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A MALARIA MEROZOITE SURFACE PROTEIN (MSP1)-STRUCTURE, PROCESSING AND FUNCTION

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Merozoite surface protein-1 (MSP-1, also referred to as P195, PMMSA or MSA 1) is one of the most studied of all malaria proteins. The protein is found in all malaria species investigated and structural studies on the gene indicate that parts of the molecule are well-conserved. Studies on Plasmodium falciparum *have shown that the protein is in a processed form on the merozoite surface, a result of proteolytic cleavage of the large precursor molecule. Recent studies have identified some of these cleavage sites. During invasion of the new red cell most of the MSP1 molecule is shed from the parasite surface except for a small C-terminal fragment which can be detected in ring stages. Analysis of the structure of this fragment suggests that it contains two growth factor-like domains that may have a functional role.*

Key words: malaria – merozoite surface protein – Plasmodium

Merozoite Surface Protein-1 (MSP1; also known as precursor to major merozoite antigens (PMMSA), merozoite surface antigen-1 (MSA1), 195 kDa glycoprotein (gp195) and other notations based on its apparent molecular weight e.g. P.195) has been identified in *Plasmodium falciparum* and in other malaria species. The proteins have been grouped together on the basis of certain common features, including size, subcellular location, time of synthesis, and structural similarities detected by antibody cross reactivity and deduced primary amino acid sequence homology. Of great interest is the role of this protein in the biology of the parasite and in the induction of host-protective immune responses. The protein is synthesised by the intracellular schizont of the asexual blood (reviewed by Holder, 1988) and liver stages (Szarfman et al., 1988; Suhrbier et al., 1989) and expressed on the surface of the merozoites released by rupture of the infected cell. Variation in the size of the protein between species and different clones or isolates of a single malaria species, and immunochemical studies demonstrating strain-specific, species-specific and conserved epitopes, suggested structural differences and these have been analysed extensively by sequence analysis of cloned MSP1 genes. A number of studies have indicated that the primary translation product is modified. Early in the biosynthesis of the protein an N-terminal signal peptide is removed and a C-terminal hydrophobic anchor sequence is replace by a glycosylphosphatidy inositol moiety. In addition, at or immediately before schizont rupture the protein is processed by the action of proteases to produce a complex of polypeptides on the merozoite surface. Early studies on MSP1 have been reviewed (Holder, 1988), this article will focus on some of the more recent studies with particular emphasis on those in our laboratory.

STRUCTURE OF THE MSP1 GENE

Since the determination of the first complete *P. falciparum* MSP1 gene sequence in 1985 many other sequences have now become available, including MSP1 sequences from *P. yoelii* (Lewis, 1989), *P. chabaudi* (Deleersnijder et al., 1990) and *P. vivax* (del Portillo et al., 1991). The original analysis by Tanabe et al., (1987) indicated that the *P. falciparum* MSP1 primary structure could be divided into 17 blocks (at the amino acid level) in which the sequence was either highly conserved, or clearly homologous or quite different in comparison

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between distinct serotypes, and that the non-highly conserved blocks fell into two distinct sets (Fig. 1). This "dimorphic allele" model has been largely substantiated by subsequent analyses, and genes representing the putative products of intragenic recombination within conserved blocks have been identified. Exceptions to this model have been identified, however; block 2 which contains a short but highly repetitive structure of variable length and composition in the genes originally sequenced, also exists in a third, essentially non repetitive form (Certa et al., 1987, Peterson et al., 1988) that is prevalent in parasite populations in some areas (Kimura et al., 1990; Scherf et al., 1991). In addition, amino acid sequence differences resulting from frame-shift mutations have been identified (Certa et al., 1987). The contribution of cloning and sequencing artifacts must also be borne in mind; the first complete sequence published (Holder et al., 1985) has recently been corrected (Tucker et al., 1991), and comparison of this revised sequence with that of Palo Alto PLF-3/B11 (Myler, 1989) and T9/94 (Blackman et al., 1991a) suggests that the conserved block 17 at the C-terminus of the protein is longer than was previously thought.

