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Molecular Identification of a Malaria Merozoite Surface Sheddase

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Proteolytic shedding of surface proteins during invasion by apicomplexan parasites is a widespread phenomenon, thought to represent a mechanism by which the parasites disengage adhesin-receptor complexes in order to gain entry into their host cell. Erythrocyte invasion by merozoites of the malaria parasite Plasmodium falciparum requires the shedding of ectodomain components of two essential surface proteins, called MSP1 and AMA1. Both are released by the same merozoite surface “sheddase,” but the molecular identity and mode of action of this protease is unknown. Here we identify it as PFSUB2, an integral membrane subtilisin-like protease (subtilase). We show that PFSUB2 is stored in apical secretory organelles called micronemes. Upon merozoite release it is secreted onto the parasite surface and translocates to its posterior pole in an actin-dependent manner, a trafficking pattern predicted of the sheddase. Subtilase propeptides are usually selective inhibitors of their cognate protease, and the PFSUB2 propeptide is no exception; we show that recombinant PFSUB2 propeptide binds specifically to mature parasite-derived PFSUB2 and is a potent, selective inhibitor of MSP1 and AMA1 shedding, directly establishing PFSUB2 as the sheddase. PFSUB2 is a new potential target for drugs designed to prevent erythrocyte invasion by the malaria parasite.


Introduction

Malaria is a devastating global health problem, responsible for up to 3 million deaths annually [1]. The disease results from cyclical replication within erythrocytes of protozoan parasites of the genus Plasmodium. The parasite divides asexually within its host cell to produce a number of progeny merozoites. Upon eventual rupture of the schizont, these are released to rapidly invade fresh red cells and perpetuate the cycle. Like all apicomplexan parasites, the Plasmodium merozoite enters its host cell by an active invasion process that is mediated by adhesive receptor–ligand interactions and driven by an actinomyosin motor [2]. Light and electron microscopic studies have shown that initial attachment to the host erythrocyte is followed by reorientation of the merozoite such that its apical end contacts the cell surface. This results in the formation of an irreversible zone of contact, or tight junction, between the apical prominence and the host cell surface. The host cell membrane then invaginates, forming a parasitophorous vacuole (PV) into which the parasite is propelled; in the process, the junction sweeps around the periphery of the parasite with concomitant “shaving” of bristle-like structures from the parasite surface [3,4], eventually sealing behind the intracellular parasite. The initial low-affinity binding appears to be mediated by a large, glycosylphosphatidyl inositol (GPI)-anchored protein complex which is uniformly distributed around the parasite surface and is composed of fragments of merozoite surface protein-1 (MSP1) plus associated partner proteins [5–7]. Many subsequent interactions in the invasion pathway are mediated by proteins released from micronemes, secretory vesicles at the apical end of the merozoite [8]. One of these proteins, apical membrane antigen-1 (AMA1), is a type I integral membrane protein that is secreted onto the merozoite surface just prior to interaction with the host cell and may play a role in reorientation, junction formation, or government of the release of a second set of apical organelles called rhoptries [9–11]. Both MSP1 and AMA1 play essential roles in the blood-stage cycle of the malaria parasite [12,13].

During invasion both AMA1 and the MSP1 complex are quantitatively shed from the parasite surface, in each case as a result of a single proteolytic cleavage at a juxtamembrane site. Shedding of MSP1 results from cleavage just distal to a tandem EGF (epidermal growth factor)-like domain called MSP119 at its C-terminus [14]. MSP119 remains bound to the parasite surface via its GPI anchor and is the only part of the MSP1 complex to be carried into the host cell. AMA1 is cleaved precisely 29 residues away from the transmembrane domain (TMD), releasing the bulk of the ectodomain and resulting in just the juxtamembrane “stub” being carried into the host cell with its cognate TMD and cytoplasmic domain [15–17]. Shedding of these proteins is required for productive invasion [7,18,19], and may be important to release adhesive interactions between the parasite and host cell surface in...
PfSUB2 Is a Merozoite Surface Sheddase

Synopsis

Malaria causes immense suffering and loss of life across the globe. In the face of growing resistance to available drugs and no licensed vaccine, new approaches are urgently required to tackle its control. Fundamental to these is an improved understanding of the basic biology of the malaria parasite. The parasite invades and replicates within red blood cells. During invasion a number of important proteins need to be shed from the parasite surface, probably in order to disengage the adhesive interactions that enable initial binding. Shedding of these surface proteins is achieved by a parasite enzyme called a protease, and compounds or antibodies that block the action of this protease prevent invasion, killing the parasite. Here the authors identify this protease as PfSUB2, a large, membrane-bound member of the subtilisin-like protease superfamily. They find that PfSUB2 is secreted from apical organelles called micronemes at the point of invasion to migrate rearwards over the surface of the parasite, and that a protein designed to be a specific inhibitor of PfSUB2 potently prevents shedding of parasite surface proteins. This work sets the scene for the development of inhibitors of PfSUB2 as a new generation of antimalarial drugs.

