

# A multifunctional serine protease primes the malaria parasite for red blood cell invasion

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The malaria parasite *Plasmodium falciparum* replicates within an intraerythrocytic parasitophorous vacuole (PV). Rupture of the host cell allows release (egress) of daughter merozoites, which invade fresh erythrocytes. We previously showed that a subtilisin-like protease called PfSUB1 regulates egress by being discharged into the PV in the final stages of merozoite development to proteolytically modify the SERA family of papain-like proteins. Here, we report that PfSUB1 has a further role in ‘priming’ the merozoite prior to invasion. The major protein complex on the merozoite surface comprises three proteins called merozoite surface protein 1 (MSP1), MSP6 and MSP7. We show that just before egress, all undergo proteolytic maturation by PfSUB1. Inhibition of PfSUB1 activity results in the accumulation of unprocessed MSPs on the merozoite surface, and erythrocyte invasion is significantly reduced. We propose that PfSUB1 is a multifunctional processing protease with an essential role in both egress of the malaria merozoite and remodelling of its surface in preparation for erythrocyte invasion.

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## Introduction

Transmission of *Plasmodium* spp., the protozoan parasite that causes malaria, occurs following the bite of an infected

Anopheline mosquito. Injected sporozoites migrate to the liver where they invade hepatocytes and replicate within a parasitophorous vacuole (PV) to yield a liver-stage schizont containing several thousand merozoites per cell. In a process called egress, the schizont then ruptures to release the merozoites, which enter the bloodstream and invade erythrocytes. This initiates the asexual erythrocytic cycle, responsible for the clinical manifestations of the disease. At each round of subsequent intraerythrocytic growth, further mitotic replication takes place, also inside a PV, producing 16–32 daughter merozoites, which egress to invade fresh erythrocytes and perpetuate the cycle.

Developing malaria merozoites, including those of the most dangerous form, *Plasmodium falciparum*, express a number of merozoite surface proteins (MSPs). MSP1 is a major malaria vaccine candidate antigen (reviewed by Vekemans and Ballou, 2008), and is indispensable in the parasite life cycle (O’Donnell *et al*, 2000; Drew *et al*, 2004). MSP1 is synthesized as an ~195-kDa glycosyl phosphatidylinositol-anchored precursor that assembles as a complex with two peripheral membrane proteins called MSP6 and MSP7 (Holder *et al*, 1985; Stafford *et al*, 1996; Pachebat *et al*, 2001; Trucco *et al*, 2001; Kauth *et al*, 2003, 2006). The MSP1/6/7 complex is abundant, uniformly coats the merozoite surface and is involved in the receptor-mediated interactions that initiate erythrocyte invasion (Su *et al*, 1993; Goel *et al*, 2003; Li *et al*, 2004). Just prior to egress, all three MSPs undergo a series of precise ‘primary’ proteolytic cleavage events (Freeman and Holder, 1983; Lyon *et al*, 1986, 1987; Holder *et al*, 1987; McBride and Heidrich, 1987; Stafford *et al*, 1994; Pachebat *et al*, 2007). The major cleavage products remain non-covalently associated on the surface of the released merozoite, until the complex is finally shed at the point of erythrocyte invasion in an essential secondary processing step by the action of a membrane-bound parasite protease called PfSUB2 (Harris *et al*, 2005). The specific nature of the primary proteolysis and the positional conservation of the cleavage sites in MSP1 orthologues across the *Plasmodium* genus (reviewed by Blackman, 2000) suggest that primary processing is important for the function of the MSP1/6/7 complex and for merozoite viability. However the protease(s) responsible for primary processing is unknown.

Parasite protease activity is required for blood-stage egress in *P. falciparum* (Delplace *et al*, 1988; Salmon *et al*, 2001; Wickham *et al*, 2003; Gelhaus *et al*, 2005), but the mechanisms by which egress is regulated are only just beginning to become clear. We recently showed that, just prior to egress, an essential subtilisin-like serine protease called PfSUB1 is discharged from specialized merozoite organelles called exo-nemes into the PV space (Yeoh *et al*, 2007). There, PfSUB1 mediates the proteolytic maturation of members of a family of abundant, papain-like putative proteases called SERA,

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previously implicated in egress (Pang *et al*, 1999; Aly and Matuschewski, 2005). Selective inhibition of PfSUB1, with either a natural compound called MRT12113 (Yeoh *et al*, 2007) or covalently modifying chloroisocoumarins (Arastu-Kapur *et al*, 2008) prevents SERA maturation and blocks egress. This indicates a role for PfSUB1 in triggering egress, probably through activation of the SERA enzymes. We also noticed in our study that, at relatively low concentrations of MRT12113, egress occurred but the released merozoites were defective in invasion (Yeoh *et al*, 2007). This implied an additional role for PfSUB1 in merozoite maturation.

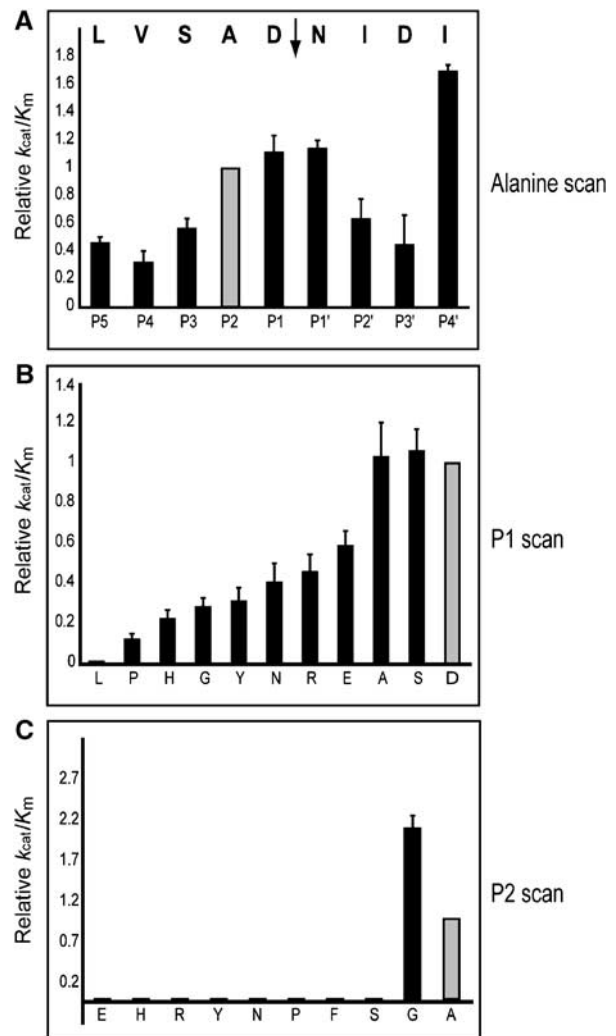
Here, we report that PfSUB1 is indeed required for the development of invasive merozoites. We show that, upon its release into the PV, PfSUB1 directly mediates the primary proteolytic processing of MSP1, MSP6 and MSP7. Inhibition of PfSUB1-mediated processing of MSP1 abrogates invasion by released merozoites, indicating a crucial role in merozoite viability. Our new insights provide a simple model to explain the strict temporal and spatial regulation of the primary and secondary processing steps to which the MSP1/6/7 complex is subjected, and show that PfSUB1 is a multifunctional processing protease of the malarial PV that regulates both egress and proteolytic remodelling of the developing merozoite in preparation for its release from the confines of the infected cell.