The gene codes for a signal sequence, followed by a short repetitive sequence (but not in the exceptions outlined above), and the rest of the sequence is largely unique although several short peptide sequence may be present more than once. Several authors have highlighted the presence of potential N-glycosy-lation sites, but the limited experimental evidence suggest that these are not modified. At the C-terminus of the deduced amino acid sequence is a hydrophobic tail sequence preceded by a cysteine-rich domain (Fig. 2). The cysteine-rich region at the C-terminus of *P. falciparum* MSP1 appears to contain two Epidermal Growth Factor (EGF)-like motifs (Appella et al., 1988; Engel, 1989), with characteristic spacing of the cysteines and a glycine residue: (Cys-X-$_{1-8}$-Cys-X_{2-7}-Cys-X_{8-11}-Cys-X-Cys-X_{5-9}-Gly-X_2-Cys, where X is any other amino acid). In MSP1 from the other malaria species both cysteines 2 and 4 (which are linked in EGF) are missing from domain 1.

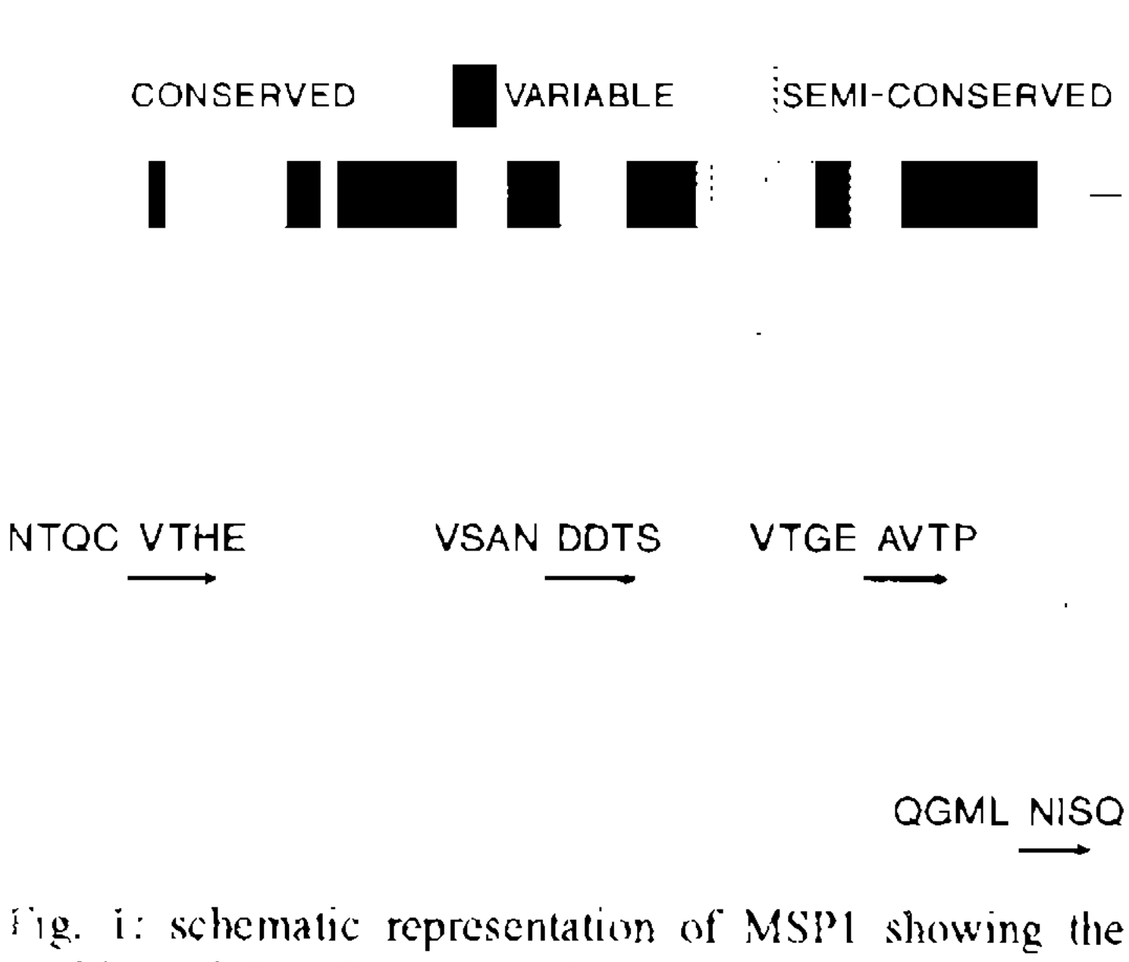

Fig. 1: schematic representation of MSP1 showing the position of the 17 sequence blocks defined by Tanabe et al. (1987) and the positions in the primary sequence of the processed fragments present on the merozoite surface. The sequence blocks have been defined as conserved, semi-conserved and variable in the comparison of genes from different "dimorphic types". The amino acid sequence, where known, at the N-terminus of each fragment is indicated on the righthand side of each of the cleavage points; the apparent molecular masses of the fragment are indicated.

PROCESSING OF THE PROTEIN ON THE MEROZOITE SURFACE

