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Subcellular Discharge of a Serine Protease Mediates Release of Invasive Malaria Parasites from Host Erythrocytes

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SUMMARY

The most virulent form of malaria is caused by waves of replication of blood stages of the protozoan pathogen *Plasmodium falciparum*. The parasite divides within an intraerythrocytic parasitophorous vacuole until rupture of the vacuole and host-cell membranes releases merozoites that invade fresh erythrocytes to repeat the cycle. Despite the importance of merozoite egress for disease progression, none of the molecular factors involved are known. We report that, just prior to egress, an essential serine protease called PfSUB1 is discharged from previously unrecognized parasite organelles (termed exonemes) into the parasitophorous vacuole space. There, PfSUB1 mediates the proteolytic maturation of at least two essential members of another enzyme family called SERA. Pharmacological blockade of PfSUB1 inhibits egress and ablates the invasive capacity of released merozoites. Our findings reveal the presence in the malarial parasitophorous vacuole of a regulated, PfSUB1-mediated proteolytic processing event required for release of viable parasites from the host erythrocyte.

INTRODUCTION

Malaria is caused by protozoan parasites of the genus *Plasmodium*. The parasite life-cycle is divided between a vertebrate host and a mosquito vector. Clinical manifestations of malaria are caused by the asexual blood stage life cycle. The parasite invades erythrocytes to form a ring which undergoes mitotic replication within a parasitophorous vacuole (PV) to become a schizont. This eventually ruptures in a process known as egress, releasing invasive merozoites which invade fresh erythrocytes to repeat the cycle. The molecular events leading to egress are obscure, but studies using broad-spectrum protease inhibitors have strongly implicated parasite protease activity (Delplace et al., 1988; Gelhaus et al., 2005; Salmon et al., 2001; Soni et al., 2005; Wickham et al., 2003). Particular interest has focused on members of the serine-rich antigen (SERA) family of malarial papain-like proteins. Disruption of the SERA8 gene prevents release of sporozoites from oocysts in the insect vector (Aly and Matuschewski, 2005). Also, antibodies against the most abundant blood-stage family member, SERA5, interfere with merozoite egress (Pang et al., 1999), and both SERA5 and SERA6 appear indispensable for blood-stage parasite growth (Miller et al., 2002; McCoubrie et al., 2007). However direct evidence for a role for these or any other known malarial proteases in egress of blood-stage merozoites is lacking, and the cellular mechanisms that govern this essential, highly regulated event are unknown.

The genome of *P. falciparum*, the species causing the most virulent form of malaria, encodes three subtilisin-like serine proteases (subtilases). Of these, PfSUB3 is not essential in asexual blood stages (R.O., unpublished data), while PfSUB2 was recently identified as the 'sheddase' responsible for the release of merozoite surface proteins during erythrocyte invasion (Harris et al., 2005). The third *P. falciparum* subtilase, PfSUB1, is maximally expressed in the final stages of schizont maturation (Blackman et al., 1998). Production of recombinant PfSUB1 has enabled studies of its substrate specificity and the development of a simple activity assay (Blackman et al., 2002; Withers-Martinez et al., 2002). Homology modeling of PfSUB1 has highlighted distinctive structural features as well as similarities to bacterial subtilisins (Withers-Martinez et al., 2002), raising the possibility that PfSUB1 may be a target for the development of selective protease inhibitor-based antimalarial drugs. However, the function of PfSUB1 has remained elusive.

Here, we show that that the *pfsub1* gene is refractory to disruption in blood stages, indicating that it performs an essential task. We demonstrate that PfSUB1 is stored in a set of parasite organelles that are distinct from those involved in invasion. Using a high-throughput screen, we isolated a selective inhibitor of PfSUB1 and used it in "chemical knockdown" studies. Our results provide the first insights into the role of PfSUB1 and reveal a hitherto unsuspected regulatory pathway in which discharge of PfSUB1 into the PV in the final stages of schizont maturation triggers a series of proteolytic events that culminate in egress of invasive merozoites.