## Results

### Characterization of PfSUB1 substrate specificity

Maturation of SERA5 by PfSUB1 involves cleavage at three positions, referred to as site 1 (386EIKAE↓TEDDD395 in the 3D7 SERA5 sequence), site 2 (882IIFGQ↓DTAGS891) and an allele-specific sequence called site 3 (196TVRGD↓TEPIS205) (Yeoh *et al*, 2007). A comparison of blood stage-expressed SERA family members showed that features of the site 1 and 2 cleavage sites—which flank the central papain-like putative catalytic domain found in all SERAs—were conserved. We then showed that both SERA4 and SERA6, two other abundant members of the SERA family, were also processed by PfSUB1 at schizont rupture. Taken together with analyses of a limited series of synthetic peptides (Withers-Martinez *et al*, 2002), these results suggested a putative consensus PfSUB1 recognition motif of Ile/Leu/Val/Thr/Phe-Xaa-Gly/Ala-Paa (not Leu)↓Xaa (where Xaa is any amino-acid residue and Paa tends to be a polar residue). We also noticed a tendency for acidic residues as well as Ser or Thr at one or more of the proximal five positions on the prime side of the scissile bond.

To seek support for this prediction and further dissect the sequence requirements for recognition by PfSUB1, we synthesized several fluorogenic peptides based on the internal PfSUB1 sequence 215LV SAD↓NID223 at which autocatalytic cleavage occurs to remove the PfSUB1 propeptide during maturation of the protease in the malarial secretory system (Blackman *et al*, 2002). After confirmation that it was cleaved as expected by recombinant PfSUB1 at the Asp–Asn bond (Supplementary Figure S1), peptide Abz-LVSADNIDIQ-EDDnp was compared with a series of similar peptides that differed only by substitution of individual residues with Ala (Figure 1A). This showed that, of the non-prime side positions examined, substitution of the Val at P4 had the greatest effect on substrate cleavage efficiency. This is consistent with the predicted hydrophobic nature of the PfSUB1 S4 pocket



**Figure 1** Analysis of PfSUB1 substrate specificity. (A) Alanine substitution scanning of fluorogenic peptide Abz-LVSADNIDIQ-EDDnp. The parent peptide sequence is shown (inset), with the cleavage site arrowed. The x-axis indicates residue positions that were individually substituted with Ala. (B) P1 scanning of the same parent peptide Abz-LVSADNIDIQ-EDDnp. The x-axis indicates the various amino-acid substitutions made at the P1 position. (C) P2 scanning of Abz-LVSADNIDIQ-EDDnp. The x-axis indicates the various amino-acid substitutions made at P2. In each plot, bars indicate the efficiency of cleavage by recombinant PfSUB1, expressed as the ratio of activity against the substituted peptide, versus activity against the parent peptide (indicated in each case by a grey bar with a value of 1). Ratios are expressed as relative  $k_{cat}/K_m$  values. In all cases, each value represents the mean of eight separate determinations. Error bars, standard deviation values.

(Withers-Martinez *et al*, 2002), and the known importance of the P4 residue in substrate recognition by subtilases generally (Siezen and Leunissen, 1997). In addition, the P5 Leu and P3' Asp positions were found to be relatively important for PfSUB1 cleavage. We next investigated the importance of the P1 position, using a further set of peptides that varied only at this position (Figure 1B). Although replacement with Ser or Ala had no significant effect on cleavage, peptides containing Glu, Arg, Asn, Tyr, Gly or His at P1 were cleaved with intermediate efficiency, Pro at P1 was cleaved poorly, and a peptide with Leu at P1 was not cleaved. Finally, the importance of the P2 position was examined in a similar

manner (Figure 1C). This showed that, of the amino-acid residues assessed, only Ala and Gly could be accommodated at P2, with an apparent preference for Gly; substitution with any of nine alternative residues resulted in peptides that were completely refractory to cleavage by PfSUB1.

These results support the consensus motif described above, confirming the importance of the P4 and—in particular—the P2 subsites in substrate cleavage by PfSUB1. The data also provide experimental evidence for a role in substrate recognition of both the P5 position and of acidic residues on the prime side of the cleavage site.

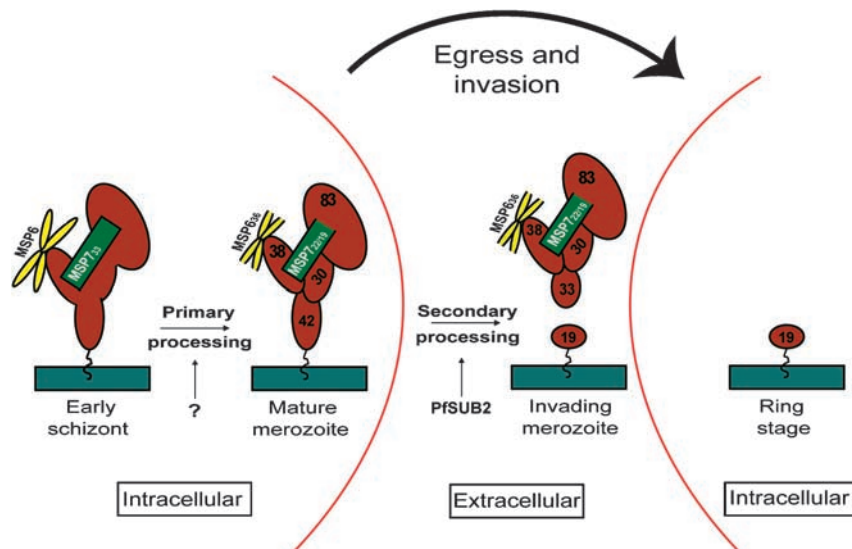
**The PfSUB1 consensus substrate recognition motif resembles all known MSP primary processing sites**

Armed with the above information, we searched for additional physiological PfSUB1 substrates that might explain the observed effects of inhibitor MRT12113 on the invasive capacity of released merozoites. We reasoned that, upon its release into the PV to exert an effect upon the SERAs just prior to schizont rupture, PfSUB1 should also have access to other PV proteins, including surface-resident merozoite proteins that are exposed to the PV lumen. Extensive work over the last 25 years has shown that several MSPs undergo dramatic proteolytic modification just prior to schizont rupture. The best-characterized examples of these are MSP1 and two peripheral membrane proteins, MSP6 and MSP7 (Figure 2). MSP1, which exists in two major allelic forms in *P. falciparum*, is converted to four polypeptide fragments of ~83, 38, 30 and 42 kDa (referred to as MSP1<sub>83</sub>, MSP1<sub>38</sub>, MSP1<sub>30</sub> and MSP1<sub>42</sub>, respectively). MSP6 and MSP7, which are also dimorphic proteins and both of which are non-covalently associated with MSP1 on the merozoite surface, are also cleaved at internal sites; in each case the C-terminal

products remain bound to the MSP1 complex, whereas the fate of the N-terminal fragments is unknown. Most of the cleavage sites have been mapped by N-terminal sequencing of endogenous cleavage products (Supplementary Figure S2). As shown in Table I, comparison of all these MSP processing sites with the internal PfSUB1 processing site, the known SERA5 processing sites, and the predicted analogous sites in SERA4 and SERA6, revealed a pattern strikingly consistent with the PfSUB1 consensus recognition motif described above. Most conspicuously, every site has either Ala or Gly at the P2 position, and all have Leu, Ile, Val or Thr at P4. In addition, none of the sites contains Pro or Leu at P1, and on the prime side of the scissile bond most exhibit an acidic tendency and/or a preference for Ser and Thr residues. Interestingly, cleavage at the MSP7<sub>19</sub> site does not occur in forms of MSP7 that contain a Lys substitution of Gly193, the residue at the P2 position in those MSP7 alleles that are processed at this site (Pachebat *et al*, 2007). Collectively, these observations raised the strong possibility that MSP1, MSP6 and MSP7 processing are all directly mediated by PfSUB1 upon its release into the PV.

