Plasmodium falciparum Merozoite Surface Protein 6 Is a Dimorphic Antigen

J. Andrew Pearce,1* Tony Triglia,1 Anthony N. Hodder,1 David C. Jackson,2 Alan F. Cowman,1 and Robin F. Anders3

Infection and Immunity Division, The Walter and Eliza Hall Institute of Medical Research,1 and Department of Microbiology and Immunology, University of Melbourne,2 Melbourne, Victoria 3050, and Department of Biochemistry, La Trobe University, Victoria 3086,3 Australia

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Merozoite surface protein 1 (MSP1) is a highly polymorphic Plasmodium falciparum merozoite surface protein implicated in the invasion of human erythrocytes during the asexual cycle. It forms a complex with MSP6 and MSP7 on the merozoite surface, and this complex is released from the parasite around the time of erythrocyte invasion. MSP1 and many other merozoite surface proteins contain dimorphic elements in their protein structures, and here we show that MSP6 is also dimorphic. The sequences of eight MSP6 genes indicate that the alleles of each dimorphic form of MSP6 are highly conserved. The smaller 3D7-type MSP6 alleles are detected in parasites from all malarial regions of the world, whereas K1-type MSP6 alleles have only been detected in parasites from mainland Southeast Asia. Cleavage of MSP6, which produces the p36 fragment in 3D7-type MSP6 and associates with MSP1, also occurs in K1-type MSP6, but at a different site in the protein. Anti-3D7 MSP6 antibodies weakly inhibited erythrocyte invasion by homologous 3D7 merozoites but did not inhibit a parasite line expressing the K1-type MSP6 allele. Antibodies from hyperimmune individuals affinity purified on an MSP3 peptide cross-reacted with MSP6; therefore, MSP6 may also be a target of antibody-dependent cellular inhibition.

Many Plasmodium falciparum merozoite surface proteins are polymorphic, and this diversity appears to have arisen as a result of the selection pressure exerted by the host immune response (13, 17, 19, 27). The effectiveness of a vaccine can potentially be compromised by diversity in the target antigen; therefore, sequence polymorphisms are a major consideration when developing an antigen as a vaccine (12, 13, 23). The merozoite surface antigens is a potential vaccine component, but potential be compromised by diversity in the target antigen; therefore, sequence polymorphisms are a major consideration when developing an antigen as a vaccine (12, 13, 23). The merozoite surface antigens is a potential vaccine component, but MSP1 has been a particular focus of much work towards a malaria vaccine.

Many studies have provided results that strongly support the use of MSP1 in an asexual-stage vaccine (4, 13, 34). Proteolytically processing of MSP1 releases all but the glycosylphosphatidylinositol-anchored 19-kDa C-terminal fragment of this antigen from the merozoite surface at the time of invasion (5–7, 25, 26). p36 (MSP636) and p22 (MSP722), two polypeptides that are not MSP1 gene products (45, 46), are associated with the shed MSP1 complex. The genes encoding p36 and p22 have recently been described as MSP6 and MSP7, respectively (37, 52).

Here we show that MSP6 is also dimorphic and that the MSP6 alleles described are highly conserved within each dimorphic form. One of the dimorphic forms is distributed globally, whereas the other has only been detected in P. falciparum lines from Southeast Asia. The expression of an MSP6 allele is not associated with MSP1 dimorphism or the dimorphism in other known merozoite surface proteins. Both dimorphic MSP6 proteins are cleaved in situ but at different proteolytic sites. Antibodies that react with recombinant MSP6 were present in a pool of human serum from blood donors living in a region of malaria endemicity, and rabbit antibodies against MSP6 weakly inhibited merozoite invasion in vitro.

MATERIALS AND METHODS

In vitro culture of P. falciparum. P. falciparum parasites were grown using standard methods (50). The P. falciparum lines used in this work were the 3D7 clone of isolate NF54 (39), D10 from the Papua New Guinean (PNG) P. falciparum isolate FC27 (2), and clone W2mef from Southeast Asia (35). Other P. falciparum parasites studied included a Honduran line, HB3 (53), K1 from Kanchanaburi in the southeast of Thailand (48), NF7 from Ghana (2), and CSL-2 (38). The origins of the parasite Ghana, 7G8, ItG2, and Malay Camp (MC) lines have been described elsewhere (21, 30). CR25 and QA-1 are lines from Vietnam and China, respectively (42). Stage-specific parasites were produced by sorbitol synchronization of cultures as described previously (28). Free merozoites were isolated from in vitro cultures of P. falciparum 3D7 parasites by centrifugation and membrane filtration (32).