RESULTS

PfSUB1 Is Indispensable in the Asexual Blood-Stage Cycle

To investigate the function of PfSUB1, we first determined whether it is required for blood-stage growth. Parasites were transfected with a construct designed to disrupt the *pfsub1* gene by double homologous recombination (Duraisingh et al., 2002). This did not integrate, as determined by Southern blot analysis, but was maintained in episomal form for up to five drug cycles (data not shown). In contrast, in parallel experiments this approach was successfully used to disrupt the *pfsub3* gene (R.O., unpublished data). We next attempted to modify the *pfsub1* gene by fusion of its 3′ end to green fluorescent protein (GFP) using a single-crossover homologous recombination strategy (Harris et al., 2005). Again, repeated transfections resulted only in long-term maintenance of episomes (data not shown).

These results suggested either that modifications of the *pfsub1* gene that interfere with its function are deleterious, or that the locus is inaccessible to homologous recombination. To test both possibilities, we attempted to more subtly modify the gene. Parasites were independently transfected with four related constructs, each designed to integrate into the genomic *pfsub1* locus by single-crossover homologous recombination with the result of reconstituting a complete open reading frame (ORF) while simultaneously introducing a C-terminal triple haemagglutinin (HA) epitope tag (Figure S1 in the Supplemental Data available online). Constructs pPfSUB1HA3 and pPfSUB1HA3-UTtrun contained targeting sequence comprising the extreme 3′ 943 bp of the authentic *pfsub1* ORF. Constructs pPfSUB1chiHA3 and pPfSUB1chiHA3 mut contained a shorter targeting fragment of the authentic *pfsub1* gene, fused in frame to synthetic "recodonised" sequence encoding the C-terminal part of PfSUB1. The synthetic gene shares low identity at the nucleotide level with the authentic *pfsub1* sequence, so these two constructs were expected to crossover only upstream of the catalytic Ser codon (Ser608), with the predicted result of creating a chimeric modified *pfsub1* locus; in the case of pPfSUB1-chiHA3 this would encode wild-type, catalytically active PfSUB1, whereas in the case of pPfSUB1chiHA3 mut the chimeric gene product would possess an Ala substitution of Ser608, and so would lack proteolytic activity (Figure S1A). PfSUB1 coding sequences in constructs pPfSUB1HA3, pPfSUB1chiHA3 and pPfSUB1chiHA3 mut were flanked by the 848 bp-long 3′ UTR of the *P. berghei* dihydrofolate reductase (*dhfr*) gene to ensure correct transcription termination and polyadenylation of the modified gene, whereas pPfSUB1HA3-UTtrun contained instead a severely truncated form of the same 3′ UTR. Truncation of 3′ UTR sequences in several eukaryotes including *Plasmodium* can reduce gene expression, so integration of this plasmid was predicted to downregulate or ablate PfSUB1 expression. After two drug cycles, PCR analysis indicated integration of pPfSUB1HA3 and pPfSUB1chiHA3 into the parasite genome (Figure S1B). In contrast, no integration of the other two constructs could be detected, suggesting that integration was detrimental to parasite growth. Since the chimeric constructs were intended solely to test the requirement for catalytically active PfSUB1, work with the pPfSUB1chiHA3-transfected line was not progressed further. The pPfSUB1HA3-transfected parasite line (called 3D7SUB1HA3) was cloned by limiting dilution. Analysis of two clones, C10 and F7, confirmed that the input plasmid had integrated through the expected single-crossover homologous recombination event, placing the HA3 tag at the 3′ end of the *pfsub1* gene (Figures S1C and S1D). Western blot confirmed expression of epitope-tagged PfSUB1 (Figure S2A). Neither clone displayed any defect in growth rate compared to the parental 3D7 line (data not shown), indicating that neither fusion to the HA3 tag nor replacement of the *pfsub1* 3′ UTR with the full-length *P. berghei dhfr* 3′ UTR affected parasite growth.

These results prove that the *pfsub1* locus is accessible to homologous recombination. Collectively, our ability to epitope-tag the *pfsub1* gene together with our inability to disrupt it, fuse it to GFP, substitute its catalytic Ser codon, or replace its 3′ UTR with a truncated 3′ UTR, strongly suggests that PfSUB1 activity is essential for maintenance of the asexual erythrocytic life cycle of the parasite.