**Recombinant PfSUB1 mediates correct primary processing of parasite-derived MSP1, MSP6 and MSP7**

To test the above conjecture, we developed an *in vitro* assay to assess the capacity of recombinant PfSUB1 (rPfSUB1) to convert MSP1, MSP6 and MSP7 precursors to species resembling those on naturally released mature merozoites. Our assay took advantage of the fact that biosynthesis of all three precursor proteins initiates at around the beginning of schizont development, whereas primary processing takes place only at the end of this process, just prior to merozoite egress. Mid-stage *P. falciparum* schizonts were treated with a cocktail



**Figure 2** Proteolytic processing of the MSP1/6/7 complex. Biosynthesis of all three MSPs begins at the start of schizogony (nuclear division). As indicated, the complex is a stoichiometric assembly of the various components, except that the MSP6 precursor (yellow) binds as a tetramer (Kauth *et al*, 2006). Primary processing converts it to MSP6<sub>36</sub>. The MSP7 precursor undergoes an early processing step during secretory transport (Pachebat *et al*, 2007) to produce MSP7<sub>33</sub> (green), the form that binds to the MSP1 precursor (brown). Primary processing converts MSP7<sub>33</sub> to both MSP7<sub>22</sub> and MSP7<sub>19</sub>. The various MSP1 processing products are numbered for clarity. Secondary processing, which occurs following egress and probably at the point of invasion, involves further cleavage within MSP1<sub>42</sub> to produce MSP1<sub>33</sub> and MSP1<sub>19</sub>. The latter is carried into the newly invaded erythrocyte on the merozoite surface (to form a so-called ring-stage parasite), whereas MSP1<sub>33</sub> is shed along with the remaining MSP1/6/7 complex. Secondary processing is mediated by a membrane-bound protease called PfSUB2, whereas the protease(s) responsible for primary processing has been hitherto unknown (indicated by a question mark).

**Table I** Homology between known processing sites in MSP1, MSP6 and MSP7 and previously identified PfSUB1 cleavage sites

Processing site identity	Sequence <sup>a</sup> (P5–P5')	References
PfSUB1 internal processing site	L V S A D ↓ N I D I S	Sajid <i>et al</i> (2000)
SERA5 site 1	E I K A E ↓ T E D D D	Debrabant <i>et al</i> (1992); Yeoh <i>et al</i> (2007)
SERA5 site 2	I I F G Q ↓ D T A G S	Debrabant <i>et al</i> (1992); Yeoh <i>et al</i> (2007)
SERA5 site 3 (allele specific)	T V R G D ↓ T E P I S	Yeoh <i>et al</i> (2007)
SERA4 site 1 <sup>b</sup>	K I T A Q ↓ D D E E S	Yeoh <i>et al</i> (2007)
SERA4 site 2 <sup>b</sup>	Y V Y G Q ↓ D T T P V	Yeoh <i>et al</i> (2007)
SERA6 site 1 <sup>b</sup>	K V K A Q ↓ D D F N P	Yeoh <i>et al</i> (2007)
SERA6 site 2 <sup>b</sup>	F V H G Q ↓ S N E S D	Yeoh <i>et al</i> (2007)
MSP1 <sub>83</sub> –MSP1 <sub>30</sub> junction (3D7)	P L V A A ↓ S E T T E	Stafford <i>et al</i> (1994)
MSP1 <sub>30</sub> –MSP1 <sub>38</sub> junction (3D7)	Q I T G T ↓ S T S S	Stafford <i>et al</i> (1994)
MSP1 <sub>30</sub> –MSP1 <sub>38</sub> junction (FCB-1)	E V S A N ↓ D D T S H	Heidrich <i>et al</i> (1989)
MSP1 <sub>38</sub> –MSP1 <sub>42</sub> junction (3D7)	V V T G E ↓ A V T P S	Stafford <i>et al</i> (1994)
MSP1 <sub>38</sub> –MSP1 <sub>42</sub> junction (FCB-1)	V V T G E ↓ A I S V T	Heidrich <i>et al</i> (1989); Blackman <i>et al</i> (1991); Cooper <i>et al</i> (1992); Stafford <i>et al</i> (1994)
MSP6 <sub>36</sub> cleavage site (3D7)	V V Q A N ↓ S E T N K	Trucco <i>et al</i> (2001)
MSP7 <sub>22</sub> cleavage site	K V K A Q ↓ S E T D T	Stafford <i>et al</i> (1996)
MSP7 <sub>19</sub> cleavage site (allele specific) <sup>c</sup>	S T Q G Q ↓ E V Q K P	Stafford <i>et al</i> (1996)

<sup>a</sup>Arrow denotes the scissile bond. P4 and P2 positions are shaded to highlight the conservation at these sites. Acidic residues on the prime side of the cleavage site are underlined.

<sup>b</sup>The SERA4 and SERA6 processing sites are predicted from sequence alignments and homology with the experimentally determined SERA5 processing sites (Yeoh *et al*, 2007). All other sites shown here were experimentally determined by amino-acid sequence analysis.

<sup>c</sup>MSP7 is not converted to the MSP7<sub>19</sub> form in certain allelic forms (including that of FCB-1), which contain a Gly-to-Lys substitution at the P2 position (Pachebat *et al*, 2007).