Identification of MSP6 in The Institute for Genome Research P. falciparum database. Preliminary sequence data for P. falciparum chromosomes 10 and 11 were obtained from The Institute for Genomic Research website (www.tigr.org). Sequencing of chromosomes 10 and 11 was part of the International Malaria Genome Sequencing Project and was supported by an award from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Deduced protein sequences were aligned using the program ClustalW (49).

Sequencing MSP6 alleles and MSP1 block 4 from P. falciparum gDNA. Genomic DNA (gDNA) was extracted from trophozoites and schizonts as described previously (51). The entire MSP6 gene was obtained by PCR amplification using the following primers: 10A5 seq (5′-ATGAATAGGTTAAAAATAATTACACAAATACAGTTACAT-3′) and 10A3 seq (5′-ATTTTAAATCAGTTACAT-3′). The PCR product was sequenced directly using the following primers: ESS10Afwd (5′-AATAACACTTTAATACAGTTACAT-3′), 10A190seq (5′-ATTACACGAA
The sequence of MSP1 block 4 was obtained from a PCR amplification product through the use of the following primers: 5'-GATAATGAAAGATTTGAAGAGATTAC-3' and 5'-GCCggatccAATAACTTTATCAGAAATGAACTT-3'. The MSP6 GST fusion protein was expressed in BL21 (Amersham Pharmacia). The MSP6 GST fusion protein was produced by immunizing New Zealand rabbits with 100 μg of protein emulsified in Montanide ISA-720 (SEPPIC, Paris, France). The primary immunization was intramuscular, and two subsequent immunizations at monthly intervals were subcutaneous. Rabbits were bled and sera was collected 12 days after the third injection.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Synchronized parasites from 3D7, D10, or W2mef cultures were harvested at the schizont stage by standard methods. The pellets were washed twice in human toxicity-phosphate-buffered saline (HT-PBS) and then resuspended in reducing sample buffer. These samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and were electrophoretically transferred to nitrocellulose of 0.45-μm pore size (Sartorius, Göttingen, Germany). Immunoblotting using human and rabbit sera at a dilution of 1:500 was performed as described previously (16). Human serum was preabsorbed with sonicated Escherichia coli to remove E. coli-reactive antibodies (41). Bound antibody was detected with horseradish peroxidase-coupled sheep anti-rabbit immunoglobulin G (IgG) (Amrad, Kew, Victoria, Australia), and the protein bands on the immunoblots were visualized using a chemiluminescent substrate (Pierce, Rockford, Ill.) according to the manufacturer's instructions.

Invasion inhibition assays. IgG was affinity purified (using protein G-Sepharose) (Pharmacia Biotech, Uppsala, Sweden) from pooled rabbit sera. Bound IgG was eluted with 0.1 M glycine (pH 2.6), neutralized, and then dialyzed with PBS. The antibody solution was sterilized by passage through a 0.22-μm-pore-size filter, adjusted to 5 mg/ml, and stored at 4 °C. P. falciparum cultures were synchronized by treatment with sorbitol to obtain early trophozoites for invasion inhibition assays (29). The parasites were diluted with uninfected erythrocytes to give a parasitemia of 0.5 to 1.5% and a hematocrit of 2% in hypoxanthine-free human tonicity medium (Sartorius, Gottingen, Germany). Invasion inhibition assays were performed at 37 °C. The parasites were diluted with uninfected erythrocytes to give a parasitemia of 0.5 to 1.5% and a hematocrit of 2% in hypoxanthine-free medium. Antisera to the MSP6-GST fusion protein was added to control wells. Cultures were incubated at 37 °C for 10 h. The cells were harvested after 48 h, and hypoxanthine incorporation was determined on a TopCount1 scintillation spectrometer. The mean value of percent invasion relative to the average of eight control wells without antibodies was calculated, and the standard error for each mean was determined from three assays in triplicate. Statistical analysis was carried out using the Student’s t test.