PfSUB1 Is Discharged from a Parasite Organelle around the Point of Schizont Rupture

Previous studies suggested that PfSUB1 accumulates in subcellular organelles that are distinct from micronemes and rhoptries (secretory organelles at the apical end of the merozoite involved in invasion) but that resemble a third class of secretory vesicles called dense granules (Blackman et al., 1998). We exploited the epitope-tagged PfSUB1 (PfSUB1HA3) expressed by the 3D7SUB1HA3 clones to re-examine this localization. Double immunofluorescence analysis (IFA) confirmed that PfSUB1HA3 resides in a set of organelles that are neither rhoptries nor micronemes. Unexpectedly, PfSUB1HA3 also did not colocalize with the dense granule marker, RESA (Figure 1A). Identical results were obtained using a specific rabbit antiserum to detect PfSUB1; importantly, the signal obtained with this colocalized with the anti-HA signal in the 3D7SUB1HA3 clones, validating the rabbit antibodies

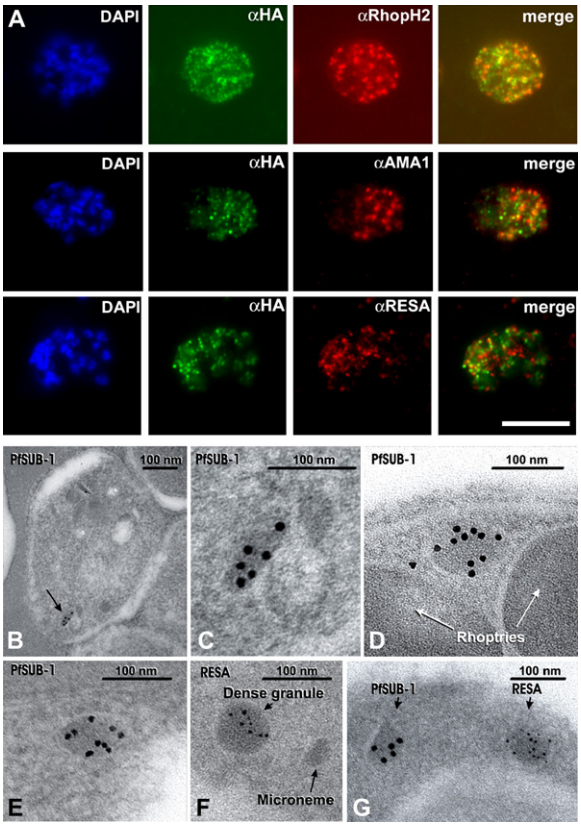

Figure 1. PfSUB1 Accumulation Does Not Overlap with Known Organelles

(A) Schizonts of 3D7SUB1HA3 clone C10 dual-labeled with the anti-HA mAb 3F10 (αHA; green) plus mAb 61.3 (αRhopH2; rhoptries, Ling et al., 2003), mAb 4G2 (αPfAMA1; micronemes, Harris et al., 2005), or mAb 28/2 (αRESA; dense granules, Aikawa et al., 1990). Nuclei were stained with DAPI (blue). Merged images (no DAPI) show that PfSUB1 localizes with none of the other markers. The scale bar represents 5 μm. Identical results were obtained with 3D7SUB1HA3 clone F7 or by using mAbs specific for RAP2 or EBA-175 as markers for rhoptries and micronemes respectively (data not shown). Note that mAb 3F10 is unreactive with parental 3D7 *P. falciparum* in IFA (Harris et al., 2005). (B–G) Immunoelectron microscopic localization of PfSUB1 in *P. falciparum* schizonts. (B and C) Labeling of an elongate organelle, arrowed in (B) and at higher magnification in (C), by anti-PfSUB1 antibodies labeled with 10 nm immunogold. (D and E) Two more examples of PfSUB1 positive organelles are shown, also labeled with 10 nm immunogold. These are less elongate than in (B) and (C), reflecting some variation in organelle shape; however, they are typically ellipsoidal, in contrast with the rounded dense granules. (F and G) Staining for RESA with mAb 28/2 and 5 nm immunogold. In (F), an unlabeled microneme is also shown for size comparison. In (G) double staining for PfSUB1 and RESA with different sizes of immunogold shows that the two proteins are in different organelles.