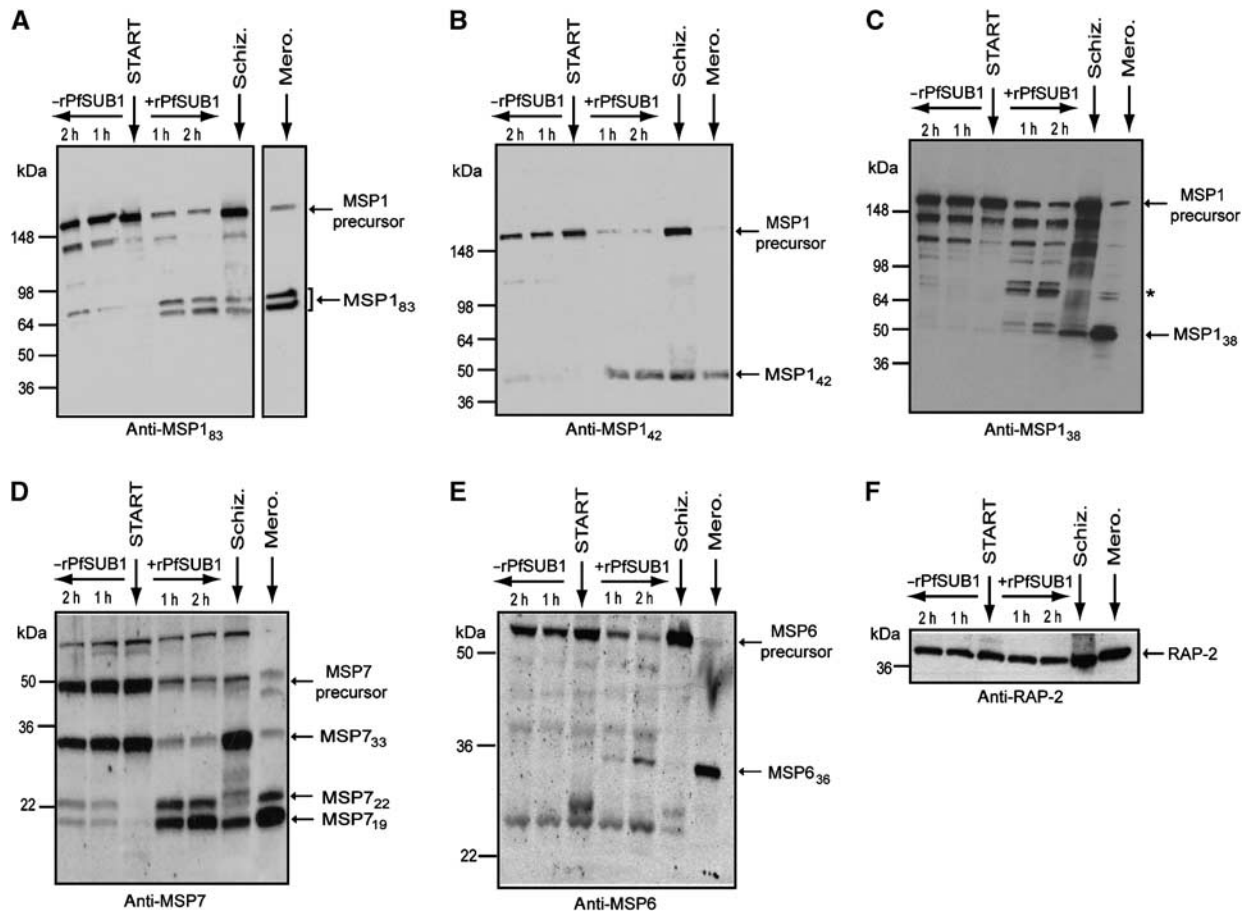
of protease inhibitors to inactivate endogenous proteases, including PfSUB1, as completely as possible. The parasites were then released from their host cells using saponin, which disrupts the erythrocyte and PV membrane (but not the parasite plasma membrane), and were finally washed to remove the protease inhibitors. Western blot showed that these preparations contained, as expected, predominantly full-length forms of all three MSPs (Figure 3, all 'START' lanes). Incubation with rPfSUB1 resulted in rapid conversion of these to smaller processed fragments indistinguishable from those present in the extracts of highly mature schizonts (harvested at around the point of egress) or purified naturally released merozoites (Figure 3A–E). Some low-level conversion to these processing fragments occurred upon prolonged incubation in the absence of added rPfSUB1, but this could be completely blocked by the presence of either MRT12113 (not shown, but see below) or recombinant PfSUB1 prodomain (Figure 4), another selective inhibitor of PfSUB1 (Jean *et al*, 2003), suggesting that it was mediated by residual endogenous PfSUB1 that had not been fully inactivated by protease inhibitor treatment of the schizonts. These data support our hypothesis that PfSUB1 regulates primary processing of all three components of the MSP1/6/7 complex.

#### **Recombinant PfSUB1 correctly processes recombinant MSP1, MSP6 and MSP7 and peptides based on processing sites**

To confirm that the MSP processing observed in parasite extracts was directly mediated by PfSUB1, and was not the result of activation by PfSUB1 of a distinct protease, we next examined the effects of rPfSUB1 on recombinant MSPs. Preliminary experiments (Supplementary Figure S3) showed that addition of rPfSUB1 to full-length recombinant MSP1 resulted in conversion to just 4–5 major fragments, consistent with cleavage at a limited number of internal sites. Attempts to define these sites by N-terminal sequencing proved unsuccessful due to the limited amounts of available protein.

Accordingly, we turned to the use of a previously described recombinant heterodimer comprising the N- and C-terminal 'halves' of MSP1 (Kauth *et al*, 2003). Although this approach did not allow the analysis of cleavage at the MSP1<sub>30</sub>–MSP1<sub>38</sub> junction, this protein was available in relatively large amounts in a pure form. As shown in Figure 5, addition of purified rPfSUB1 to this, or to full-length recombinant MSP6 or MSP7, resulted in all cases in conversion to products of approximately the expected size. N-terminal sequencing of the processed products confirmed cleavage at the expected sites. These included the 3D7 MSP7<sub>19</sub> site, confirming that PfSUB1 can cleave this sequence that possesses a Thr at P4 (see Table I). In addition, the analysis identified the only two previously unknown MSP1 primary processing sites: TTKGA ↓ SAQSG, a fourth site that lies at the beginning of a repetitive sequence within the 3D7 form of MSP1<sub>83</sub> and so could not previously be precisely defined (Stafford *et al*, 1994); and KTEGQ ↓ SDNSE, at the N terminus of the FCB-1 MSP1<sub>30</sub>. The latter lies within a polymorphic region of MSP1 and hence is different from the analogous site in the 3D7 allelic form; nonetheless, it is positionally conserved in the MSP1 primary sequence (Supplementary Figure S4). Both sites are completely consistent with the putative PfSUB1 consensus recognition motif established above. These results showed that PfSUB1 can directly mediate correct processing of all three MSPs, without the involvement of another protease.

On the basis of the above, we predicted that peptides based on the processing sites would be good substrates for rPfSUB1. Accordingly, decapeptides based on some of the MSP processing sites were examined for susceptibility to cleavage by rPfSUB1. All were correctly cleaved only at the expected bond (Supplementary Figure S5; Supplementary Table S1), confirming the capacity of PfSUB1 to correctly target these sequences. A comparative analysis of all these peptides, plus similar peptides based on the known and predicted SERA4, SERA5 and SERA6 processing sites (Yeoh *et al*,



**Figure 3** Correct processing of parasite-derived MSP1, MSP6 and MSP7 by rPfSUB1. (A) Mid-stage *P. falciparum* 3D7 schizonts were treated with protease inhibitors, released from host erythrocytes with saponin, then sampled at once (START) or following further incubation at 37°C in the presence or absence of added rPfSUB1. Samples were analysed by western blot in parallel with extracts of mature schizonts (Schiz.) or purified naturally released merozoites (Mero.), using the MSP1<sub>83</sub>-specific monoclonal antibody (mAb) 89.1 to probe the blot. Positions of the MSP1 precursor and the primary processing product MSP1<sub>83</sub>, which migrates as a doublet in the case of 3D7 (Stafford *et al*, 1994), are indicated. Positions of molecular mass marker proteins are shown. (B) As in (A), but using MSP1<sub>42</sub>-reactive mAb X509 to probe the blot. The MSP1<sub>42</sub> primary processing product is indicated. (C) As above, but using anti-MSP1<sub>38</sub> antibodies to probe the blot. The MSP1<sub>38</sub> primary processing product is indicated (note that the 3D7 form of this fragment generally migrates at 46–50 kDa on SDS-PAGE). Less abundant species also evident (asterisked) are probably processing intermediates. (D) As above, but using anti-MSP7 antibodies to probe the blot. Positions of the MSP7 precursor, the intermediate MSP7<sub>33</sub> processed form, and the MSP7<sub>22</sub> and MSP7<sub>19</sub> processing products are indicated. (E) As above, but using anti-MSP6 antibodies to probe the blot. Positions of the MSP6 precursor and the MSP6<sub>36</sub> processing product are indicated. (F) As above, but using the RAP-2-specific mAb H5 to probe the blot. RAP-2 is not known to undergo proteolytic processing, and so acted as a control for these assays to demonstrate the specificity of the PfSUB1-mediated processing.