MSP3b synthesis and affinity purification of human antibodies. A synthetic peptide, MSP3b, was used to affinity purify human antibodies from plasma (24). The peptide is identical to that used by others in an antibody-dependent cellular inhibition assay and corresponds to amino acids 184 to 210 of 3D7 MSP3 (36). MSP3b was synthesized using Fmoc (9-fluorenylemethyl) chemistry in a Milligen 9050 Pico automatic peptide synthesizer (20). The peptide was cleaved from the resin, and side chain protecting groups were removed simultaneously by treatment with 88% trifluoroacetic acid (TFA) containing 5% phenol, 2% triisopropylsilane, and 5% water. Crude peptide was precipitated and washed in cold diethyl ether and purified by reversed-phase chromatography using a C4 PepRPC column (1.6 by 10 cm) installed in a fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden). Chromatograms were developed in a gradient of 0.1% TFA in water with 0.1% TFA in acetonitrile as the limit solvent. Appropriate fractions were collected, and the purity of the peptide was assessed by analytical reversed-phase chromatography using a C4 Vydac column (0.46 by 25 cm) installed in a Waters high-performance liquid chromatography system. MSP3b was bound to Maxisorp polystyrene immunoplates (Nunc, Roskilde, Denmark) as described previously, with some modifications (11). A total of 200 μl of peptide per well in HT-PBS at 10 μg/ml was bound to the well overnight at 4 °C. The immunoplates were then washed three times with 0.05% Tween 20-HT-PBS. Bound antibody was eluted with 0.2 M glycine (pH 2.5), transferred directly into dialysis tubing with a 15,000 molecular weight cutoff, and dialyzed against PBS for 24 h. The dialyzed eluates were concentrated using Centricron 30 concentrators (Amicon) and then stored at −20 °C before use.

RESULTS

Identification of a gene with homology to MSP3. Three P. vivax merozoite surface proteins contain alanine-rich heptad repeats which are homologous to the alanine-rich heptad repeats found in MSP3 (22). On the assumption that P. falciparum might also contain MSP3 homologues, we used MSP3 sequences and TBLASTN software (1) to search the P. falciparum genome database (http://www.tigr.org/BlastSearch/pfalciparum_11.html). An open reading frame was identified from the database, but it did not contain repeats of any kind. The sequence of the ORF of 1,113 bp was found to be identical to that of the MSP6 gene reported and partially characterized by Trucco et al. (52). This group also established that MSP6 is present in parasites in a complex with MSP1, the dimorphic asexual-stage vaccine candidate (45, 52).

MSP6 is dimorphic. PCR amplification of MSP6 from the gDNA of 13 strains of P. falciparum isolates produced DNA fragments either 1.1 or 1.3 kb in length (Fig. 1). Sequences of PCR products from eight P. falciparum isolates revealed that five have ORFs 1,113 bp in length (designated 3D7-type MSP6). The sequences of these five alleles are almost identical to the published 3D7 MSP6 sequence (52). The sequences of the other three MSP6 sequences, MC, W2mef, and K1 (designated K1-type MSP6), were also almost identical to each other but were longer than the 3D7-type sequences due to three blocks of additional sequence (Fig. 2 and 3). These three blocks of
sequence were 15, 24, and 129 bp in length, respectively, and are multiples of whole sense codons. The 15- and 24-bp blocks were inserted in frame with the MSP6 reading frame, whereas the 129-bp block of sequence appears to have been inserted between positions 1 and 2 of codon 125 (3D7 type). No significant matches to the 129-bp block were found in the P. falciparum genome database. A fourth block of additional sequence (consisting of 6 bp at nucleotides 893 to 899) was unique to the MC gene and encoded two sense codons. The W2mef and K1 MSP6 alleles were 1,272 bp in length, whereas the MC allele consisted of 1,278 bp.