Results

Epitope Tagging of PfSUB2 by Targeted Homologous Recombination

PfSUB2 is a poorly abundant parasite protein, hindering our earlier attempts at precise sub-cellular localization of the mature protein. To overcome this obstacle, we set out to modify the endogenous pfsub2 gene by fusing it to a sequence encoding three consecutive haemagglutinin (HA) epitope tags using targeted homologous recombination. Parasites transfected with construct pH11-T996HA3 and the control plasmid pH11-T996w (Figure 1A) were cultured in the presence of the antifolate WR99210 followed by rounds of drug cycling to remove parasites harbouring free episomes (which are rapidly lost in the absence of drug pressure). The resulting parasite lines, called PfSUB2HA and PfSUB2w, were cloned by limiting dilution and maintained in the presence of WR99210. Southern blot analysis (Figure 1B) showed that all transgenic clones contained more than one copy of the targeting plasmid integrated into the pfsub2 locus, as is common in single-crossover homologous recombination in P. falciparum as a result of the plasmids being maintained as concatamers (e.g., [13]). Note that despite this, only one copy of the modified gene is expected to be functional, because all downstream copies contain either the targeting fragment only or a promoterless, truncated remnant of the endogenous locus lacking the epitope tag. In over 6 mo of continuous culture, none of the clones exhibited any growth defect compared to the parental D10 clone (not shown), indicating that neither the addition of the HA tags to the C-terminus of PfSUB2 nor replacement of the pfsub2 3' UTR with the heterologous P. berghei sequence was detrimental to parasite replication.

Analysis of the transgenic clones by Western blot using two different HA-specific monoclonal antibodies (mAbs), 12CA5 and 3F10, confirmed the in-frame fusion of the epitope tag to PfSUB2. Pulse-chase studies have shown that PfSUB2 maturation involves two post-translational processing steps in which the approximately 150-kDa primary translation product (called PfSUB2p) is converted first to a 75-kDa intermediate form (PfSUB2i) and finally to a 72-kDa terminal intracellular form (PfSUB2c) [23,25]. All were detected in schizont extracts probed with the anti-HA mAbs (Figure 1C; the closely spaced PfSUB2c/PfSUB2i doublet is not easily discernible here, but see below), consistent with our earlier evidence that maturation of PfSUB2 involves truncation from the N-terminus of the protein. An additional anti-HA reactive species of 55 kDa, also often evident on Western blots, has previously been observed as a minor component in pulse-chase experiments, and is likely a non-specific degradation product of PfSUB2.

PfSUB2 Is a Microneme Protein

To establish the sub-cellular location of PfSUB2, parasites were then analysed by indirect immunofluorescence (IFA) using both of the anti-HA mAbs. Neither mAb showed any reactivity with any stages of the PfSUB2w line or clones thereof, or with the parental D10 clone (not shown). In contrast, both mAbs produced a strong punctate pattern in mature schizonts of both the uncloned PfSUB2HA line (not shown) and its clones. To examine this localisation in detail, dual labelling was performed using mAbs specific for a plasma membrane marker (MSP1), two different rhoptry markers (RhopH2 and RAP2; data not shown for RAP2), and a microneme protein (AMA1). The results were clear (Figure 2A); the anti-HA signal was quite different from that of MSP1, it was adjacent to but distinct from that of the rhoptry-specific signals, but it co-localised completely with that of AMA1, indicating that in mature schizonts, PfSUB2 accumulates in micronemes.

These findings were confirmed by immuno-electron microscopy (IEM) using the anti-HA mAb 3F10, which showed light but consistent labelling of typical elongated
micronemes within late-stage schizonts (Figure 2B), similar to that of AMA1 (Figure 2C). No labelling with mAb 3F10 was observed in less-mature schizont stages lacking micronemes, or apically in free merozoites, and labelling was also absent from wild-type schizont controls (not shown). There was no evidence of any dense granule labelling with the anti-HA antibody.