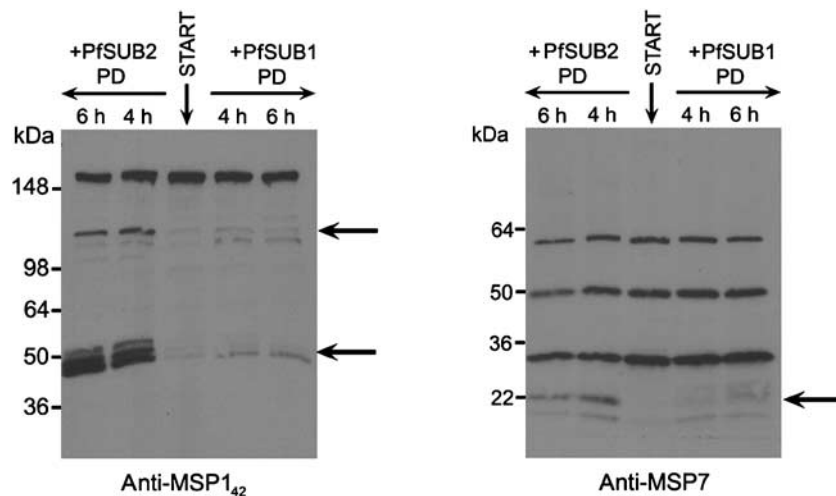
2007), indicated substantial differences in turnover rates, but identified the most efficiently cleaved peptide as that based on SERA4 site 1, Ac-KITAQDDEES-OH (Supplementary Figure S6). This site is particularly rich in prime side acidic residues, consistent with a preference for cleavage at such sites.

In contrast to the results with peptides based on the primary processing sites, a peptide based on the secondary MSP1 processing site known to be targeted by PfSUB2 (FQDML↓NISQH) was completely refractory to cleavage by rPfSUB1 (data not shown). This was not surprising, given our evidence that PfSUB1 cannot cleave on the C-terminal side of a Leu residue.

#### **Inhibition of parasite PfSUB1 activity interferes with MSP1 primary processing and results in invasive-defective merozoites**

Identification of PfSUB1 as the enzyme responsible for primary processing of the MSP1/6/7 complex suggested that the

previously observed effects of MRT12113 on invasion might be a result of impaired MSP processing on released merozoites. To seek evidence that MRT12113 inhibits MSP processing under *in vitro* culture conditions, we first examined the effects of treating parasites with 150 μM MRT12113 during the final stages of schizont maturation. Detailed dose-response experiments have shown that at this concentration of MRT12113, egress is only partly blocked (Yeoh *et al*, 2007), enabling examination of proteins released into culture supernatants upon egress. Examination of the protein profile of the culture supernatants revealed that, as well as the effects on SERA5 processing noted previously, a species of ~80 kDa was significantly reduced in abundance in the presence of MRT12113 (Figure 6A). Western blot (not shown) and tryptic peptide mapping (Supplementary Table S3) identified this as MSP1<sub>83</sub>. Shedding of this protein normally occurs upon secondary processing of the MSP1/6/7 complex by PfSUB2 following egress (Figure 2), but as MRT12113 does not



**Figure 4** Recombinant PfSUB1 prodomain selectively blocks endogenous processing of parasite-derived MSP1 and MSP7. Extracellular schizonts prepared as described in Figure 3 were sampled at once (START) or after incubation at 37°C for 4 or 6 h in the presence of purified recombinant PfSUB1 or PfSUB2 prodomain (PD, ~84 nM final concentration; see Supplementary Figure S8 for an indication of prodomain purity). Samples were analysed by western blot using mAb X509 (anti-MSP1<sub>42</sub>) or anti-MSP7 antibodies to probe the blots. Processing products (arrowed) appear in the presence of PfSUB2 prodomain (which does not inhibit PfSUB1; Harris *et al*, 2005), but not in the presence of PfSUB1 prodomain. Positions of molecular mass marker proteins are shown.

directly inhibit PfSUB2 activity (Yeoh *et al*, 2007) the reduction in MSP1<sub>83</sub> production must result from a defect in primary processing. This demonstrates that MRT12113 can indeed interfere with MSP processing by PfSUB1 under culture conditions.

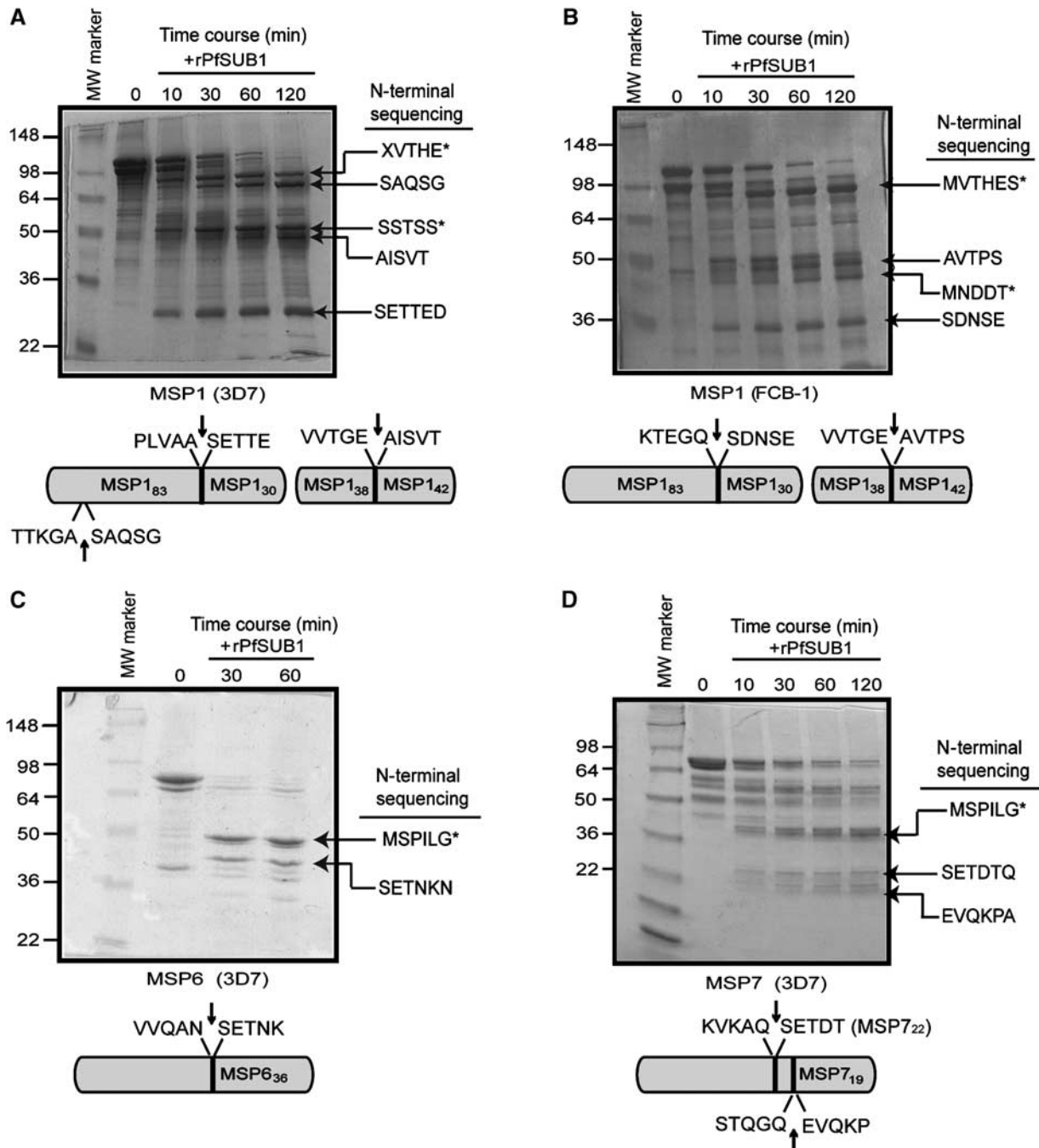
The ED<sub>50</sub> of MRT12113 against egress is ~180 μM, whereas its activity against invasion is much higher (ED<sub>50</sub> ~25 μM). We have previously postulated (Yeoh *et al*, 2007) that this apparent discrepancy may be a result of both the unusual abundance of the SERA proteins and fundamental differences between the requirements for egress and invasion. Specifically, if complete inhibition of SERA maturation is needed to block egress, schizonts in which this is only partially blocked would not exhibit a rupture defect. On the other hand, if efficient or complete processing of MSPs is required for invasion, even modest inhibition of MSP1/6/7 maturation might prevent invasion. To address the possibility of a link between invasion and MSP processing, merozoites released in the presence of egress-permissive but invasion-inhibitory levels of MRT12113 (50–100 μM) were analysed by western blot. Figure 6B shows that although only trace amounts of MSP1 precursor were detectable in control naturally released merozoites (see also Figure 3), those released in the presence of MRT12113 possessed significantly higher levels of unprocessed MSP1. These results support our hypothesis that the invasion defect produced by MRT12113 is a direct result of its inhibition of MSP maturation. Correct and complete proteolytic maturation of the MSP1/6/7 complex by PfSUB1 may be a prerequisite for invasion.

