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Short communication

An additional primary proteolytic processing site in merozoite surface protein-1 of *Plasmodium berghei*

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The merozoite surface protein-1 (MSP-1) is a relatively abundant protein which has been found in all *Plasmodium* species examined [1,2]. MSP-1 is synthesized as a high molecular mass (~ 200 kDa) precursor protein which is proteolytically processed into smaller fragments. Proteolytic processing of MSP-1 is a two-step procedure characterized by primary and secondary processing events [3]. Primary processing occurs at, or just before, terminal merozoite differentiation and release and results in the formation of a noncovalent polypeptide complex with fragments of approximately 83, 30, 38 and 42 kDa [4]. The 42 kDa C-terminal fragment is further processed to 33 and 19 kDa fragments at the time of merozoite invasion [5]. This secondary processing is catalyzed by a Ca^{2+}-activated serine protease which results in the shedding of a soluble MSP-1 complex [6]. The remaining glycosyl-phosphatidylinositol-anchored 19 kDa fragment [7] contains two epidermal growth factor-like modules [8] and is carried into the erythrocyte upon merozoite invasion [9].

Comparison of the MSP-1 sequence from *P. berghei* with other rodent parasite MSP-1 sequences [10–12] revealed four major interspecies variable regions, or blocks (Jennings et al., in preparation). The first (i.e., most N-terminal) of these variable blocks was cloned from a λgt11 cDNA expression library screened with two different mAbs against PbMSP-1 [13]. The mAbs, designated as mAb-F4.4 and mAb-L1.6, were generated from mice immune to *P. berghei* [14]. Variable block 1 (VB1) contains seven degenerate

Abbreviations: LT-B, B subunit of *E. coli* labile toxin; MSP-1, merozoite surface protein-1; VB1, variable block 1.

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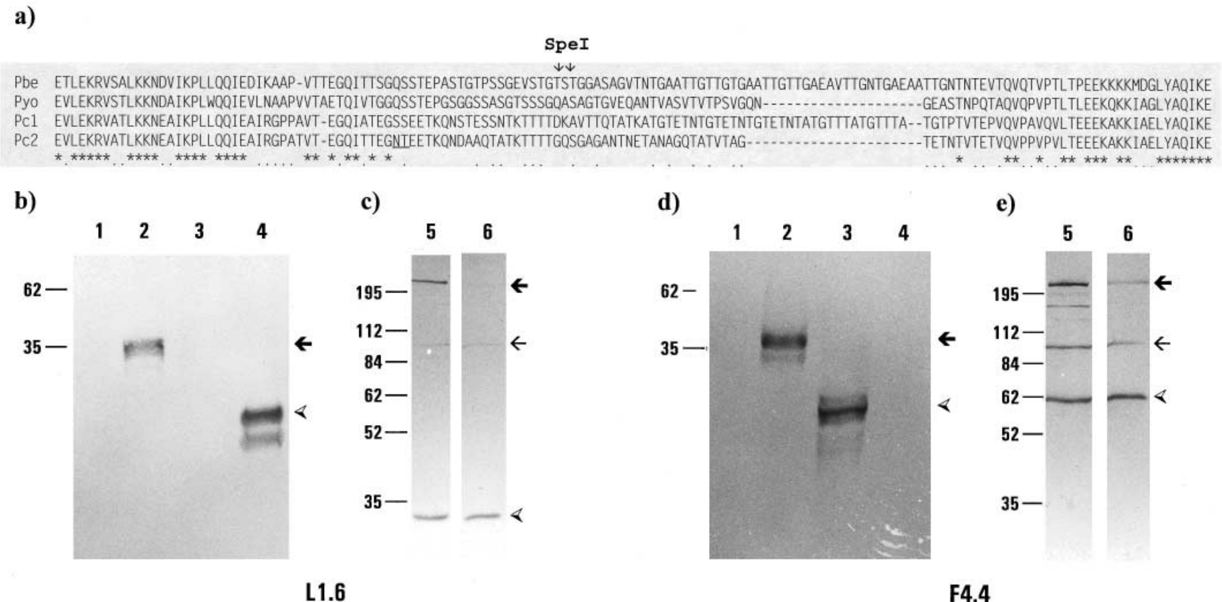