MSP1 is processed at the end of schizogony just prior to the release of merozoites from the mature schizont and a number of fragments are present on the merozoite surface. The exact timing of the processing is unclear, and its relevance has been questioned (Pirson & Perkins, 1985; Perkins & Rocco, 1988); nevertheless only a single fragment remains on the merozoite surface during invasion of a new erythrocyte (Blackman et al., 1990). On the merozoite surface at least four discrete fragments resulting from protease activity, are held together in a noncovalent complex (McBride & Heidrich, 1987). Processing schemes, in which the location of the fragments in the linear deduced protein sequence have been assigned are in broad agreement (Lyon et al., 1986; Holder et al., 1987). The size of the fragments depends upon the amino acid sequence of the particular allele, but the complex consists of polypeptides with the approximate size of 83 kDa, 28-30 kDA, 38 kDa and 42 kDa, and for convenience will be referred to as $MSP1_{83}$, $MSP1_{30}$, $MSP1_{38}$ and $MSP1_{42}$ (Figure 1). The C-terminal membrane-bound fragment, $MSP1_{42}$, undergoes a second proteolytic event to produce $MSP1_{33}$ and $MSP1_{19}$ (see below).

To identify the exact location of the proteolytic processing events N-terminal amino acid sequencing of merozoite surface fragments has been carried out. The N-terminal sequences of $MSP1_{83}$ (Wellcome and FCB1 strains: Holder et al., 1985; Strych et al., 1987), $MSP1_{38}$ (FCB1 strain, Heidrich et al., 1989) and $MSP1_{42}$ (FCB1 strain and T9/94 clone: Heidrich et al., 1989;