PfSUB2 Is Secreted to Translocate across the Surface of Free Merozoites

To follow the fate of PfSUB2 upon merozoite release, naturally released merozoites were next examined by IFA as described above. As with intracellular parasites in mature schizonts, all merozoites exhibited an intense, punctate anti-HA signal. Unexpectedly, however, in the great majority of
cases (≥70% of free merozoites), it comprised a single tight focus of fluorescence that clearly localised to the opposite pole of the merozoite to that recognised by the anti-rhoptry mAbs. This posterior localisation was also found in the free merozoite by IEM, labelling being present just interior to the cell surface, consistent with antibodies binding to the epitope tag at the extreme C-terminus of the PfSUB2 cytoplasmic domain (Figure 2D and 2E; Figure 3A, top row). There are no known organelles situated at the posterior of the merozoite, so this suggested that, just after schizont rupture, PfSUB2 is secreted from the micronemes onto the merozoite surface and redistributed rearwards to accumulate at its posterior end. In a portion of cases (~10%), the anti-HA IFA signal presented as two separate foci that lay lateral to the anterior–posterior axis of the merozoite, suggesting the presence of a mid-bodied ring of fluorescence encircling the parasite at right angles to this axis and perhaps representing PfSUB2 en route to the posterior (Figure 3A, middle row). Only in less than 20% of cases did the anti-HA IFA signal remain adjacent to the rhoptries as reproducibly observed in intact schizonts. In contrast, the anti-AMA1 IFA signal in free merozoites took the form of a uniform circumferential pattern (Figure 3A, bottom row) as observed previously (e.g., [16]). In no case did the anti-AMA1 IFA signal concentrate at the posterior pole of the parasite as seen for the anti-HA signal. Several apicomplexan microneme proteins translocate onto the parasite surface upon release from the host cell. The best-studied example, the Toxoplasma adhesin TgMIC2/M2AP, is secreted spontaneously at low basal levels and capped to the posterior pole of the free tachyzoite. As the parasite invades a host cell, secretion is markedly up-regulated and driven rapidly to completion [26]. Our observations suggest that PfSUB2 behaves in a somewhat similar manner, although complete translocation of PfSUB2 can apparently occur even in the absence of host cell invasion. These findings document the first demonstration of capping of a Plasmodium merozoite surface protein. Importantly, the trafficking pattern observed indicates that PfSUB2 has the capacity to track along the merozoite surface, a characteristic predicted of a membrane-bound protease involved in shedding of merozoite surface proteins.

Translocation of PfSUB2 Is Actin Dependent

The unique form of substrate-dependent gliding motility exhibited by many Apicomplexa is mediated by interactions between the cytoplasmic domains of transmembrane adhesins, such as TgMIC2/M2AP or its Plasmodium sporozoite homologue, TRAP, and a subplasmalemmal actinomyosin motor [2]. Compounds that interfere with this motor block motility and invasion, and prevent capping of several micronemal adhesins, though not their secretion per se (e.g., [27,28]). To test the possibility that this or a similar actin-dependent system might drive PfSUB2 translocation, we investigated the effects of latrunculin A and cytochalasin D, two inhibitors of actin polymerisation with distinct mechanisms of action. As shown in Figure 3B, 5 μM latrunculin A clearly inhibited posterior accumulation of PfSUB2 on released merozoites, with approximately 90% of free parasites exhibiting instead a diffuse distribution of the anti-HA signal at or around the apical region of the merozoite. This result indicates that rearward translocation of PfSUB2 is actin dependent. In contrast, cytochalasin D had no effect on PfSUB2 capping, even at concentrations as high as 4 μM.

Figure 2. PfSUB2 Is a Microneme Protein
(A) IFA images of schizonts of PfSUB2HA clone 2D dual-labelled with mAbs X509 (anti-MSP1), 61.3 (anti-RhopH2), or 4G2 (anti-AMA1), plus in each case mAb 3F10 (anti-HA). The anti-HA signal co-localised only with the anti-AMA1 signal. Identical results were obtained with the uncloned transgenic PfSUB2HA line, and/or when anti-RAP2 mAb H5 was used as the rhoptry marker instead of mAb 61.3 (not shown). Parasite nuclei are stained throughout with DAPI (blue). Scale bar represents 2 μm. (B and C) Electron micrographs showing immuno-gold labelling of micronemes within late-stage schizonts of PfSUB2HA clone 2D using: (B) anti-HA mAb 3F10, detecting epitope-tagged PfSUB2; and (C) a polyclonal antibody specific for PfAMA1. The inset in (B) shows another example of micronemal staining with mAb 3F10 from another schizont. Rh, rhoptry.

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This was surprising given the known inhibitory effect of cytochalasins on invasion by the malaria merozoite [29], but identical results were obtained in three independent experiments using two different batches of the drug.

**PfSUB2 Remains on the Plasma Membrane of Invading Parasites**

Probing newly invaded ring-stage parasites from highly synchronised cultures of the PfSUB2HA clones with mAb 3F10 produced a “ring-shaped” signal that co-localised with that of a mAb specific for MSP1, the fragment of MSP1 that remains on the parasite surface after invasion (Figure 3C). By 4–6 h post invasion, the anti-HA IFA signal was no longer detectable, only to appear again in a punctate form in the late stages of schizont maturation (not shown). PfSUB2 expression peaks in the latter stages of intraerythrocytic maturation [22,30,31], so the signal observed in early rings must derive from PfSUB2 carried into the cell on the merozoite surface. Collectively, the developmental profile of PfSUB2 expression and sub-cellular trafficking, its ability to mobilise onto and across the merozoite surface, and its presence at the plasma membrane of newly invaded ring stages are entirely consistent with an involvement in MESH activity.