Fig. 1. Monoclonal antibodies F4.4 and L1.6 recognize different MSP-1 fragments. (a) Variable block I and the flanking conserved regions from MSP-1 of rodent *Plasmodium* species were aligned. The sequences are: Pbe, residues 251–393 (AC # U43521) from *P. berghei* (K173 strain); Pyo, residues 251–374 (AC # J04668) of *P. yoelli* (YM strain); Pc1, residues 251–391 (AC # M34947) of *P. chabaudi* (IP-PC1 strain); and Pc2, residues 251–370 (AC # L22982) of *P. chabaudi* (AS strain). Identical residues are denoted with an asterisk and similar residues with a period. The two amino acid residues spanning the *Spe*I restriction site in *P. berghei* are denoted with arrows. The proteolytic processing site in *P. chabaudi* [17] is underlined. Recombinant constructs, (b) and (d), expressing this region of PbMSP-1 as a fusion protein with LT-B were prepared [13] and tested for reactivity against mAb-L1.6 and mAb-F4.4 [14]. *E. coli* (lysate from 5×10^7 cells per lane) expressing various recombinant proteins were electrophoresed on 11% polyacrylamide SDS gels, transferred to Immobilon® membranes and analyzed by immunoblotting with the indicated mAb. The recombinant constructs express LT-B alone (lanes 1), LT-B fused with the entire region (lanes 2), LT-B fused with the portion which is to the C-terminal side of the *Spe*I site (lanes 3), and LT-B fused with the portion which is to the N-terminal side of the *Spe*I site (lanes 4). Bold arrows denote recombinant proteins recognized by both mAbs and the arrowheads denote recombinant proteins recognized by a single mAb. mAb-L1.6 recognizes an epitope to the N-terminal side of the *Spe*I site and mAb-F4.4 recognizes and epitope to the C-terminal side of the *Spe*I site. Segmenters, (c) and (e), and merozoites were isolated by differential centrifugation and analyzed by immunoblotting with the indicated mAb. Infected blood was centrifuged for 20 min at $10\,000 \times g$ on 60% Percoll® gradients and the uppermost layer, predominantly schizont- and segmenter-infected erythrocytes, were cultured in RPMI-1640 medium containing 10% fetal calf serum for 2.5 h. Following the incubation, infected erythrocytes were collected by centrifugation at $500 \times g$ for 4 min and solubilized in SDS gel electrophoresis sample buffer at a concentration of 0.1 μl packed infected erythrocytes per μl. Approximately 2.5×10^6 (2.5 μl) infected erythrocytes were electrophoresed on 9% polyacrylamide gels, transferred to Immobilon® and analyzed by immunoblotting with the indicated mAb (lanes 5). The supernatant from 25 μl packed infected erythrocytes was recentrifuged at $6000 \times g$ for 10 min to collected the released merozoites. Similar differential centrifugation procedures have been previously utilized for the isolation of merozoites [17,21]. The resulting pellet was solubilized in 60 μl of SDS gel electrophoresis sample buffer and 20 μl was analyzed by SDS gel electrophoresis and immunoblotting (lanes 6). Because of the small size of the second pellet, the merozoites were not quantitated. Bold arrows denote intact 230 kDa PbMSP-1 and the small arrows denote the 90 kDa fragment recognized by both mAbs. Arrowheads denote the 30 kDa fragment recognized exclusively by L1.6 and the 60 kDa fragment recognized exclusively by mAb-F4.4. The persistence of the 230 kDa protein in the merozoite-enriched pellet is probably due to the inability of differential centrifugation to completely separate the infected- erythrocytes from the merozoites.

tandem repeats of 10 amino acids. A series of recombinant constructs, possessing or lacking the tandem repeats, were prepared by taking advantage of a *Spe*I restriction site (Fig. 1a), and were tested as vaccines (C.S. Toebe, Ph.D. Dissertation, Tulane University). Immunoblotting of the proteins expressed by these recombinant constructs indicates that the epitope recognized by mAb-L1.6 is to the N-terminal side of the *Spe*I restriction site (Fig. 1b), and the epitope recognized by mAb-F4.4 is the C-terminal side of the *Spe*I restriction site (Fig. 1d).

Analysis of schizont-infected erythrocytes and enriched merozoites by SDS gel electrophoresis and immunoblotting reveals that mAb-L1.6 recognizes an approximately 30 kDa polypeptide not recognized by mAb-F4.4 (Fig. 1c), and mAb-F4.4 recognizes an approximately 60 kDa polypeptide not recognized by mAb-L1.6 (Fig. 1e). Both mAb-L1.6 and mAb-F4.4 recognize the intact 230 kDa MSP-1 and an approximately 90 kDa polypeptide (Fig. 1c,e). This 90 kDa polypeptide is homologous to the 83 kDa processing fragment of *P. falciparum* MSP-1 as demonstrated by alignment of the rodent *Plasmodium* MSP-1 sequences with PfMSP-1 sequences [13]. The immunoblotting results indicate that the 90 kDa PbMSP-1 fragment is further processed into 30 kDa and 60 kDa polypeptides and the protease site is between the L1.6 and F4.4 epitopes. A further processing of the 83 kDa fragment to 73 or 67 kDa fragments has been occasionally reported for PfMSP-1 [15,16]. This further processing of the 83 kDa fragment, however, appears to be a minor event in that the 83 kDa polypeptide is usually the predominant processing product. Therefore MSP-1 from *P. berghei* has a proteolytic processing site that is absent in PfMSP-1.