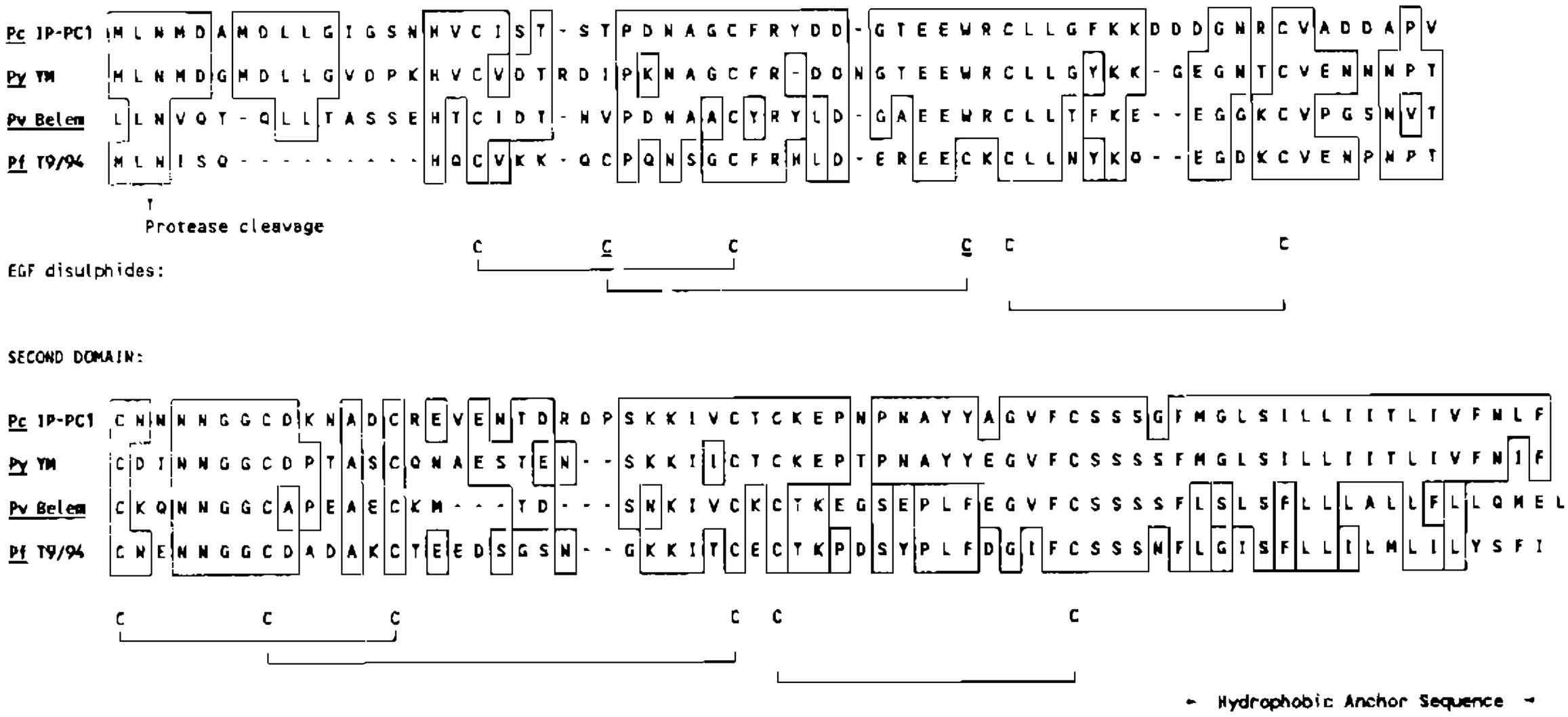

Fig. 2: comparison of the MSP1 C-terminal sequence from *Plasmodium chabaudi* IP-PC1 (Deleersnijder et al., 1990). *P. yoelii* YM (Burns et al., 1988; Lewis, 1989), *P. vivax* Belem (del Portillo et al., 1991) and *P. falciparum* T9/94 (Blackman et al., 1991a). The sequences are aligned from the protease cleavage site producing PfMSP1$_{19}$ and are divided into two domains corresponding to the growth factor domains (Blackman et al., 1991a) each containing 6 cysteines in the *P. falciparum* sequence. The second domain terminates with the hydrophobic anchor sequence thought to be replaced by a glycosylphosphatidylinositol moiety. The position of the cysteines and the disulphide bond arrangement in epidermal growth factor (EGF) are indicated; note that cysteines 2 and 4 in domain 1 are only present in the *P. falciparum* sequence. Identical residues in each sequence are boxed, some gaps have been included to optimise alignment.

Blackman et al., 1991a) have been determined by direct amino acid sequencing of the purified fragments (Fig. 1).