In addition to the three blocks of extra sequence, the dimorphic forms of MSP6 are distinguished by numerous polymorphic sites. The majority of the differences among the alleles are centrally located in the coding sequence, the first 240 and the last 386 bp of sequence being entirely conserved in all eight alleles (Fig. 2). There are two synonymous mutations (one twofold and one fourfold) conserved within each dimorphic group of alleles. A twofold synonymous mutation occurs in a nucleotide site of a codon at which one of three possible nucleotide changes is synonymous, whereas a fourfold synonymous mutation is a mutation in which all possible substitutions are synonymous. These mutations indicate that the alleles within each dimorphic group are most likely derived from a common precursor rather than evolving convergently.

The deduced protein sequences of K1 and W2mef MSP6 are 427 amino acids in length and MC MSP6 is 429 amino acids long, whereas 3D7 MSP6 has 371 amino acids (Fig. 2 and 3). The calculated molecular mass of K1 MSP6 is 48,393 Da (compared to 42,250 Da for 3D7 MSP6). The pI of K1-type MSP6 is 4.4 and is very similar to the pI of 4.2 calculated for the 3D7-type MSP6. A disproportionate increase in positively charged residues in the sequence inserts contributes to the slightly higher pI in K1-type MSP6.

The residues immediately to either side of the site that is cleaved to generate MSP6 36 in 3D7-type alleles are conserved in K1-type MSP6 alleles, but the amino acids from P2 to P6 are

FIG. 2. ClustalW (49) alignment of eight MSP6 protein sequences that were deduced from sequenced DNA. The proteins fall into two distinct groups, the 3D7-type MSP6 group (which includes 3D7, NF7, D10, 7G8, and HB3) and the larger K1-type MSP6 group (which includes MC, W2mef, and K1 P. falciparum lines). A “2” or “4” above the alignments indicates the position of a codon containing a twofold or fourfold synonymous mutation, respectively. The 3D7-type MSP6 36 cleavage site is indicated by an arrow. Lines of dots indicate the positions of sequences corresponding to those of the synthetic peptide MSP3b.
not conserved (Fig. 4). When these sequences are aligned with those of MSP130, MSP736, and MSP344 cleavage sites (J. A. Pearce, A. N. Hodder, and R. Anders, submitted for publication), the Glu residue at P2 appears as a radical substitution in the K1-type MSP6 sequence (Fig. 4). In addition, residues P9, P10, and P12 are also unique to each dimorphic form (Fig. 2).

The average nucleotide diversity (H) value for the eight sequenced MSP6 alleles is 0.01633 (standard deviation, 0.0036). The ratio of nonsynonymous to synonymous mutations suggested that MSP6 sequences have been under diverging selection. To examine this further, we performed three tests of neutrality on the MSP6 data with DNASP version 3.52 software (40). Both the Tajima’s test and the Fu and Li D* statistic indicated a departure from neutrality, but neither test gave a statistically significant result. However, the Fu and Li F* statistic of 1.69 was significant (P < 0.05) and indicates that the polymorphisms present in the eight MSP6 alleles are not selectively neutral. A Strobeck’s S statistic of 0.101 indicates the probability of obtaining a sample of MSP6 alleles with a number of haplotypes equal to or less than the five observed in this sample and strongly predicts the existence of more MSP6 haplotypes in the parasite population.

**MSP6 protein dimorphism is detected in parasite samples.** Rabbit antibodies to recombinant 3D7 MSP6-GST (used in immunoblot analysis of 3D7 parasite samples) detected full-length MSP6 of 48 kDa in schizont pellets and the processed form of 36 kDa (MSP636) in culture supernatants as reported previously (52) (Fig. 5A). Identical proteins were detected in D10 parasites, in consistency with the results seen with the 3D7-type MSP6 allele that was identified in D10 gDNA. In the W2mef parasite line a full-length MSP6 product of 59 kDa was detected, a result which corresponds to findings obtained with the larger K1-type MSP6 gene found in W2mef. A larger processed form of MSP6 of 43 kDa was detected in the culture supernatant of the W2mef line. To confirm that the truncated W2mef MSP6 product corresponded to the C-terminal MSP636 fragment seen in 3D7, identical parasite samples to those used as described for Fig. 5A were probed with rabbit antibodies raised against recombinant 3D7 MSP636 (Fig. 5B). Identical MSP6 products were detected, indicating that the 43-kDa fragment of W2mef MSP6 was the C-terminal fragment. As was the case for the 3D7 and D10 parasite lines, the processed form of MSP6 in W2mef was the dominant form of the antigen detected in culture supernatants (Fig. 5).