## Discussion

It has long been recognized that the final stages of intracellular development of the *P. falciparum* merozoite are accompanied by extensive proteolytic modification of MSPs. Particular interest has focused on the MSP1/6/7 complex because of its abundance, its essential nature and its involvement in the initial interactions between the merozoite and its

host erythrocyte. We have now shown that all primary processing events to which this complex is subjected are directly mediated by PfSUB1, a serine protease released into the PV lumen just prior to egress. Together with our earlier findings, our work implicates PfSUB1 not only in regulation of egress, but also in preparing the merozoite for release from the host cell. Our results provide the opportunity to dissect the consequences of MSP processing and its functional significance for erythrocyte invasion. Moreover, they explain how maturation of the MSP1/6/7 complex is temporally regulated.

Our peptide scanning analysis, together with our identification of MSP1, MSP6 and MSP7 as authentic macromolecular substrates for PfSUB1, has expanded our picture of the substrate specificity of PfSUB1 (Supplementary Figure S7). As well as substantiating the importance of Gly or Ala at the P2 subsite, our results confirm the preference for acidic residues, Ser or Thr on the prime side of the scissile bond and allow us to confidently add Thr to the list of residues that can be accommodated at P4. It is worth noting that Thr and Phe are the only other residues apart from Ile, Val and Leu that are observed at the P4 position of the predicted site 1 and site 2 processing sites in those blood-stage SERA family members (SERA1, 2, 3, 7 and 9) that have not been experimentally demonstrated to be PfSUB1 substrates (Yeoh *et al*, 2007), supporting our prediction that all these SERAs are likely processed by PfSUB1. The role of the P5 position in substrate recognition by PfSUB1 remains unclear; the alanine scanning data suggest that it may be important, and consistent with this we have yet to observe Ala at P5 in any of the physiological cleavage sites identified to date. PfSUB1 has orthologues in all *Plasmodium* species examined, and the active-site cleft residues predicted to be involved in substrate recognition by the protease are highly conserved (Withers-Martinez *et al*, 2004). The few MSP1 primary processing sites that have been mapped in other species (reviewed by Blackman, 2000) are very similar to those in *P. falciparum*. However, none is identical. Attempts to perform an allelic replacement of the