(Figure S2B). Dual-labeling immunoelectron microscopy (Figures 1B–1G and S3) confirmed the presence of PfSUB1 and RESA in different organelles. Although these shared the electron-dense character typical of dense granules, the PfSUB1-containing structures appeared less numerous than the RESA-containing organelles and were typically larger and elongate (mean dimensions from a sample of 10 was 101 × 60 nm, in contrast to the more spheroidal RESA-containing dense granules with mean dimensions of 72 × 63 nm).

A characteristic of dense granules in apicomplexan parasites is that they are discharged predominantly following host cell invasion. Thus, RESA is released from merozoites soon after invasion and translocates to the erythrocyte cytoskeleton (Aikawa et al., 1990; Culvenor et al., 1991). A number of rhoptry proteins are also present in the newly-invaded host cell, including RAP2 (Baldi et al., 2000). As expected, IFA of newly-invaded ring stage parasites of the 3D7SUB1HA3 clones confirmed the presence of RESA at the host erythrocyte membrane; however, PfSUB1HA3 was not detected (Figure 2A), despite the fact that the IFA signals for both proteins were of similar intensity in schizonts. Western blot confirmed this; whereas both RAP2 and RESA (Figures 2B and 2C) were readily detected in ring-stage extracts, no PfSUB1HA3 signal was evident. In contrast, culture supernatants sampled following schizont rupture contained a strong PfSUB1HA3 signal (Figure 2C), suggesting that PfSUB1 is quantitatively released into culture media at schizont rupture.

The intraerythrocytic malaria parasite undergoes nuclear division as a syncytium, until cytokinesis ("segmentation") and budding of individual merozoites in the final stages of schizogony. To establish more precisely the point at which PfSUB1 release takes place, we examined highly mature, segmented 3D7SUB1HA3 schizonts by IFA. This revealed that late in schizogony the PfSUB1HA3 IFA signal became less dot-like, more diffuse and largely located to the periphery of intracellular merozoites. Dual labeling with an antibody to the merozoite plasma membrane marker MSP1 supported this, suggesting that near the end of schizogony PfSUB1 can translocate from its previous organellar location into the PV (Figure 2D). Additionally, where bursting schizonts were visible, the free merozoites were surrounded by a "cloud" of anti-HA signal, suggesting that at the point of egress PfSUB1 discharge had already begun (Figure 2E).

Our results show that PfSUB1 defines a set of subcellular vesicles that are distinct from the three previously described types of *Plasmodium* secretory organelle. The fact that PfSUB1 is released from these vesicles, which we term "exonemes," just prior to schizont rupture suggested that the role of PfSUB1 is related not to invasion, but to some pre-invasion proteolytic event.

A Selective Inhibitor of PfSUB1 Interferes with Schizont Rupture and Merozoite Invasion and Blocks Proteolytic Maturation of SERA5

We previously described an assay for PfSUB1 activity using a fluorogenic peptide substrate (Blackman et al., 2002). To identify small molecule inhibitors of PfSUB1 suitable for investigation of its function, we adapted this assay to an automated, 384-well microplate format and used it to screen over 170,000 low molecular weight compounds from a number of commercial and proprietary sources