**MSP6 dimorphism is not associated with dimorphism in MSP1.** Analyses of the MSP1 and MSP6 alleles expressed by different *P. falciparum* lines indicated that the expression of 3D7-type MSP6 is not linked to the expression of a particular dimorphic form of MSP1 (Table 1). Also, the expression of the K1-type MSP6 is not linked to the dimorphism in block 3 and blocks 6 to 16 of MSP1. However, the data set is too small to allow determination of whether there is an association between K1-type MSP6 and a particular dimorphic form of blocks 2 and 4 of MSP1.

We also examined whether MSP6 allele expression was linked to the expression of a particular MSP7 allele, as MSP7 is also a component of the MSP1 complex (37, 46). Sequencing of 3D7, NF7, W2mef, and K1 MSP7 PCR products detected no sequence variation in these lines, two of which contain 3D7-type MSP6 (3D7 and NF7) and two of which contain K1-type MSP6 (W2mef and K1). This result indicates that the expression of a particular MSP6 allele is not linked to the expression of a particular allelic type of MSP7.

Examination of the known dimorphic genes GLURP and MSP3, which are located close to the MSP6 locus on chromosome 10 (8, 31), also failed to identify an association with the expression of MSP6 dimorphic alleles (data not shown).

The known geographical origins of the 13 lines used in this study indicate that the parasite lines containing the 3D7-type MSP6 allele are present in all malarious regions sampled. However, the three parasite lines that contained the K1-type MSP6 allele were only detected in Thailand and Malaysia. This region also contains parasites (CR25 and CSL2) that have the 3D7-type MSP6 gene (Fig. 1). The preliminary conclusion that K1-type MSP6 lines are geographically restricted needs to be confirmed by determining the MSP6 genotypes of a larger number of parasite isolates.

**Human antibodies react with MSP6.** The antigenicity of MSP6 was investigated by using recombinant 3D7 MSP6-GST fusion protein in immunoblot analysis and by probing with plasma collected from blood donors living in a region of PNG where *P. falciparum* is endemic. After absorption to remove antibodies to *E. coli* was performed, antibodies in the plasma reacted with MSP6-GST but not with GST alone (Fig. 6A),
indicating that MSP6-reactive antibodies are generated in individuals naturally infected with *P. falciparum*.

A subset of human antibodies to MSP3 was used to examine antigenic cross-reactivity between MSP6 and MSP3. A synthetic peptide of 27 residues (MSP3b) was used to affinity purify antibodies from PNG serum. MSP3b corresponds to residues 211 to 237 of D10 MSP3 and is identical to the MSP3b used previously (36). MSP3b antibodies reacted strongly with recombinant MSP3 expressed in *E. coli* with a six-His tag (MSP3hh) and to a lesser extent with MSP6-GST, but not with r5Pehh, an unrelated *P. falciparum* recombinant protein expressed in *E. coli* with a six-His tag through the use of the same vector (Fig. 6B). GST alone was not detected with MSP3b antibodies, indicating that the MSP6 portion of MSP6-GST was responsible for the reaction with the MSP3b antibodies. These results suggest that MSP6 might contribute to the inhibition of *P. falciparum* growth seen in the ADCI assay (36).

**W2mef (K1-type MSP6) parasites are less inhibited by 3D7-MSP6 antibodies than 3D7 parasites.** IgG from rabbits immunized with recombinant 3D7 MSP6-GST produced a small (20%) but significant (*P* = 0.009) inhibition of 3D7 *P. falciparum* merozoite invasion compared to the results seen for invasion in the presence of the same concentration (500 μg/ml) of IgG from nonimmunized rabbits (Fig. 7). No significant reduction in invasion was detected with anti-MSP6 IgG at concentrations of 120 or 250 μg/ml. When the experiment was repeated using W2mef parasites (which express K1-type MSP6), however, the anti-3D7-MSP6 antibodies did not significantly reduce merozoite invasion at any concentration of antibodies used. Rabbit IgG against 3D7 AMA-1 used as a positive control was strongly inhibitory for invasion by 3D7 merozoites but not for that by W2mef merozoites (23a).

