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ScienceDirect
Molecular & Biochemical Parasitology 151 (2007) 59–69
MOLECULAR & BIOCHEMICAL PARASITOLOGY

# Extensive proteolytic processing of the malaria parasite merozoite surface protein 7 during biosynthesis and parasite release from erythrocytes☆

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Received 25 July 2006; received in revised form 15 September 2006; accepted 11 October 2006
Available online 2 November 2006

**Abstract**

In *Plasmodium falciparum*, merozoite surface protein 7 (MSP7) was originally identified as a 22 kDa protein on the merozoite surface and associated with the MSP1 complex shed during erythrocyte invasion. MSP7 is synthesised in schizonts as a 351-amino acid precursor that undergoes proteolytic processing. During biosynthesis the MSP1 and MSP7 precursors form a complex that is targeted to the surface of developing merozoites. In the sequential proteolytic processing of MSP7, N- and C-terminal 20 and 33 kDa products of primary processing, $MSP7_{20}$ and $MSP7_{33}$ are formed and $MSP7_{33}$ remains bound to full length MSP1. Later in the mature schizont, $MSP7_{20}$ disappears from the merozoite surface and on merozoite release $MSP7_{33}$ undergoes a secondary cleavage yielding the 22 kDa $MSP7_{22}$ associated with MSP1. In free merozoites, both $MSP7_{22}$ and a further cleaved product, $MSP7_{19}$ present only in some parasite lines, were detected; these two derivatives are shed as part of the protein complex with MSP1 fragments during erythrocyte invasion. Primary processing of MSP7 is brefeldin A-sensitive while secondary processing is resistant to both calcium chelators and serine protease inhibitors. Primary processing of MSP7 occurs prior to that of MSP1 in a post-Golgi compartment, whereas the secondary cleavage occurs on the surface of the developing merozoite, possibly at the time of MSP1 primary processing and well before the secondary processing of MSP1.


*Keywords:* Malaria; Merozoite; Plasmodium; Protein processing

## 1. Introduction

Merozoites are invasive blood-stage forms of the malarial parasite, *Plasmodium falciparum* that invade erythrocytes leading to the pathological consequences of this most lethal form of malaria in humans. The merozoite surface and apical organelles such as rhoptries and micronemes are of interest to elucidate the mechanism of invasion of erythrocytes. Many merozoite surface proteins have been identified, including four proteins (MSP1, -2, -4 and -5) that are linked to the membrane via glycosyl phosphatidyl inositol (GPI) anchors [1] and some, such as MSP3 and MSP6 that are soluble in the parasitophorous vacuole and partially associated with membrane-bound proteins [2]. MSP7 is a peripheral membrane protein tightly associated with MSP1 [3,4]. Certain organellar proteins also appear to move to the surface either during or immediately after invasion. The

*Abbreviations:* BFA, brefeldin A; GPI, glycosyl phosphatidyl inositol; GST, glutathione *S*-transferase; hpi, hours post-invasion; MSP, merozoite surface protein

☆ *Note*: nucleotide sequence data reported in this paper are available in the GenBank™ database under accession nos. DQ987537 (D10), DQ987539 (HB3), DQ987538 (A4), DQ987540 (W7) and DQ987541 (7G8).

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doi:10.1016/j.molbiopara.2006.10.006

apical membrane antigen 1 (AMA1) primarily located in the micronemes has a transmembrane domain [5] and this protein is transferred on to the surface after merozoite release and prior to invasion [6]. These merozoite surface proteins have been implicated in red cell invasion. Several of these proteins undergo extensive proteolytic processing and upon red cell invasion many are shed from the surface by the action of a protease (a 'sheddase'), for example the MSP1 complex and AMA1. Both MSP1 and AMA1 undergo processing and only a small C-terminal region enters the red cell [7,8]. On the merozoite surface, the MSP1 complex is comprised of at least six proteins, four of which are derived by primary processing of MSP1 ($MSP1_{83}$, $MSP1_{30}$, $MSP1_{38}$ and $MSP1_{42}$), and the other two derived from MSP6 [2] and MSP7 [3]. The secondary processing of MSP1 (i.e., cleavage of $MSP1_{42}$ to $MSP1_{33}$ and $MSP1_{19}$) occurs when merozoites invade erythrocytes resulting in the release of most of the MSP1 complex [9], while the GPI-anchored C-terminal polypeptide ($MSP1_{19}$) remains on the surface of the new ring-stage parasite [10]. Previous studies on the shed MSP1 complex led to the discovery of a 22 kDa polypeptide (p22) and a 36 kDa polypeptide (p36) that were not derived from MSP1 [4]; later identified as derivatives of MSP7 [3] and MSP6 [2], respectively. MSP7 encodes a 351 amino acid polypeptide, although only the C-terminal region of this protein (p22) seemed to be present on the merozoite surface [3,4].