We determined the sequence at the 3' end of the MSP1 gene present in the T9/94 clone of *P. falciparum* and compared the deduced amino acid sequence with sequences determined by direct analysis of MSP1$_{42}$ and MSP1$_{19}$ purified from merozoites. The sequence of 1200 nucleotides representing the 3' coding region of the T9/94 MSP1 gene was obtained and was identical with that of the Palo Alto PLF-3/B11 clone (Myler, 1989) and the corrected version of the Wellcome strain (Tucker et al., 1991). MSP1$_{42}$ and MSP1$_{19}$ were purified on a Mab 111.4 column (this monoclonal antibody recognized a reduction-sensitive determinant in the C-terminal cysteine-rich domain of MSP1; Holder et al., 1987), separated by SDS-PAGE, transferred to Immobilon and then subjected to Edman degradation. The start of the two sequences and that derived from the analysis of the gene are incorporated in Figure 1. The N-terminus of MSP1$_{42}$, is 376 amino acids away from the stop codon. The N-terminus of MSP1$_{19}$ suggests that this fragment results from a chymotrypsin-like activity between a leucine and an asparagine residue 114 amino acids away from the end of the primary translation product and close to the start of the conserved block 17. It is difficult to predict the size of MSP1$_{19}$ because the protein is modified by glycosylphosphatidylinositol addition (reviewed in Holder, 1988), but the mobility of the protein on SDS-PAGE is less than would be expected. This discrepancy is unlikely to be accounted for by N-glycosylation since a clear asparagine was detected in the first cycle of Edman degradation and this is the only residue in the Asn-X-Ser/Thr motif (where X is any amino acid except proline) expected to be a target for N-glycosylation. In fact, this direct sequence analysis provides evidence against the notion that MSP1$_{19}$ is N-glycosylated.

The cleavage point between MSP1$_{83}$ and MSP1$_{30}$ as well as the exact sites in MSP1 of the "MAD 20"-type still need to be identified.

Examination of the C-terminal sequence of MSP1 in other *Plasmodium* species suggests that this chymotrypsin-like cleavage site may be conserved (Fig. 2). In each sequence there is a leucine-asparagine motif just upstream of the growth factor domains.

Secondary processing of $MSP1_{42}$

Only $MSP1_{19}$ is carried with an invading merozoite into the infected red cell and this fragment is derived from the C-terminal, membrane-bound end of $MSP1_{42}$ (Blackman et al., 1990, see Figs. 1 and 3), therefore an obvious question is what happens to the remainder of MSP1? Using an MSP1-reactive human Mab, X509, reacting with the N-terminal region of $MSP1_{42}$, we have investigated the secondary processing of $MSP1_{42}$ and detected a 33 kDa product ($MSP1_{33}$) that is released from the merozoite surface.

X509 reacts with MSP1 in an isolate-specific manner on immunoblots or by immunoprecipitation, and by immunofluorescence there was no detectable reactivity of Mab X509 with ring stages of the parasite (Blackman et al., 1990, 1991b). In merozoites extracts Mab X509 reacted with a 43 kDa polypeptide with the characteristics of $MSP1_{42}$. The reactivity of Mab X509 with $MSP1_{42}$ in both reduced and nonreduced forms, together with its complete lack of reactivity with $MSP1_{19}$, indicated that the X509 epitope is situated outside the C-terminal region of $MSP1_{42}$. Epitope mapping studies localized the binding site to within the dimorphic amino acid sequence block 16. When culture supernatants, collected after reinvasion from schizonts that had been radiolabelled with (^{35}S)methionine were analysed by immunoprecipitation, Mab X509 precipitated a 33 kDa labelled protein ($MSP1_{33}$), the mobility of which was not reduction sensitive. Similarly, $MSP1_{33}$ could be detected in samples of culture supernatants on immunoblots probed with Mab X509. Immunochemical analyses showed that $MSP1_{33}$ cross reacted with $MSP1_{42}$, but not with $MSP1_{19}$; antibodies selected from a human immune serum by $MSP1_{33}$ reacted with the $MSP1_{42}$, but not with $MSP1_{19}$, whereas antibodies affinity-adsorbed on $MSP1_{19}$ reacted with both $MSP1_{42}$ and $MSP1_{19}$, but not with $MSP1_{33}$. The relationship between $MSP1_{42}$ and the 33 kDa species was further confirmed by the identical maps obtained with the chymotryptic peptides of (^{35}S)-methionine labelled $MSP1_{42}$ and $MSP1_{33}$. Based on the amino acid sequence studies described above, the sequence of $MSP1_{33}$ is expected to run from Ala_{1348} to Leu_{1630}; with a predicted molecular weight of 32,456 daltons for the MAD-20 allele, close to the estimated size of T9/96 $MSP1_{33}$, and a predicted size of 30506 daltons for the T9/94 $MSP1_{33}$.