**DISCUSSION**

Here we show MSP6 to be dimorphic (as are several other well-characterized protein antigens associated with the *P. falciparum* merozoite surface) (31, 44, 47, 54). Polymorphisms in MSP6 were not described in a previous study, as the MSP6 dimorphic-block sections of MSP1 are also assigned a dimorphic character (K1 or MAD). The table indicates that the expression of an MSP6 dimorphic allele is not correlated with the expression of a dimorphic-block section of MSP1. The geographical origin of each line is also indicated.

### TABLE 1. Table showing the clone or line of parasite and the MSP6 dimorphic type (3D7 or K1)

<table>
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<th>MSP1 block 2 allele</th>
<th>MSP1 block 3 allele</th>
<th>MSP1 block 4 allele</th>
<th>MSP1 block 6-16 allele</th>
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<td>MAD</td>
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<sup>a</sup> The dimorphic-block sections of MSP1 are also assigned a dimorphic character (K1 or MAD). The table indicates that the expression of an MSP6 dimorphic allele is not correlated with the expression of a dimorphic-block section of MSP1. The geographical origin of each line is also indicated.

<sup>b</sup> NA, not available.

**FIG. 6.** Two panels of immunoblots that demonstrate MSP6 antigenicity. (A) Immunoblot of samples of GST alone and samples of recombinant MSP6-GST that were probed with *E. coli* antibody-absorbed human PNG serum shows that antibodies that react with MSP6-GST are present in human serum from areas where *P. falciparum* is endemic. (B) Samples used as described for panel A and, in addition, samples of recombinant MSP3 with a six-His tag (MSP3hh) and an unrelated six-His protein (r5Pehh) were probed with antibodies isolated using the synthetic MSP3-based peptide (MSP3b) from serum from areas of PNG where malaria is endemic. The PNG antibodies react with recombinant MSP3 and MSP6 but not with the negative controls.

**FIG. 7.** Histogram of parasite invasion of red blood cells (RBC) in the presence of IgG antibodies at various concentrations. IgG was isolated from rabbit antisera to 3D7 MSP6 and AMA1 and also from prebleeds. The antibodies were used to inhibit invasion of 3D7 or W2mef *P. falciparum* parasites. Invasion in 3D7 parasites was inhibited significantly (*P* = 0.009) with a 500 μg/ml concentration of IgG MSP6 serum antibodies (•) by 20% compared to prebleed results but was not significantly reduced in W2mef parasites. AMA1 antibodies were used as a positive control with 3D7 parasites, but 3D7 AMA1 antibodies did not inhibit invasion by W2mef parasites.
genes in the three parasite lines examined belonged to the same dimorphic type and had identical sequences (52). There are marked differences between the dimorphic forms identified in this study, whereas only small differences were found among the alleles within each dimorphic group. The high level of sequence conservation (including a twofold and a fourfold synonymous mutation in the alleles of each dimorphic group) is a strong indicator that each group of alleles is descended from a different progenitor. The geographical localization of the K1-type alleles to mainland Southeast Asia is also consistent with there being a recent K1-type progenitor, although sample numbers need to be increased to determine whether this is the case.

The apparent restricted geographical range of the parasite lines that contain K1-type MSP6 contrasts with the results seen with other polymorphic asexual-stage antigens (MSP1, MSP2, and S antigen), which show a more uniform geographic distribution of alleles (3, 12). However, geographical variation of P. falciparum genotype frequencies has been reported; one example is the sexual-stage antigen Pf48/45 (14, 15). The KK allele of Pf48/45 was present in all P. falciparum lines from Thailand and Malaysia, whereas the KE allele was the only allele present in the South America lines sampled. In contrast, a mix of the two alleles was detected in Africa. It is suspected that Pf48/45 plays a role in gamete recognition and compatibility and hence may have evolved in conjunction with other genes present in the local parasite population (14).