**Recombinant PfSUB2 Propeptide Is Folded and Binds Specifically to Mature Parasite-Derived PfSUB2**

Subtilases are synthesised as zymogens that comprise minimally a secretory signal peptide, a propeptide, and a catalytic domain. The propeptide acts as an intramolecular chaperone, being essential for folding of the catalytic domain. Subtilase propeptides are also highly potent, selective inhibitors of their cognate proteases (e.g., [32–34]), a property that is probably important in regulating subtilase activation during secretion. Prompted by the finding that PfSUB2 is actively distributed across the free merozoite surface, we investigated whether its propeptide could interfere with MESH activity.

By analogy with processing of PfSUB1 [33] and other subtilases, conversion of PfSUB2 to the 75-kDa PfSUB2I form likely represents autocatalytic cleavage of the propeptide. From the mass of this species we calculated that propeptide cleavage occurs between residues Tyr680 and Lys720 of the PfSUB2 sequence. We therefore expressed in *Escherichia coli* a series of propeptide constructs of varying size, in each case extending from Asn22 (the predicted N-terminus after signal peptide removal) and terminating at different residues within the above range. Constructs were fused to a short N-terminal peptide containing a His6 tag and an S-tag (Figure 4A), an approach previously used successfully to express and characterise the PfSUB1 propeptide [33]. The protein comprising Asn22-Leu687 (called PfSUB2PD) was expressed at the highest levels, and so further work focused exclusively on this. Preliminary purification trials showed PfSUB2PD to be susceptible to degradation, so to optimise yields, much of the purification was routinely performed in the presence of 8 M urea (Figure 4B). Purified PfSUB2PD was refolded into aqueous buffer and its identity confirmed by Western blot and mass spectrometry (not shown).

Circular dichroism (CD) analysis revealed that PfSUB2PD possesses substantial secondary structure (Figure 4C). Subtilisin propeptides broadly divide into two groups according to their folding capacity; whereas some are unfolded when (Figure 3B). This was surprising given the known inhibitory effect of cytochalasins on invasion by the malaria merozoite [29], but identical results were obtained in three independent experiments using two different batches of the drug.

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expressed on their own (e.g., [35]), others—including the PfSUB1 propeptide—adopt significant secondary structure in isolation [33,34,36]. The CD data indicate that the propeptide of PfSUB2 falls into this second class. Subtilase propeptides vary considerably in size and exhibit little sequence homology across evolution, but structural determination of folded propeptides from bacteria [37–39] and mammals [36] have shown that they share a core structure with a simple $\beta$β fold. The observed $\beta$β content of PfSUB2PD is consistent with such a structure.

To seek direct evidence that PfSUB2PD was capable of binding to its cognate protease, purified PfSUB2PD was incubated with detergent extracts of PfSUB2HA clone 2D then recovered using S-protein agarose beads. Purified PfSUB1 propeptide (PfSUB1PD) was used as a control in these experiments. Figure 5 shows that of the four PfSUB2-derived species present in the detergent extracts, only PfSUB2$^I$ and PfSUB2$^T$—those forms predicted to lack the propeptide—were significantly bound by PfSUB2PD. Only traces of all four proteins were bound by PfSUB1PD. Reprobing these Western blots with high-titre polyclonal antibodies specific for AMA1, MSP1, or RAP2, all abundant schizont proteins, revealed no non-specific binding of any of these proteins to PfSUB2PD (not shown). These results demonstrate that PfSUB2PD can form a specific molecular complex with the mature forms of PfSUB2.

The PfSUB2 Propeptide Is a Potent and Selective Inhibitor of MESH Activity

Purified PfSUB2PD was then tested for its effect on MESH activity in isolated intact merozoites, taking advantage of an assay previously used extensively to characterise the inhibitor sensitivity of the sheddase. Figure 6A shows that PfSUB2PD potently inhibited shedding of both AMA1 and MSP1 in a dose-dependent manner, with a calculated IC50 (inhibitory concentration 50%) of approximately 300 nM, whilst PfSUB1PD had no detectable effect. Identical levels of inhibition were obtained with slightly less-pure preparations of PfSUB2PD, similar to those in lane 2 in Figure 4B, that had been purified in the absence of urea and had not been subjected to reversed phase–high performance liquid chromatography (RP-HPLC) (not shown).