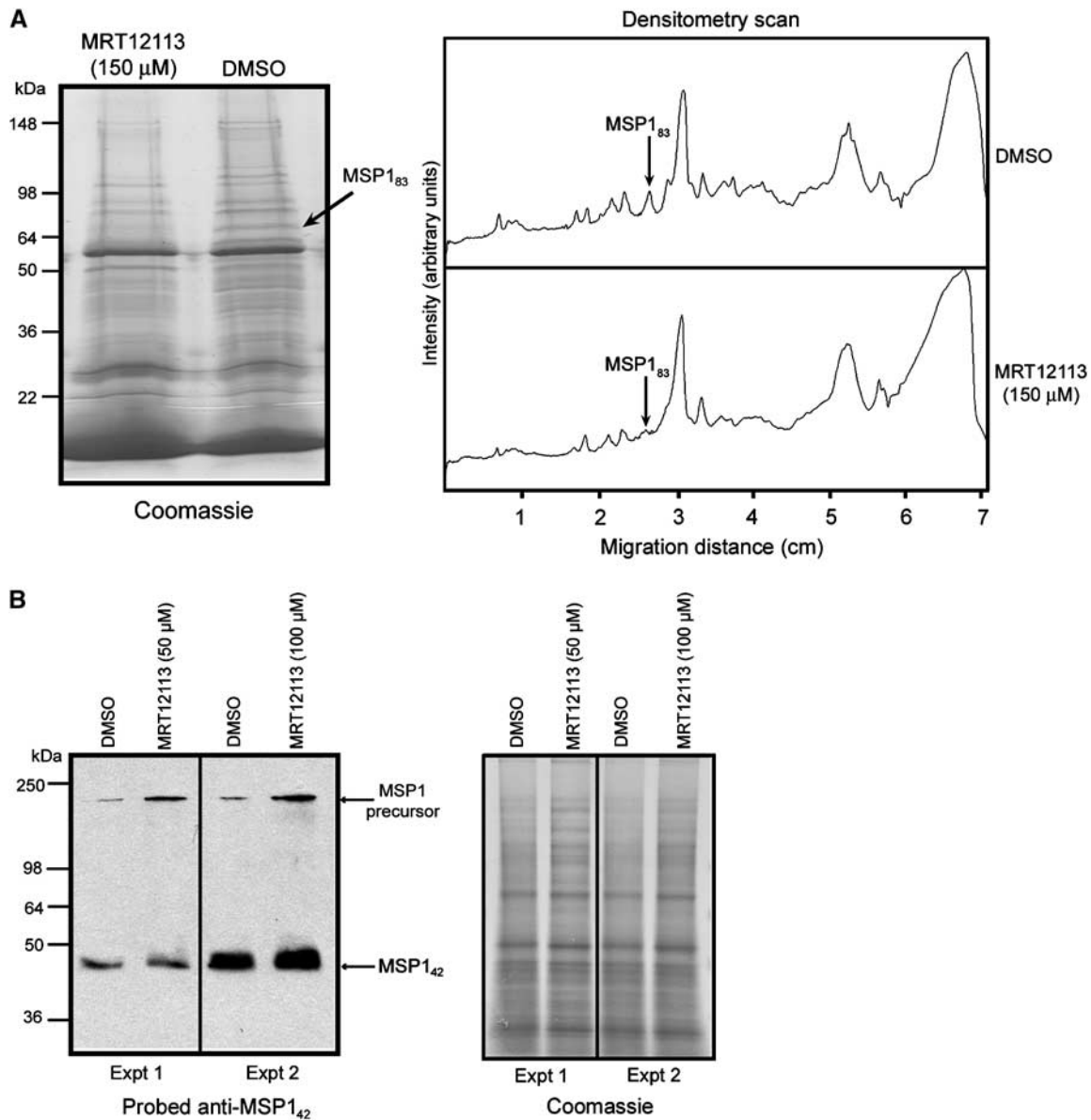


**Figure 5** Correct processing of recombinant MSP1, MSP6 and MSP7 by rPfSUB1. (A) Time course of processing of recombinant MSP1 (3D7 type). (B) Time course of processing of recombinant MSP1 (FCB-1 type). (C) Time course of processing of recombinant MSP6 (3D7 type). (D) Time course of processing of recombinant MSP7 (3D7 type). Gels are stained with Coomassie blue. Major processing products are arrowed, alongside the first five residues of their determined N-terminal amino-acid sequences. Unidentified residues are indicated by 'X'. Asterisked sequences correspond to the N termini of the recombinant protein precursors. Beneath each gel is shown a schematic of the relevant precursor (not drawn to scale, and heterodimers in the case of the MSP1 proteins), with relative positions of the identified cleavage sites indicated.

*P. berghei* *sub1* gene with *psub1* have been unsuccessful (R Tewari *et al*, unpublished), suggesting that PfSUB1 cannot fully complement the function of its *P. berghei* orthologue. SUB1 orthologues in different *Plasmodium* species may have co-evolved with their cognate substrates such that they are optimally matched.

Following merozoite egress, the *P. falciparum* MSP1/6/7 complex undergoes a secondary processing step in which MSP1<sub>42</sub> is cleaved at a Leu-Asn within the motif

FQDML↓NISQH to produce MSP1<sub>19</sub> and MSP1<sub>33</sub>. This is mediated by the membrane-bound subtilase PfSUB2 upon its release from secretory organelles called micronemes at the apical end of the merozoite (Harris *et al*, 2005). We found here that this sequence is completely refractory to cleavage by PfSUB1, providing a plausible model to explain the observed strict temporal and spatial regulation of the two processing steps (Figure 2); whereas primary processing of the MSP1/6/7 complex can take place only upon release of



**Figure 6** Inhibition of MSP1 primary processing by invasion-inhibitory levels of the PfSUB1 inhibitor MRT12113. (A) Left-hand side: supernatants from schizonts incubated for 12 h in protein-free medium supplemented with 150  $\mu$ M MRT12113 or 1% (v/v) DMSO (vehicle) only, analysed by SDS-PAGE and Coomassie blue staining. An  $\sim$ 80-kDa species (arrowed), which decreased in abundance in the presence of MRT12113, was identified by tryptic peptide mapping as MSP1<sub>83</sub> (Supplementary Table S3). The band just below this probably corresponds to the lower band of the MSP1<sub>83</sub> doublet (see Figure 3A), but it could not be identified by mass spectrometry because it migrated very close to other bands on the gel. Right-hand side: densitometric analysis of both tracks of the stained gel. The signal at the position of MSP1<sub>83</sub> in each track is arrowed. Quantitation of the relative levels of this signal in the presence and absence of 150  $\mu$ M MRT12113 showed a 75% mean reduction in intensity in the presence of the drug (three separate experiments). (B) Left-hand side: western blot of merozoites released in the presence or absence of two different concentrations of MRT12113 (two separate experiments). Increased levels of unprocessed merozoite surface MSP1 (arrowed) in the presence of the inhibitor are clearly visible. Right-hand side: Coomassie blue-stained gel of the same protein samples showing that equivalent levels of protein were loaded on each track.

PfSUB1 from exonemes into the PV lumen just before egress, secondary processing—which ultimately releases the bulk of the complex from the merozoite surface—cannot be mediated by PfSUB1, so can only occur upon subsequent release of PfSUB2 from micronemes following egress. The entire pathway is therefore tightly regulated by a combination of the differentially timed discharge of exonemes and micronemes, and the distinct substrate specificities of PfSUB1 and PfSUB2. It is conceivable that primary processing is a prerequisite for secondary processing by PfSUB2. Further work required to

address this will be aided by successful expression of active recombinant PfSUB2, which is yet to be achieved (Harris *et al*, 2005).

Proteolytic maturation of proteins involved in cell invasion by pathogens is well documented, being especially commonplace in viruses, where the final stage of capsid assembly often involves substantial, protease-regulated conformational rearrangements in critical structural proteins that convert a non-infectious precursor form into an infectious particle. Well-studied examples include that of HIV-1, where complete,



ordered and accurate cleavage of the Gag polyprotein by the virus-encoded aspartic protease during capsid assembly and budding is strictly required for virus infectivity (Kaplan *et al*, 1993; Pettit *et al*, 1994; Wiegers *et al*, 1998). Proteolytic modification of the MSP1/6/7 complex by PfsUB1 may represent an analogous process, functionally activating important merozoite surface molecules and so effectively priming the parasite for invasion. Although caution is required in the interpretation of experiments relying on the use of a small compound inhibitor of only modest potency (raising the possibility of off-target effects), our data also suggest that—similar to the situation with HIV Gag—even partial inhibition of MSP processing may impact on invasion efficiency. The different susceptibility to PfsUB1 of peptides based on the various MSP and SERA cleavage sites may also have analogies with Gag processing. Assuming that it reflects differences in cleavage efficiency in the context of the intact proteins—a postulate supported by our previous observations that cleavage at SERA5 site 1 occurs faster than cleavage at site 2, a distinction shared by peptide substrates based on these sites (Yeoh *et al*, 2007)—it implies a degree of kinetic regulation of PfsUB1-mediated processing. The relative rates of proteolysis at different sites, both within and between individual protein substrates, may be important. In the moments just before egress, for example, SERA processing may need to occur more rapidly than processing of the MSPs, as it likely forms the initial step in a multistage cascade that needs to be completed for egress to proceed.

It is informative to contrast our results with aspects of sporozoite invasion. Sporozoites are injected by the mosquito vector into the host dermis, from where they migrate the often considerable distance to the liver. During this journey, the sporozoite can either non-productively penetrate and migrate straight through tissue cells, or alternatively can productively invade them with the formation of a PV (Mota *et al*, 2001). Productive invasion is associated with proteolytic cleavage of the major sporozoite surface circumsporozoite protein. Cleavage is markedly upregulated upon interaction with the highly sulphated heparin sulphate proteoglycans expressed by host hepatocytes, but not by contact with low-sulphated forms expressed by other cell types (Coppi *et al*, 2007). Thus, as it is important that the sporozoite does not engage productively with the many non-permissive cells it comes into contact with during its journey between the dermis and the liver, it becomes infection-competent only upon reaching the appropriate cells, hepatocytes. Merozoites are released directly into the bloodstream, where new host cells are immediately available, but in contrast to the sporozoite the merozoite needs to productively invade without delay, as its invasive half-life is extremely short and in immune or semi-immune hosts it is immediately exposed to high levels of serum antibodies that can block invasion. This may explain why it is advantageous for the merozoite to be 'prepared' for invasion during the moments leading up to egress, rather than after it.