An examination of the blocks of dimorphic sequence in MSP1 did not reveal an association between the expression of MSP1 and MSP6 dimorphisms in eight parasite lines despite the established association between MSP1 and MSP6 on the merozoite surface (45, 52). Thus, the interaction between MSP1 and MSP6 is likely to be mediated by conserved regions of each protein. MSP7 also forms a complex with MSP1 and MSP6 (37, 46); however, the MSP7 gene was found to be entirely conserved in both 3D7-type and K1-type MSP6 parasite lines and therefore presumably does not influence the viability of parasites expressing a particular MSP6 dimorphic allele. Sequence analysis of MSP6 alleles suggests that the polymorphisms present are due to positive selection. Our data also indicate that MSP6-reactive antibodies are generated in a natural human infection (Fig. 6A), which may provide the selection pressure necessary to generate the polymorphisms seen in MSP6 alleles. These results are consistent with MSP6 having a role in influencing the growth rate of parasites in vivo. Furthermore, antibodies to recombinant 3D7-MSP6 weakly inhibited 3D7 merozoite invasion in vitro whereas there was no inhibition with W2mef (K1-type) parasites (Fig. 7). Therefore, K1-type MSP6-expressing parasites may have a slight growth advantage in a host that has been previously exposed to 3D7-type MSP6 parasites. Some evidence that host responses exert differential selective pressure on dimorphic forms of a merozoite antigen has come from studies of MSP2. Individuals previously infected with a P. falciparum line bearing a specific MSP2 allele did not become infected when exposed to a parasite line expressing the same MSP2 allele 29 months later (18), suggesting that allele-specific resistance is involved in the development of immunity. A recent phase 1-2b vaccine trial in PNG that used combination B, which included the 3D7 allele of MSP2, demonstrated a specific effect against the development of 3D7-type infections and a higher incidence of morbidity episodes associated with parasites of the other (FC27-type) MSP2 dimorphic form (23).

Like many other proteins associated with the merozoite surface, MSP6 is proteolytically processed. Cleavage occurs in both dimorphic forms of MSP6 but at different sites in the protein. The conservation of the processing event in K1-type MSP6 suggests that cleavage is important for MSP6 function. The two dimorphic forms are almost identical with respect to the C-terminal half of MSP6, and this level of homology would presumably allow the K1-type MSP6 C-terminal fragment (MSP6_{C2}) to function similarly to the 3D7-type MSP6_{C2} as a component of the MSP1 complex. It remains to be established whether cleavage of MSP6 occurs before or after association with the MSP1 complex.

The sequence polymorphisms, which include several radical amino acid substitutions close to the 3D7 cleavage site in the K1-type alleles, appear to block cleavage at this site in W2mef (K1-type) MSP6 (Fig. 5). The radical substitution of Glu for Ala in position P2 appears stark when aligned with the cleavage sites of other merozoite surface proteins and could be particularly important for preventing cleavage at this site in K1-type MSP6. The cleavage site in K1-type MSP6 has not been identified, but it may be generated from the inserted sequences or substitutions that are common to K1-type alleles. Alternatively, cleavage site generation and abolition may have resulted from conformational changes that stem from sequence differences between the two dimorphic forms.

It has been proposed that one antibody-mediated effector mechanism against asexual blood stages of P. falciparum involves cooperation between cytophilic antibodies and monocytes (9, 10). The antibody-dependent cellular inhibition assay has been used to assess the inhibitory capacity of such cytophilic antibodies in vitro (36). Antibodies against an MSP3-based synthetic peptide (MSP3b) inhibited growth by 70% when used in the ADCI assay (36). This is a high level of inhibition mediated by a small subset of antibodies to a conserved region of MSP3. We prepared an equivalent set of antibodies to MSP3b and found that they reacted with recombinant 3D7-type MSP6 in an immunoblot analysis (Fig. 6B). This cross-reaction was not surprising, as 9 of the central 11 amino acid residues in MSP3b are also found in both MSP6 dimorphic alleles. This result raises the possibility that the affinity-purified anti-MSP3b antibodies might mediate their ADCI effect by reacting with MSP6 in addition to MSP3.

MSP6 appears to have a regional variant that has only been detected in mainland Southeast Asia. Preliminary analysis indicates that this polymorphism might provide antigenic diversity and also a small growth advantage to the parasite in specific circumstances. MSP6 possesses some characteristics of a blood-stage vaccine candidate, but further work is required to establish its vaccine potential.

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