMSP1P
387 in the pocket domain and the domain surrounding the pocket domain, respectively.

388 To identify the residues that are critical for PvMSP1P-19 binding, a large-scale wheat germ
389 cell-free (WGCF) expression system (Cell Free Sciences, Matsuyama, Japan) with Ni-affinity
390 purification was used. The PvMSP1P-19 (original or mutant fragment) amplicons (the primer
391 described in Table S2) were inserted into the pEU-E01-His-TEV-MSC-N2 vector using the In-
392 fusion Cloning System (Clontech) according to the manufacturer's protocol. Large-scale
393 protein expression was conducted using WGCF expression in accordance with the
394 manufacturer's protocol. The soluble fraction of the expressed material was applied to a Ni-
395 affinity column (Qiagen) and eluted with 500 mM imidazole. To determine the expression
396 levels and purity of the recombinant proteins separated by 13% SDS-PAGE and stained with
397 Coomassie Brilliant Blue.

398

399 **Fluorescence-activated cell sorting (FACS)-based reticulocyte binding assay**

400 The reticulocytes were used in a flow cytometry-based binding assay. Briefly, the
401 reticulocytes were suspended at 1×10^6 reticulocytes/mL and incubated with 20 μ g of hexa-
402 His-tagged recombinant protein. The samples were washed with PBS containing 1% BSA and
403 incubated with an Alexa Fluor 647-conjugated mouse monoclonal antibody against penta-His

(Qiagen) for 1 hour. The samples were washed with PBS-BSA and incubated with Thiazole Orange (TO) Retic-COUNT reagent (Becton-Dickinson) for 30 minutes at 25°C. For the fluorescence detection of single RBCs, a total of 100,000 events were acquired per sample using a FACS Accuri™ C6 Flow Cytometer (Becton-Dickinson). The flow cytometric results were analysed using FlowJo 7.6. Unstained cells and cells singly stained with TO were used to separate the normocytes and reticulocytes, respectively.

***P. vivax* invasion inhibition assay**

The invasion inhibition assay has been described previously (22, 51). Briefly, a preparation enriched in reticulocytes was obtained from cord blood by magnetic-activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Reticulocytes were magnetically labelled with CD71-microbeads and sorted on an LS column (Miltenyi Biotec). The concentrated late stage of the vivax parasite was half-cycle co-cultured with enriched and carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained reticulocytes at 37°C for 24 hours in an incubator containing 5% O₂. The parasite culture was mixed with rabbit antibodies against PvMSP1-19, PvMSP1P-19 and PvDBPII at a final dilution of 1:10. The monoclonal antibody 2C3 (murine anti-Fy6, a kind gift from Renia L, Singapore Immunology Network-BMSI-A STAR) was used as an invasion inhibition control (52), and rabbit pre-immune sera were used as negative controls. The post-invasion rate was analysed by FACS with 80,000 cells. The nuclei of newly growing vivax (ring-trophozoite) were stained with 4',6-diamidino-2-phenylindole (DAPI).

Statistical analysis

The data were analysed using GraphPad Prism (GraphPad Software) and Microsoft Excel 2013. For the erythrocyte binding and binding inhibition assays, Student's *t*-test was used to

compare the means of each group. For the invasion inhibition assay, one-way ANOVA with Tukey's post-test was used. Statistically significant differences are indicated by single asterisks ($p < 0.05$), double asterisks ($p < 0.01$) and triple asterisks ($p < 0.001$).

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FIGURE LEGENDS

Figure 1. Schematic structure of PvMSP1P and EGF-like domain sequence homology in

variant malaria-causing species. (A) Schematic diagram of PvMSP1P-19 at the amino acid (aa.) level. Two EGF-like domains (EGF, grey box) are encoded at the 1,751-1,789 and 1,792-1,834 aa. positions. The signal peptide (SP, black box), tandem repeat region (TR, orange), Glu/Gln polymorphic region (PR, yellow box) and GPI-anchored domain (GPI, blue box) are indicated. The synthesized peptides (18-mers) are shown in the black bar within the EGF-like domain. The nucleotide sequence is shown in Table S1. (B) Sequence alignment of the MSP1P-EGF-like domains of various *Plasmodium* species. The red bar represents sequences that are identical in all species. The sequence similarity is indicated by the strength of the bar as four (orange), three (green), or two (blue) identical species. The cysteine residue connection line represents the typical EGF-like domain disulphide bond. (C) Three-dimensional ribbon diagram of PvMSP1P-19. EGF-like domain 1 is shown in red, domain 2 is shown in grey, and cysteine residues are shown in yellow. The number on the cysteine residues represents the disulphide bond. (D) Three-dimensional surface diagram of PvMSP1P-19. The electrostatic surface of PvMSP1P-19 with positive (blue) and negative (red) charges is shown.

Figure 2. Reticulocyte binding preference. (A) Reticulocytes that were serially diluted from

80% to 0.5% were stained with new methylene blue and observed under light microscopy (upper panel). Reticulocyte binding was confirmed by PvMSP1P-19 or PvDBPII expressed COS-7 cell rosette formation (middle and bottom panel, Fig. S1). Red arrow heads indicate typical rosette formation. (B) Relative binding ability at different reticulocyte concentrations was calculated and normalized to PvDBPII (100% COS-7 cell transfection efficiency) with 0.5% reticulocyte (peripheral blood condition) binding ability as a standard (100%). The data are presented as the mean \pm S.D. of the relative binding (%) observed in three independent

experiments. PvDBPII and PvMSP1P-19 showed significant differences in binding ability under other conditions compared with the 0.5% reticulocyte condition in the PvDBPII and PvMSP1P-19 fractions, respectively. p values were calculated using Student's t -test. Significant differences are indicated by single asterisks ($p < 0.05$), double asterisks ($p < 0.01$) and triple asterisks ($p < 0.001$).