To assess the selectivity of the inhibition mediated by PfSUB2PD, we examined its effect on the activity of recombinant PfSUB1 (Figure 6B). PfSUB2PD had no effect at the highest concentrations tested (1.2 $\mu$M), whereas PfSUB1PD rapidly abolished protease activity as reported previously [33]. To further assess whether PfSUB2PD had any capacity to act as a general serine protease inhibitor, we examined its activity against a range of other serine proteases using standard colorimetric or fluorescence-based assays. At concentrations equivalent to between 12- and 300-fold molar excess over protease, PfSUB2PD had no effect on the activity of chymotrypsin, trypsin, and elastase, though it exhibited just-detectable inhibitory activity against subtilisin Carlsberg with a calculated $K_i$ value of 1.2 $\mu$M (data not shown). Similar inhibition of heterologous subtilases by subtilase propeptides has been noted previously (e.g., [32,34]). Taken together with the binding data above, these results provide compelling evidence that the inhibition of AMA1 and MSP1 shedding mediated by recombinant PfSUB2PD is a direct result of it.
binding to and inhibiting the activity of mature PISUB2 on the merozoite surface.

Discussion

Proteolytic shedding of surface proteins during invasion by apicomplexan parasites is thought to be a strategy by which adhesion–receptor complexes are removed as the parasite enters its host cell. Shedding of MSP1 by cleavage just upstream of its C-terminal EGF-like region occurs in all species of Plasmodium examined, and new interest in the protease responsible was sparked by the revelation that the protease perhaps concentrating at the moving junction. IEM studies of merozoites “captured” in the process of invasion will be required to test this postulate. Unfortunately this is a highly ambitious goal in P. falciparum merozoites as PfSUB2 is secreted onto the parasite surface where it is stored until schizont rupture. At merozoite release PfSUB2 is secreted onto the parasite surface where it engages with an actin-based motor. This could occur through its cytoplasmic domain or through ectoplasmic interactions with other micronemal proteins that are themselves connected to the motor. PfSUB2 is then shuttled towards the posterior pole of the merozoite, in the process encountering and cleaving the MSP1 complex and AMA1 from the surface of the parasite. Our present results show that this capping process can occur efficiently in the absence of invasion, satisfactorily explaining the limited shedding of MSP1 and AMA1 seen in preparations of free merozoites. However, our observations may not accurately reflect the dynamics of PfSUB2 translocation at invasion. The invasive half-life of P. falciparum merozoites is extremely short, and shedding of MSP1 is known to go to completion during the brief (~30 s) time course of erythrocyte invasion [7]. We therefore propose that—similar to TgMIC2/M2AP—capping of PfSUB2 on the invading merozoite likely occurs predominantly concomitant with invasion, the protease perhaps concentrating at the moving junction.

An additional prediction of our model is that PfSUB2 may not be substantially exposed at the surface of the invasive merozoite prior to the point of junction formation. In support of this, we have found that addition of purified PISUB2PD to parasite cultures has no effect on invasion (P. Harris, unpublished data). In an analogous example, antibodies against sporozoite TRAP or TgMIC2 have no effect on invasion, because these adhesins are not displayed at high levels on the parasite surface until the point of invasion, and only those molecules inhabiting the moving junction are functional during invasion [42] (V. Carruthers, personal communication). Nonetheless, we expect that it should be possible to target PISUB2 with appropriately selective low-molecular weight, membrane-permeable compounds.

Capping of PISUB2 to the merozoite posterior could be prevented by latrunculin A but not by cytochalasin D, a
puzzling finding in view of the latter’s known potent effects on invasion by the malaria merozoite and the fact that both compounds prevent invasion at the concentrations used here. Both drugs induce rapid disruption of the actin cytoskeleton in mammalian cells, but through distinct mechanisms; whereas cytochalasin D binds to filamentous actin, inhibiting polymerization at both barbed and pointed ends [43], latrunculin binds to and sequesters actin monomers, preventing their de novo polymerization [44,45]. One consequence of this is that cytochalasin D does not induce net depolymerization of actin, whereas latrunculin does [46,47], and there are now numerous examples of cellular actin-based processes that exhibit differential sensitivity to these drugs (e.g., [47,48]). Only a single actin gene is expressed in *P. falciparum* merozoites, and a recent study has shown that filaments formed by this actin are unusually short [49]. One explanation for the discrepancy between the effects observed with the two actin antagonists used in our experiments is that the degree of actin polymerisation required to provide the traction for invasion may be much greater that than required to translocate the bulk of secreted PfSUB2 the short distance (~1.2 μm) from the apical region of the merozoite to its posterior. This could explain why invasion is more sensitive than capping of PfSUB2 to perturbation by cytochalasin D.

The motility of apicomplexan zoites is stringently regulated, primarily by control of actin polymerization [50], and in highly motile apicomplexan zoites such as the *Toxoplasma* tachyzoite, motility ceases abruptly after invasion, implying rapid disassembly of the actinomyosin motor. The malaria merozoite is one of the smallest known eukaryotic cells, and so removal of the forces powering and maintaining retrograde flow of PfSUB2 after invasion would allow subsequent redistribution of the protease by simple diffusion in the parasite membrane. Our observation that PfSUB2 uniformly decorates the plasma membrane of the intracellular ring is consistent with this.