Proteolytic processing has an important role in red cell invasion by the merozoite. For example, the inhibition of MSP1 secondary processing by the binding of suramin or monoclonal antibodies (mAbs) to $MSP1_{42}$ reduces invasion [11,12]. Protease inhibitors also reduce merozoite invasion of red cells, presumably by inhibiting processing of MSP1 [13] and AMA1 [14]. AMA1 seems to require proteolytic processing to reach the merozoite surface from the apical organelles [6]. Thus, such processing events may be essential for the function of merozoite surface proteins, for example by facilitating their interaction with host cell surface receptors. It has been suggested that $MSP1_{42}$ and $MSP1_{19}$ interact with erythrocyte Band 3 protein [15].

In this article, we show that MSP7 undergoes extensive sequential proteolytic processing. MSP7 is present in late blood stages of the parasite co-located with MSP1 and antibodies to MSP7 or MSP1 (except mAb 89.1) co-precipitate both these proteins from extracts of parasites prepared using a mild non-ionic detergent. The MSP1 and MSP7 precursors form the complex prior to the processing and brefeldin A treatment leads to the accumulation of this precursor complex. Following proteolytic cleavage of MSP7, the C-terminal domain remains associated with MSP1.

## 2. Materials and methods

### 2.1. Over-expression and purification of MSP7 and production of antibodies

MSP7 polypeptides A (N-terminal region corresponding to amino acids 23–176) and B (C-terminal region corresponding to amino acids 177–351) were expressed fused to glutathione *S*-transferase (GST) using the pGEX-3X plasmid (Amersham Biosciences). MSP7B was also expressed as a hexaHis-tagged protein using the pTrcHisC plasmid (Invitrogen). The oligonucleotide primers used in this study have been described earlier [3]; Prep22Fexp and Prep22Rexp were used to amplify MSP7A and the primers, p22Fexp and p22Rexp were used for MSP7B. The amplified products were restricted with *Bam*HI and *Eco*RI and cloned into pGEX-3X. For His-tagged MSP7B, *Bam*HI and *Eco*RI restricted *msp7b* was cloned into pTrcHisC. The fusion proteins were expressed in *E. coli* BL21 Gold$^{TM}$ (Stratagene) and purified according to the supplier's instructions by GST- or Ni-NTA-agarose chromatography under non-denaturing conditions. The GST–MSP7A and GST–MSP7B fusion proteins were used to immunise Balb/c mice for the production of polyclonal antibodies (referred to as anti-MSP7A and anti-MSP7B antibodies, respectively). Antibodies to hexaHis-tagged MSP7B were also raised in rabbits.

Antibodies specific for MSP1, including rabbit polyclonal and mAbs 89.1 and X509 have been described previously [16–18].

### 2.2. Parasites and preparation of proteins for immunoblot analysis

*P. falciparum* lines 3D7 (The Netherlands), FCB1 (Columbia), D10 (Papua New Guinea), T9-96 (Thailand), 7G8, HB3 (Honduras), A4 (Brazil) and W7 (Gambia) were maintained in culture as described previously [17]. Schizonts were purified by using Percoll [19], saponin treated and lysed in SDS-PAGE sample buffer. Merozoites, prepared as described elsewhere [8], were lysed in the same way. Shed MSP1 complex from parasite culture supernatants was prepared as described [4]. To obtain parasite populations synchronised for development at various times post-invasion, magnet-purified 3D7 schizonts were allowed to invade erythrocytes for 2 h, sorbitol treated to eliminate mature forms and then cultured in normal medium. At 3-h intervals samples of cells were harvested and lysed in hypotonic buffer (25 mM Tris–HCl, pH 8.0, 5 mM EDTA and 5 mM EGTA). The lysates were centrifuged in a refrigerated table-top centrifuge and the pellet was used for further analysis.

The protein preparations were separated by 12.5% SDS-PAGE or 12% NuPAGE (Invitrogen), transferred on to Protran membrane (Schleicher and Schuell) and probed with anti-MSP7A and anti-MSP7B antibodies. The immunoblots were developed by using HRP-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents (Amersham International).

### 2.3. Sequence analysis of the MSP7 gene

DNA was prepared from laboratory lines of *P. falciparum* and 1.7 kb DNA from the MSP7 locus was PCR amplified by using TaKaRa Taq polymerase (Takara Bio Inc., Japan) and the primers CGACACACGCATGAATAGAAATTAGACATG and CCTATTAATACTTATATAAAGGTACACAATTTAACCG. Internal primers were used to obtain the sequence of both strands.