Figure 3. Inhibition of erythrocyte and reticulocyte binding and *P. vivax* invasion ability by antibodies. (A) Inhibition of PvDBPII binding by antibodies against PvDBPII and (B) inhibition of PvMSP1P-19 binding by antibodies against PvMSP1P-19 (upper panels). Confocal microscopy images show PvDBPII and PvMSP1P-19 expression on the surface of COS-7 cells; this expression was confirmed by GFP and specific antibody recognition using antibodies against PvDBPII and antibodies against PvMSP1P-19, respectively. The data are shown as the mean \pm S.D. of the binding inhibition measured in three independent experiments. Significant differences in the effects of PI sera and those of other antibodies were calculated using Student's t -test; single asterisks, $p < 0.05$; double asterisks, $p < 0.01$; triple asterisks, $p < 0.001$. (C) Inhibitory activity of serial dilutions of sera from vivax-infected patients (ROK+) or from uninfected persons (ROK-) on binding to erythrocytes and reticulocytes. Significant differences in the effects of ROK- and ROK+ sera were calculated using Student's t -test; single asterisks, $p < 0.05$; double asterisks, $p < 0.01$; triple asterisks, $p < 0.001$. (D) Vivax parasite invasion inhibition efficacy was confirmed in invasion inhibition assays. The data are presented as the mean \pm S.D. of the invasion inhibition rate obtained with pre-immune sera (PI, $n = 7$), anti-2C3 antibody (murine anti-Fy6, $n = 7$), anti-PvMSP1-19 sera ($n = 7$), anti-PvMSP1P-19 sera ($n = 7$) and PvDBPII ($n = 5$). Significant differences between PI sera and anti-2C3, anti-PvMSP1-19, anti-PvMSP1P-19 and anti-PvDBPII immunized sera were calculated using one-

way ANOVA with Tukey's post-test; single asterisks, $p < 0.05$; double asterisks, $p < 0.01$; triple asterisks, $p < 0.001$.

Figure 4. Erythrocyte binding motif and residue identification. (A) Erythrocyte and (B) reticulocyte competition binding assays were performed with PvMSP1P-19 peptides as competitors. PvDBPII was also included in an experiment to confirm nonspecific masking on RBCs. The data were analysed using Student's t -test; single asterisks, $p < 0.05$; double asterisks, $p < 0.01$. The mean \pm S.D. of the binding inhibition rate obtained in three independent experiments is shown. (B) Inhibition of the binding of PvMSP1P-19 to erythrocytes and reticulocytes via the serial dilution of S1764 (no inhibition detected at 100 μ M) and N1791 (highest inhibition detected at 100 μ M) peptides. The data are presented as the mean \pm S.D. of the binding inhibition observed in three independent experiments. (D) Reticulocyte binding ability of mutant PvMSP1P-19 protein. The data are presented as the mean \pm S.D. of the percentage of relative binding of PvMSP1P-19 observed in three independent experiments using the Sal-1 strain. Significant differences were calculated using one-way ANOVA with Tukey's post-test; single asterisks, $p < 0.05$; double asterisks, $p < 0.01$. (E) Sites of amino acid mutation are highlighted in the sequence alignment. The blue box shows the position of an unimportant peptide (S1764). The red box represents an important region for reticulocyte binding as confirmed by peptide competition assays. The red star indicates a critical residue for reticulocyte binding. (F) Three-dimensional structure of the reticulocyte binding region of PvMSP1P-19.

Figure 5. Reticulocyte-specific receptors for PvMSP1P-19 binding. (A) PvDBPII and (B) PvMSP1P-19 binding assays were performed using enzyme-treated erythrocytes and reticulocytes. Untreated (Un) erythrocytes and reticulocytes and erythrocytes and reticulocytes

treated with neuraminidase (Nm), trypsin (T) and chymotrypsin (Ct), respectively, were used to confirm receptor specificity. The data are shown as the mean \pm S.D. of the binding inhibition rate measured in three independent experiments. Significant differences compared to untreated erythrocytes or reticulocytes after the enzymatic treatment of erythrocytes and reticulocytes were calculated using Student's *t*-test and are denoted by triple asterisks, $p < 0.001$. (C) Binding specificity of PvMSP1P-19 and PvDBPII to Duffy-positive and Duffy-negative erythrocytes. The relative binding of PvMSP1P-19 to Duffy-positive and Duffy-negative erythrocytes was normalized to that of PvDBPII binding to Duffy-positive erythrocytes as a standard (100%). The data are shown as the mean \pm S.D. of the relative binding (%) measured in four independent experiments. Significant differences between Duffy-positive and Duffy-negative erythrocytes in PvDBPII and PvMSP1P-19, respectively, were observed. *p* values were calculated using Student's *t*-test; significant differences are indicated by triple asterisks, $p < 0.001$; no significance, ns.

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