The estimated IC50 of approximately 300 nM for inhibition of MESH by PfSUB2PD is somewhat higher than *K* values measured for other subtilase propeptides against their cognate enzymes, which range from around 1–6 nM [32,33] to around 200 nM [51]. However, the combination of a relatively large molecular mass and the fact that it has to interact with membrane-bound protease may preclude ready access of PfSUB2PD to merozoite surface PfSUB2 in our assay. Moreover, PfSUB2PD may not represent the optimal propeptide construct; the exact internal sites at which processing takes place during PfSUB2 maturation are unknown, and it is well documented that even small truncations of propeptide constructs can compromise their inhibitory capacity (e.g., [32,33]). Despite this, PfSUB2PD exhibited clear selectivity, showing no effect on a number of serine proteases and only weak inhibition of a phylogenetically related bacterial subtilisin. A total of only three subtilases are encoded in the *Plasmodium* genome [25].

Previously, we have conclusively excluded a role for PfSUB1 in the shedding of PfSUB2. We now report the first example of a merozoite protease that acts on an apical protease, which appears to be both necessary and sufficient for its shedding, and indeed, this is the first example of a merozoite surface sheddase.

**Figure 6.** PfSUB2PD Inhibits MESH in a Selective, Dose-Dependent Manner

(A) Shedding of AMA1 and MSP1 from isolated merozoites was assayed in the presence of the indicated additives. Two different batches (1 and 2) of purified PfSUB2PD were tested, in each case alongside a similar dilution of the membrane flow-through (Conc FT) from the final step of concentration by ultrafiltration. Control additives used were bovine serum albumin (BSA), purified PfSUB1PD, and EDTA. No effect on processing was seen if PfSUB2PD was added to buffer control reactions at the end of the incubation period (extreme right-hand lane). The lower panel shows a titration of inhibition of MSP1 shedding in the presence of 2-fold serially decreasing concentrations of PfSUB2PD (from 1.2 μM to 0.075 μM). (B) Effect of propeptides on PfSUB1 activity. Progress curves showing cleavage of substrate pepF1-6R by recombinant PfSUB1 before and following addition of 600 nM purified PfSUB2PD or PfSUB1PD, or BSA. Note that this concentration is in at least 300-fold molar excess over the amount of recombinant PfSUB1 present in the assay. The time point of addition of test proteins is arrowed.

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in MESH activity, but one criticism of our present conclusions is that the inhibition of MESH mediated by PISUB2PD could be the result of interactions with PISUB3, a subtilase that is poorly characterised but known to be transcribed in parasite blood-stages [25]. However, we have recently successfully disrupted the pfsub3 gene in blood-stages of P. falciparum; the resulting parasites are viable, and processing of both MSP1 and AMA1 occurs normally (R. O’Donnell and M. Blackman, unpublished data), demonstrating that PISUB3 is not essential for MESH activity.

In Toxoplasma gondii, several integral membrane microneme adhesins that cap to the parasite posterior through interactions with the actomyosin motor are shed in the final seconds of invasion via cleavage within their TMD by a class of intramembrane proteases called rhomboids [52,53]. A malaria merozoite rhomboid-like activity has also recently been identified [17], the role of which may be to shed merozoite adhesins that are functional homologues of these transmembrane adhesins [20]. The merozoite rhomboid(s) cannot be involved in juxtaplasmic shedding or shedding of GPI-anchored proteins such as MSP1, because rhomboids can only cleave within a TMD [54]. A Toxoplasma rhomboid, TgROM5, was recently localised to the posterior surface of the tachyzoite [55], suggesting that temporal and spatial regulation of release of capped microneme proteins is achieved through the simple expedient of maintaining the protease at the rear of the parasite; substrates are effectively recruited to the protease by the capping process. Our present findings show that PISUB2 has evolved to function in a converse fashion; because its substrates (MSP1 and AMA1) are uniformly distributed about the parasite circumference, the sheddase must be tracked across the parasite surface in order to efficiently engage with them. PISUB2 probably acts in concert with the merozoite rhomboid(s) during invasion.

Do functional homologues of PISUB2 exist in other Apicomplexa? Aside from the rhomboid studies alluded to above, little is known of the molecular details of shedding of surface and microneme proteins in other apicomplexan genera. Two subtilisin-like serine proteases, TgSUB1 and TgSUB2, have been identified in T. gondii tachyzoites, but neither of these likely performs a role analogous to that of PISUB2; TgSUB1 is a nonessential GPI-anchored microneme protease, whereas TgSUB2 is located in rhoptries in which it appears to be involved in maturation of other rhoptry proteins [56]. It remains to be seen whether Apicomplexa other than Plasmodium use a subtilisin-like sheddase at invasion.