MSP1 is essential to blood-stage parasite viability, and the MSP1/6/7 complex has been shown to have a function in the initial stages of interaction between merozoite and host erythrocyte. The molecular details of this role remain obscure. It is likely that processing of at least some of the sites examined here is essential, although in cases where polymorphism results in allele-specific cleavage—as in the case of

the MSP7<sub>19</sub> site—it seems reasonable to assume that processing at this site is not critical for merozoite viability. The next challenge will be to identify the key cleavage events in the MSP1/6/7 complex, and future work will attempt to dissect the importance of individual processing events for parasite viability. In addition, we now have the tools—rPfsUB1 plus soluble recombinant MSPs—to use *in vitro* approaches to recapitulate and characterize conformational changes that may be induced in MSPs by processing, and to investigate their functional significance.

New antimalarial drugs are urgently required in the face of increasing resistance to available therapeutics, and our findings have implications for the potential of PfsUB1 as a drug target. We now know that PfsUB1 has multiple physiological substrates, most of which are cleaved at more than one site. We would therefore predict that the capacity for simultaneous co-evolution of the protease and its substrates in response to protease inhibitor-based drugs is minimal, limiting the emergence of parasites resistant to anti-PfsUB1 drugs. Furthermore, the fact that both MSP1 and SERAs are also expressed in liver stages (Szarfman *et al*, 1988; Tarun *et al*, 2008) makes it likely that PfsUB1 has a function in development and egress of liver-stage merozoites. As well as preventing blood-stage parasite replication, drugs targeting PfsUB1 might therefore also block the parasite life cycle at an early, non-symptomatic stage in progression of the infection.

## Materials and methods

### Parasite culture

Maintenance *in vitro* of asexual blood stages of *P. falciparum* clone 3D7, purification of schizonts and merozoites, treatment with compound MRT12113 and identification by tryptic peptide fingerprinting of parasite proteins released into serum-free culture medium were as described (Blackman, 1994; Yeoh *et al*, 2007). Densitometric analysis of Coomassie blue-stained, SDS-PAGE-fractionated proteins was performed using ImageJ (<http://rsb.info.nih.gov/ij/index.html>).

### Peptide substrate assays

Internally quenched fluorogenic peptides of the general structure Abz-(Xaa)*n*-Q-EDDnp (where Abz is ortho-aminobenzoic acid, EDDnp is *N*-(ethylenediamine)-2,4-dinitrophenyl amide and Xaa are natural amino acids followed by a glutamine [Q]) were synthesized as described (Portaro *et al*, 2000; Melo *et al*, 2001). Concentrations of stock solutions in DMSO were calculated by colorimetric determination of the 2,4-dinitrophenyl group ( $\epsilon_{365} = 17\,300\text{ M}^{-1}\text{ cm}^{-1}$ ) and then adjusted to 1 mM and stored in the dark at  $-20^\circ\text{C}$ . Unlabelled *N*-acetylated decapeptides based on MSP and SERA processing sites were synthesized using standard Fmoc chemistry, and stock solutions (80 mM in DMSO) stored at  $-20^\circ\text{C}$ .

Working solutions of all peptides were prepared by dilution into PfsUB1 digestion buffer (50 mM Tris-HCl pH 7.6, 15 mM CaCl<sub>2</sub>, 25 mM (3-[(3cholamidopropyl)-dimethylammonio] propanesulphonate) (CHAPS)). For relative  $k_{\text{cat}}/K_{\text{m}}$  determination of hydrolysis of fluorogenic peptides, samples (195  $\mu\text{l}$ ) of peptide solution at concentrations of 10, 5, 2.5 and 1.25  $\mu\text{M}$  ( $\ll K_{\text{m}}$ ) were dispensed into white 96-well microplates (FluoroNunc, NUNC) and supplemented with purified rPfsUB1 ( $\sim 40\text{ nM}$ ). Hydrolysis was monitored at room temperature using a Varian Cary Eclipse fluorescence spectrophotometer by measuring the increase in fluorescence of Abz (using excitation and emission wavelengths of 320 and 420 nm, respectively). Once a steady-state initial rate was determined, reactions were driven to completion by the addition of pronase and fluorescence values converted to moles of substrate hydrolysed as described (Blackman *et al*, 2002). For comparing cleavage efficiencies of unlabelled peptides, mixtures containing equal concentrations ( $\sim 2\text{ mM}$ ) of two peptides were supplemented with

rPfSUB1 (~40 nM) and initial rates of cleavage were determined by analytical RP-HPLC as described (Withers-Martinez *et al*, 2002). Under these conditions, the relative initial rates of hydrolysis of the two peptides are directly proportional to their relative  $k_{cat}/K_m$  values. To determine which of the collection of unlabelled peptides was most efficiently cleaved, a 'league table'-type approach was used. Peptides were initially randomly compared pairwise as above, and the most rapidly cleaved of each pair was selected. These were then compared pairwise with each other, and the best cleaved of each pair was again selected. This process was repeated until the most rapidly cleaved peptide of the entire set was identified. For determination of cleavage specificity by rPfSUB1, partially digested peptides were fractionated by RP-HPLC and digestion products were identified by electrospray mass spectrometry as described (Blackman *et al*, 2002; Withers-Martinez *et al*, 2002).

#### Parasite MSP processing assays

Purified mid-stage 3D7 schizonts were washed in protein-free RPMI 1640 medium and resuspended in phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors (listed in Supplementary Table S2). After incubation on ice for 10 min, the parasite suspension was supplemented with 0.1 volume of 1.5% (w/v) saponin in PBS to disrupt the erythrocyte and PV membranes. Released parasites were washed, resuspended in 25 mM HEPES pH 6.5, 15 mM CaCl<sub>2</sub> and frozen in aliquots at -70°C. For processing assays, aliquots were divided into two and supplemented with either purified rPfSUB1 or an equal volume of control buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl and 10% glycerol). Samples were incubated at 37°C and reactions were stopped by boiling in SDS sample buffer prior to western blot analysis.

#### Antibodies and western blot

Western blots were carried out as described (Jean *et al*, 2003), probing with mAb 89.1 to detect MSP1<sub>83</sub> (Holder *et al*, 1985), mAb X509 to detect MSP1<sub>42</sub> (Blackman *et al*, 1991), or a rabbit anti-MSP1<sub>38</sub> (Woehlbier *et al*, 2006). To detect MSP6 and 7, blots were probed with rabbit antibodies to MSP6 (Kauth *et al*, 2006) or MSP7

(Pachebat *et al*, 2007). The anti-RAP-2 mAb H5 has been described (Harris *et al*, 2005).

#### Recombinant proteins and N-terminal sequencing

Recombinant PfSUB1 and PfSUB2 prodomains were expressed in insect cells or *Escherichia coli*, respectively, and purified and quantified as described (Withers-Martinez *et al*, 2002; Jean *et al*, 2003; Harris *et al*, 2005). Production and purification of recombinant full-length MSP1 (MSP-1D corresponding to the 3D7 allelic form and MSP-1F to the FCB-1 type), recombinant MSP1 heterodimer (a reconstituted complex corresponding to MSP1<sub>83/30</sub> complexed with MSP1<sub>38/42</sub>) for both allelic forms, as well as recombinant MSP6 and MSP7, have been described (Epp *et al*, 2003; Kauth *et al*, 2003, 2006).

Recombinant MSPs in 50 mM Tris-HCl pH 7.6, 15 mM CaCl<sub>2</sub> were supplemented with rPfSUB1 or control buffer as above, incubated at 37°C and sampled over a period of 2 h. Samples were analysed by SDS-PAGE and staining with Coomassie blue. For N-terminal sequencing, SDS-PAGE fractionated proteins were transferred to polyvinylidene difluoride membrane and Edman degradation performed at the Protein and Nucleic Acid Chemistry Facility, University of Cambridge, UK.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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