The processing of $MSP1_{42}$ results in the formation of two differentially targeted polypeptide products: $MSP1_{33}$ and $MSP1_{19}$. Further studies have shown that $MSP1_{33}$, which is a soluble fragment appearing in culture supernatants following schizogony, is shed in the form of a noncovalently-associated complex with a number of other proteins, including the MSP1-derived species $MSP1_{38}$ and $MSP1_{83}$ (Blackman & Holder, 1992). A schematic representation of the MSP1 proteolytic processing is shown in Fig. 3. The membrane-bound precursor MSP1 is processed at or just before merozoite release to a series of fragments, held on the surface of the merozoite through the membrane-bound fragment $MSP1_{42}$. At or before invasion secondary processing of $MSP1_{42}$ allows the complex to be shed from the merozoite surface except for $MSP1_{19}$, which is carried with the invading merozoite into the erythrocyte, and probably remains membrane-bound throughout the process (Blackman et al., 1990).

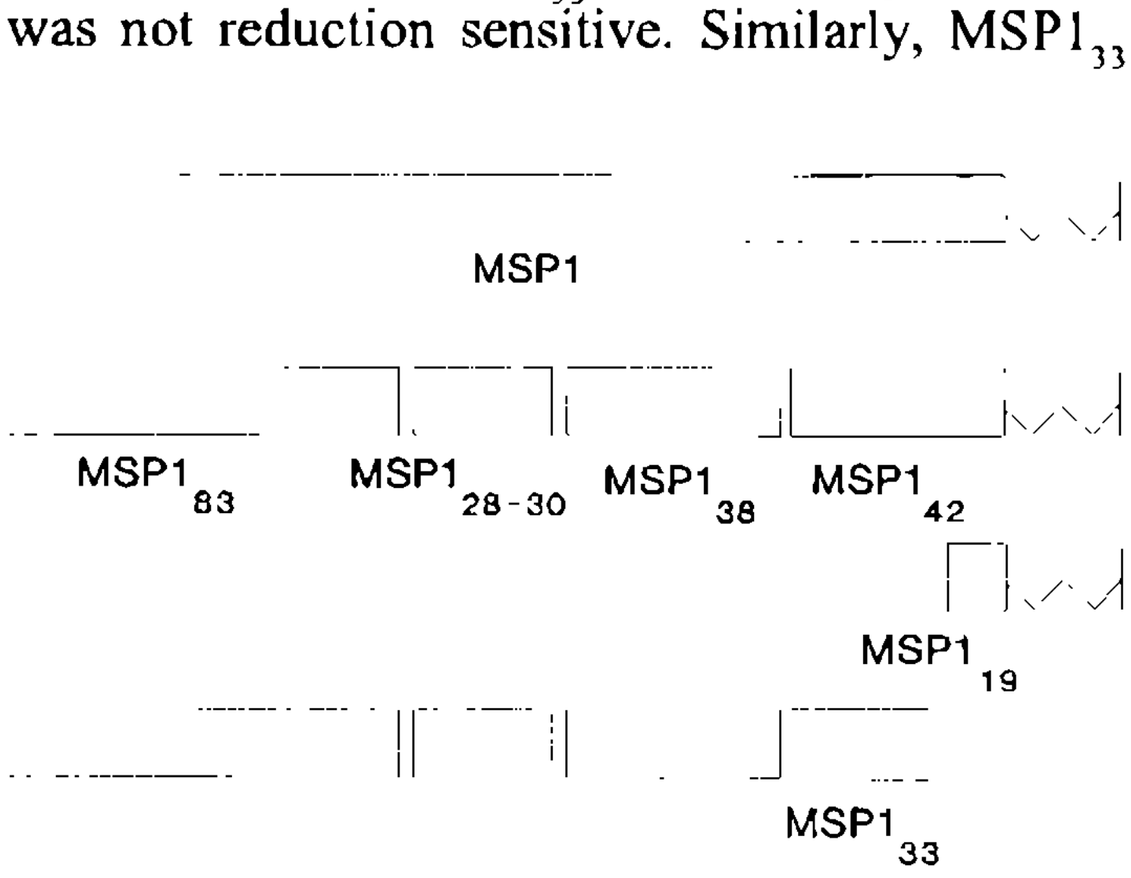

Fig. 3: processing of MSP1. The membrane-bound intact MSP1 is processed into discrete fragments present on the surface of the free merozoite. At or before red cell invasion a secondary processing step cleaves $MSP1_{42}$ to produce $MSP1_{33}$ and $MSP1_{19}$. $MSP1_{19}$ (still membrane-bound) is carried into the newly invaded erythrocyte whereas $MSP1_{33}$ is shed from the merozoite surface as part of a complex with the other MSP1-derived polypeptides.

Secondary processing of $MSP1_{42}$ is dependent upon the presence of extracellular calcium

Through the use of the chelating agent ethyleneglycolbis (β-amino-ethylether) N,N'-tetra acetic (EGTA) it has been shown that extracellular calcium is indispensable for erythrocyte invasion (Wasserman et al., 1982; McCallum-Deighton & Holder, 1992). When invasion was measured after four hours in calcium-depleted medium, supplemented with the

cations calcium, magnesium, manganese and zinc it was found that none of the cations, except for calcium, induced any significant increase in invasion rate. As calcium was added back into the medium to physiological levels invasion increased until it reached a rate comparable with that in the control assays (McCallum-Deighton & Holder, 1992). We have investigated the dependence of the secondary processing of $MSP1_{42}$ on the presence of extracellular cations.

The processing of $MSP1_{42}$ is inhibited by the chelating agents EDTA and EGTA, and this inhibition is reversed by the addition of excess calcium. This is illustrated in Fig. 4, which shows that addition of EGTA to cultures inhibits the secondary processing of $MSP1_{42}$, an effect that can be reversed by the addition of equimolar calcium, but not magnesium. This figure also illustrates that these treatments have no effect upon the primary processing of MSP1 to the fragments found on the merozoite surface.