Erythrocyte invasion is an obvious but thus far under-exploited target for drugs designed to block the malarial lifecycle. PISUB2 may represent an excellent target for new protease inhibitor-based drugs, and to this end, recombinant expression of the protease in an enzymatic assay is now a priority.

Materials and Methods

Parasite culture and transfection. P. falciparum clones D10, 3D7, and T996 were maintained and free merozoites produced as described [37]. For some experiments, medium was supplemented with 1-μM α-tocopherol, 25 μM actinomycin D or latrunculin A (Sigma-Aldrich, Poole, United Kingdom) added from stock solutions in DMSO. To obtain highly synchronised preparations of newly invaded ring-stage parasites, mature schizonts isolated by centrifugation on cushions of 65% isotonic Percoll were cultured with fresh erythrocytes for 2 h to allow rupture and reinvasion before removal of residual schizonts as described previously [14]. For transfection, ring-stage parasites were electroporated with plasmid DNA using standard procedures [38]. Parasites containing integrated plasmid were selected by drug cycling in the presence of 10 nM WR99210 (Jacobus Pharmaceuticals, Princeton, New Jersey, United States), then cloned by limiting dilution.

Transfection constructs. The 5′HA sequence was amplified from pH1-HA3 using primers HA-1 5′-GCC CCA GGT CAT ACC TTC GAG TT-3′ and HA-2 5′-GGG CAT TGG TCG ACT GTA TAT TCT AAT TT-3′, and 5′-GGG CAT TGG TCG ACT GTA TAT TCT AAT TT-3′, XhoI site underlined; primers were designed to remove the stop codon from the end of the pfsub2 coding sequence and form a continuous reading frame into the 5′HA region. The ser8 gene fragment was excised from pH1-HA3 using BglII and XhoI and replaced with the BglII/HindIII-digested PCR fragment to create pH1-H96HA3. Control plasmid pH1-96 was created in a similar manner but using a product amplified from T996 genomic DNA with primers T996I (5′-GGG CAT TGG TCG ACT GTA TAT TCT AAT TT-3′ and T996E, 5′-TCG AGT TTT CAT AAA CAT ATC ATC AAG TTG ATT CAT TGC-3′, XhoI site underlined); this amplifies a similar fragment of the pfsub2 gene but including the stop codon. The ser8 gene fragment was excised from pH1-H96AS4 using BglII and XhoI and replaced with the BglII/HindIII-digested pfsub2 fragment. Nucleotide sequences of all cloned products were confirmed by sequencing on both strands.

Southern blot. Parasite genomic DNA was prepared using the Proteinase K-Tissue Kit (Qiagen, Valencia, California, United States) and digested using Scal (Roche). Digested products were electrophoresed on a 0.7% agarose gel and transferred to Hybond N+ Nylon membrane (Amersham Biosciences, Buckinghamshire, United Kingdom). The blot was probed with a 5′-[32P]-labelled, gel-purified, 569-bp base pair PCR product extending from nucleotides 3,047 to 3,615 of the T996 pfsub2 gene.

Indirect immunofluorescence assay and fluorescence microscopy. Thin films of P. falciparum cultures were acetone-fixed and incubated with 1:500 dilution of the anti-HA mAb 12CA5 (mouse) or 3F10 (rat; Roche, Basel, Switzerland) for 30 min, then with a biotinylated goat anti-mouse or anti-rat IgG (Chemicon, Temecula, California, United States) diluted 1:500, followed by incubation with FITC streptavidin conjugate (Vector Laboratories, Burlingame, California, United States) diluted 1:500. For dual labelling, samples were additionally probed with mAb 462 (anti-PIAMA1; [10]), mAb 61.3 (anti-RhopH2; [61]), mAb H5 (anti-PRP1; L. Ling, unpublished data), or mAb 1E1 (anti-AMA1; [62]), in each case conjugated directly to Alexa Fluor 594 (Molecular Probes, Invitrogen, Carlsbad, California, United States), or mAb S509 (anti-MSPI1; [63]) followed by incubation with Alexa Fluor 394-conjugated anti-human IgG (Molecular Probes) diluted 1:500. Slides were stained with DAPI (4,6-diamidino-2-phenylindole) and mounted in Citifluor (Citifluor, Canterbury, United Kingdom). Images were collected using AxioVision 3.1 software on an Axioplan 2 Imaging system (Zeiss, Oberkochen, Germany) using a Plan-APochromat 100×/1.4 oil immersion objective, and annotated using Adobe PhotoShop.