O'Dea et al. [17] have also proposed an additional protease processing site in the N-terminal region of MSP-1 from *P. chabaudi* which gives rise to 35 and 52 kDa fragments. N-terminal sequencing of the 52 kDa fragment from PcMSP-1 reveals that cleavage occurs between an asparagine and a threonine residue (denoted by underlining in Fig. 1a). Alignment of the MSP-1 sequences from other rodent parasites reveals that

Table 1
Distribution of amino acids in the primary processing sites of MSP-1 from rodent *Plasmodium* species

P6	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′
6I	4V	6A	4S	10G	8E	15S	12E	6E	5E
4Q	4I	5T	4T	6A	6Q	1T	2S	5D	3A
3P	3T	3V	3R		1N		2T	2Q	3T
2T	2A	1I	2E		1S			2T	2D
1H	1Q	1S	2N					1N	2V
	1R		1G						1G
	1K								

The four rodent *Plasmodium* sequences were aligned with regard to the four primary processing sites of *P. chabaudi* [17]. Accession numbers for the sequences are listed in the legend to Fig. 1. The number of occurrences of particular amino acids (single-letter code) found at positions surrounding the 16 (four strains × four primary processing sites) scissile bonds (between P1 and P1′ according to the nomenclature of Schechter and Berger [22]) are shown.

the scissile bond is on the boundary between a conserved region and VB1 (Fig. 1a) and that the scissile bonds differ between species and strains. However, the homologous residues in *P. berghei* and *P. yoelii*, glutamine and serine, are chemically similar to asparagine and threonine. A mAb against PyMSP-1 immunoprecipitates polypeptides of 230, 90 and 56 kDa and pulse-chase experiments demonstrate that the 230 kDa protein is converted to the 90 and 56 kDa fragments [18]. The similarity in the sizes of the *P. berghei* and *P. yoelii* MSP-1 fragments (i.e. 60 vs. 56 kDa) suggests that this additional proteolytic processing also occurs in *P. yoelii*. No information is available on the processing of MSP-1 from the IP-PC1 strain of *P. chabaudi*.

The amino acids flanking all four primary processing sites [17] in the MSP-1 sequences from four rodent parasites were analyzed (Table 1). The amino acids surrounding the scissile bonds are somewhat conserved and have a strong predisposition for certain types of amino acids. Polar and/or charged amino acids are found in the P1 and P3 positions, whereas the P2 and P4-P6 positions tend to be aliphatic. On the P′ side of the scissile bond the amino acids tend to have hydroxyl or acidic side groups giving rise to somewhat negatively charged domain. We propose a

consensus sequence of h-h-h-p-G/A-E/Q↓S-E-n-n, where the three lower case h's refer to a stretch of generally non-polar amino acids in which at least one is hydrophobic, the lower case p refers to a polar amino acid and the two lower case n's refer to a tendency to be negatively charged. The primary processing sites of PfMSP-1 [17] also exhibit this same general consensus sequence, except that the most C-terminal primary processing sites of PfMSP-1 lacks the strong negative tendency on the P′ side of the proposed consensus sequence. However, alternative processing sites for this position, which perfectly match the consensus sequence, have been proposed [19]. In addition, some minor processing fragments have been sequenced [16] and these protease sites also show good, but imperfect, homology to the consensus sequence. Cooper and Bujard [20] have described a schizont membrane associated protease which can cleave recombinant MSP-1. The sequences recognized by this protease all comply with the proposed consensus sequence.

In summary, the collective data indicate that MSP-1 from rodent *Plasmodium* species has an additional primary processing site that is likely absent in *P. falciparum*. The evidence includes: the similar sizes of the MSP-1 proteolytic fragments (30–35 kDa + 52–60 kDa), the strong amino acid homology in this region (Fig. 1a), and the conservation of the sequence flanking the scissile bond as compared to the other primary processing sites (Table 1). Furthermore, the similarity in the sequences of the primary processing sites suggest that a single protease is responsible for the primary processing. In addition, since they recognize different processing fragments, mAb-L1.6 and mAb-F4.4 will be useful reagents for the study of MSP-1 processing.

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