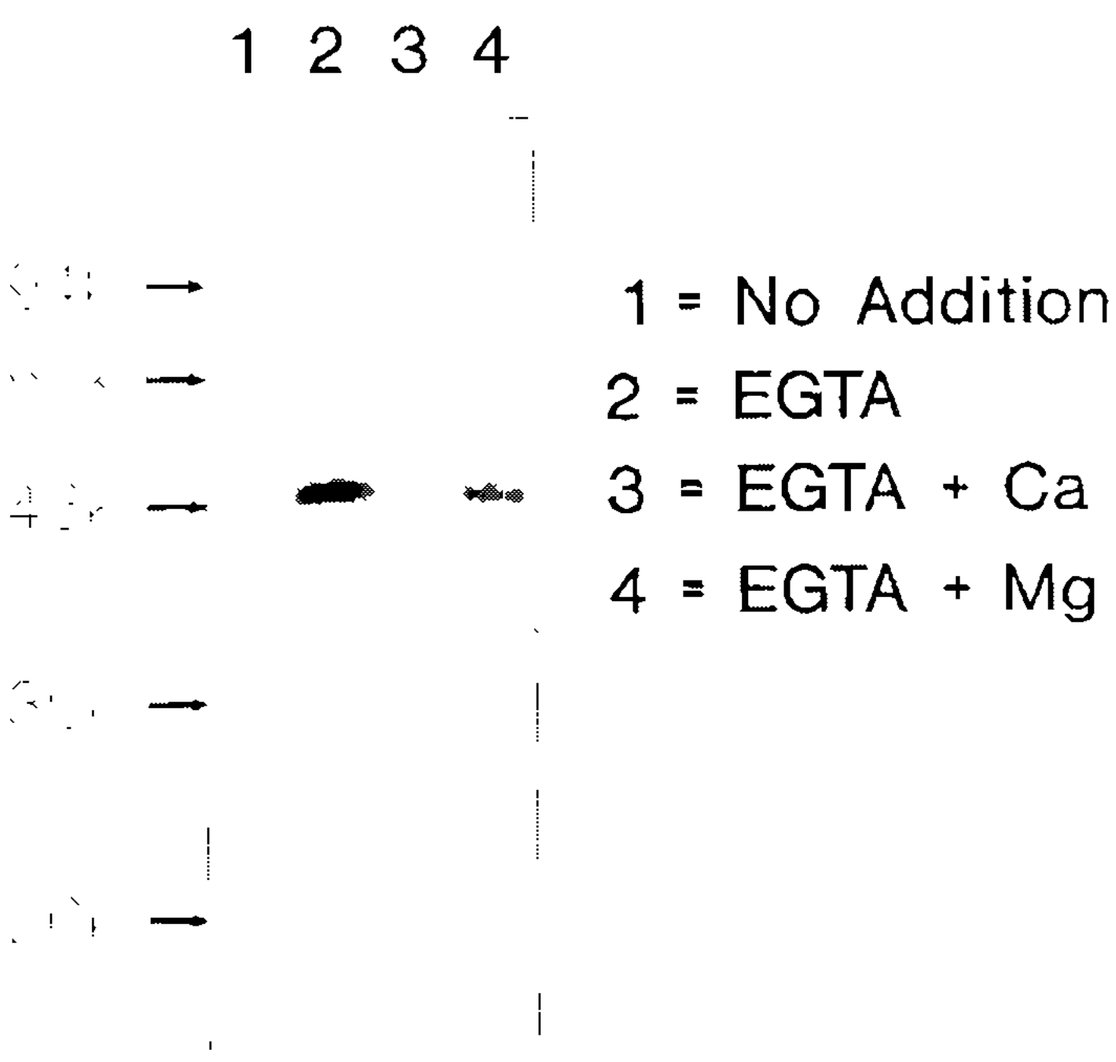

Fig. 4: chelating agents inhibit the secondary processing of $MSP1_{42}$ on the surface of merozoites. calcium ions reverse the inhibition. (^{35}S)methionine-labelled T9/96 schizonts were cultured in the presence of no additions (lane 1), 5mM EGTA (lane 2), 5mM EGTA plus 5mM $CaCl_2$ (lane 3), and 5mM EGTA plus 5mM $MgCl_2$ (lane 4). Released merozoites where harvested at hourly intervals, and detergent extracts were prepared. Immunoprecipitates with Mab 12.8 were then electrophoresed on a 12.5% gel and labelled proteins were detected by fluorography. The mobility of marker proteins is indicated. Cleavage of $MSP1_{42}$ is indicated by disappearance of the labelled polypeptide; $MSP1_{19}$, the membrane-bound product, contains no methionine residues and therefore is undetected in this experiment.

Function of MSP1 and its processing

There are few data on the role of MSP1 in erythrocyte invasion. Perkins & Rocco (1988) showed that intact MSP1 bound in a sialic acid dependent manner to human erythrocytes, suggesting that the protein may be involved as a receptor for ligand recognition on the red cell surface. It has also been suggested that MSP1 processing is a prerequisite for successful invasion, and in particular that the secondary protease cleavage of $MSP1_{42}$ must proceed to completion for a merozoite to invade an erythrocyte (Blackman et al., 1990). Depletion of calcium, a treatment that inhibits invasion also inhibits this secondary processing, but it has not been firmly established that the protease is calcium-dependent. However it has been established that the proteolytic activity responsible for the cleavage of $MSP1_{42}$ is highly site-specific. The effect of non-toxic inhibitors of this proteolytic activity upon erythrocyte invasion might provide some clues as to its importance in the invasion process.

EGF-like structures have been identified in $MSP1_{19}$ and since many proteins containing these structural motifs are involved in receptor binding or other cell surface interactions and protein adhesion it is possible that this may be the function of $MSP1_{19}$. The purpose of the proteolytic processing may be to reveal this membrane-bound activity during red cell invasion. The importance of this part of MSP1 during invasion is highlighted by the ability of antibodies to it to inhibit parasite growth (Blackman et al., 1990), but direct experimental evidence of a function is not available at present.

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