IEM. Samples of late schizonts were fixed in 0.075% (v/v) double-distilled glutaraldehyde and 4% (w/v) paraformaldehyde made up in culture medium (RMPI, pH 7.2), for 20 min on ice, then washed four times in ice-cold RPMI and dehydrated through a progressively low temperature ethanol series, infiltrated with LR White resin (EM-SCOPE, London, United Kingdom) and polymerized by ultraviolet light at room temperature for 48 h. For PISUB2HA detection, sections were immunostained with the anti-HA mAb 3F10 (diluted 1:50 or 1:100) followed by biotinylated goat anti-rat antibody (diluted 1:100), then streptavidin conjugated to 10 nm gold particles (British Biocell International, Cardiff, United Kingdom) diluted 1:100. Detection of PIAMA1 was carried out as described in a previous study [64] using a rabbit polyclonal antiserum (*680) raised against recombinant PIAMA1 (kindly supplied by C. Kocken and A. D. Palmer). Biocell International, Cardiff, United Kingdom) diluted 1:100. Detection of PIAMA1 was carried out as described in a previous study [64] using a rabbit polyclonal antiserum (*680) raised against recombinant PIAMA1 (kindly supplied by C. Kocken and A. D. Palmer) diluted 1:100. Detection of PIAMA1 was carried out as described in a previous study [64] using a rabbit polyclonal antiserum (*680) raised against recombinant PIAMA1 (kindly supplied by C. Kocken and A. D. Palmer) diluted 1:100.
Expression and purification of the PfsUB2 propeptide. A wholly synthetic gene called *pfsub2* was generated by PCR cloning into the BamH1 site of *Plasmodium* vector pAR200. The gene was transformed into the *E. coli* strain BL21-Gold (Novagen, Madison, Wisconsin, United States). The encoded propeptide construct was expressed in *E. coli* BL21-Gold (DE3) (Strategene, La Jolla, California, United States). Cells were disrupted by sonication in 20 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole (pH 8.2) supplemented with protease inhibitors, and the PfsUB2PD bound to Ni-NTA agarose (Qagen) then eluted in the same buffer containing 250 mM imidazole. Solid, usual urea was then added to 8 M and the protein chromatographed on a Superdex 200 prep grade 26/60 column equilibrated in 20 mM Tris-HCl, 150 mM NaCl, 8 M urea, 1 mM DTT (pH 8.2). Polishing was performed on a Vydac 4.6 × 150 mm C4 RP-HPLC column eluted at 1 mL min⁻¹ with a 45%–63% (v/v) gradient of acetonitrile in 0.1% (v/v) TFA over 20 min, monitoring elution at 215 nm. Eluted protein was taken up into gel filtration buffer and refolded by rapid 400-fold dilution into 20 mM Tris-HCl, 100 mM NaCl (pH 8.2). Gel filtration buffer and refolded sample was mixed into 10 volumes of 20 mM Tris-HCl, 150 mM NaCl, 8 M urea, and 0.1% (v/v) TFA buffer for subsequent experiments. The identity of purified PfsUB2PD was confirmed by Western blot using an anti-polyhistidine antibody (mAb HIS-1; Sigma) and mass spectrometric peptide fingerprinting of tryptic digests [15]. Its concentration was calculated from absorbance measurements at 280 nm, based on the tyrosine and tryptophan content that predicts a molar extinction coefficient of 55,305 M⁻¹ cm⁻¹ [66]. Purification of PfsUB1 propeptide was as described previously [33].

**Protease assays.** Production of recombinant PfsUB1 and an assay of activity based on cleavage of the fluorogenic substrate pepF1-6R has been described previously [68]. Cleavage was monitored at room temperature with a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, California, United States) equipped with a microplate reader accessory, using excitation and emission wavelengths of 352 nm and 580 nm respectively. Starting concentrations of substrate and PfsUB1 in these assays were 0.2 mM (well below the Kₐ for this substrate) and approximately 2 nM respectively. For other protease assays, enzymes and substrates were obtained from Sigma. Subtilisins BPN’ and Carlsberg were assayed using *N*-succinyl-1-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide as described previously [33]. The Kₐ value for hydrolysis of this substrate by subtilisin BPN’ under these conditions was 242 ± 8 nM. Kₐ determinations were performed as described [33]. Assays of bovine trypsin, α-chymotrypsin and porcine pancreatic elastase were performed using published procedures with substrates *N*-benzoyl-DL-Arg-7-amido-4-methylcoumarin [69], *N*-succinyl-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide [70], and *N*-succinyl-Ala-Ala-p-nitroanilide [71] respectively.

**Supporting Information**

**Accession Numbers**

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) accession numbers for the genes discussed in this paper are *pfsub2* (A1J32422) and *pfsub3* (AY999616).

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**Author contributions.** PKH, SY, and MJB conceived and designed the experiments. PKH, SY, ARD, LHB, GHM, and MJB performed the experiments. PKH, SY, ARD, LHB, GHM, and MJB analyzed the data. PKH, SY, RAOD, FH, LHB, and MJB contributed reagents/materials/analysis tools. PKH, SY, and MJB wrote the paper.

**References**