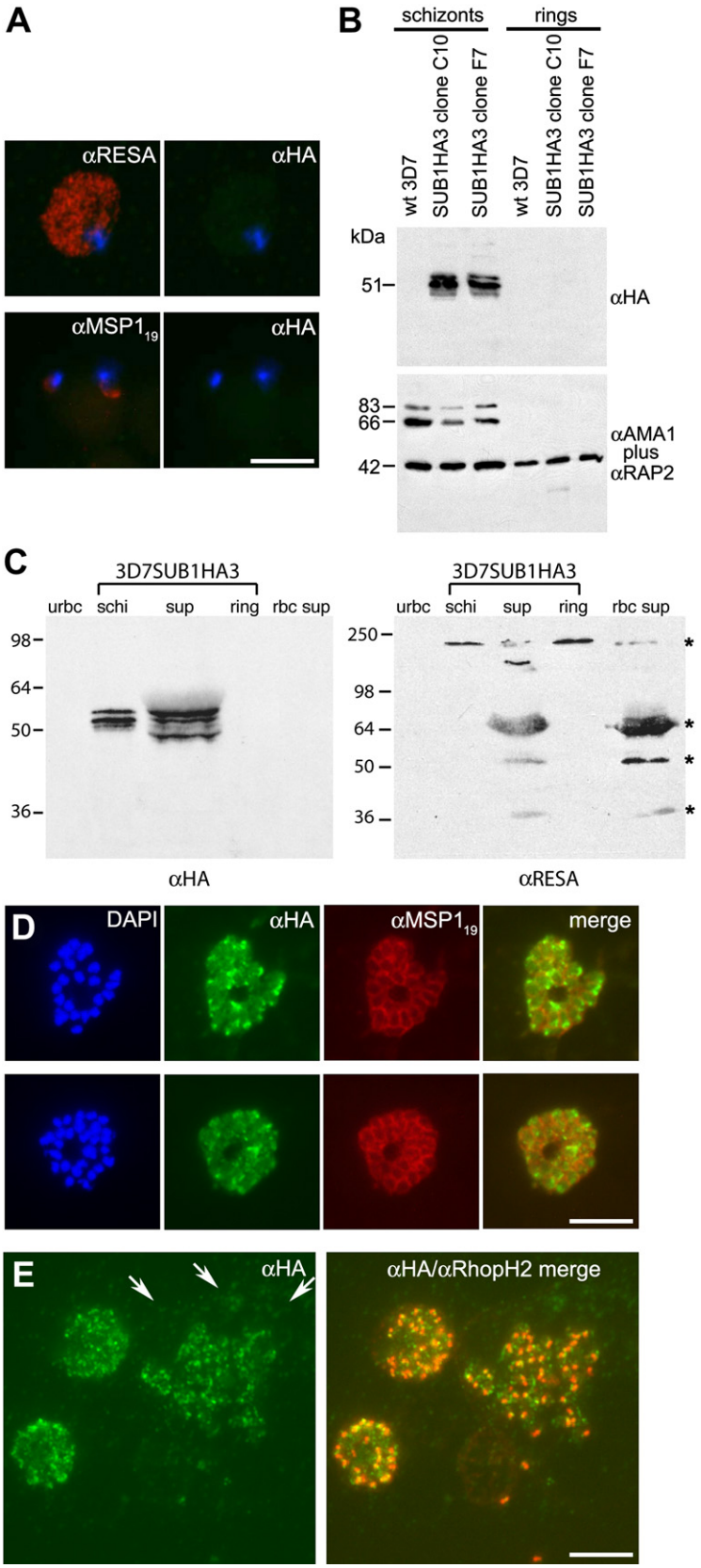

Figure 2. PfSUB1 Is Discharged Prior to Erythrocyte Invasion

(A) Ring stages of 3D7SUB1HA3 clone C10 dual-labeled with mAb 28/2 (αRESA; red) or mAb 1E1 (αMSP1$_{19}$; red) plus mAb 3F10 (αHA; green). Whereas RESA and MSP1$_{19}$ are detectable at the infected erythrocyte membrane and parasite plasma membrane, respectively, no PfSUB1HA3 signal is evident.

(B) Western blot of schizont and ring-stage extracts of parental 3D7 and 3D7SUB1HA3 clones C10 and F7 probed with mAb 3F10 (αHA) or a mixture of anti-RAP2 mAb H5 and a mouse anti-PfAMA1 serum (αAMA1 plus αRAP2). Molecular masses of species detected (in kDa) are shown on the left. The epitope-tagged mature form of PfSUB1HA3 (~50 kDa) is detected only in 3D7SUB1HA3 schizonts. The 83 kDa and 66 kDa forms of PfAMA1 are present in schizont extracts but not in rings as expected, since these are shed at invasion (Howell et al., 2001). RAP2 is present in both schizonts and rings.

(C) Western blots of schizont extracts (schi), equivalent amounts of culture supernatant following schizont rupture and erythrocyte invasion (sup), and ring-stage extracts (ring) of 3D7SUB1HA3 clone C10 probed with mAb 3F10 (αHA) or mAb 28/2 (αRESA). PfSUB1HA3 is quantitatively released in a soluble form prior to invasion, in contrast to RESA (~155 kDa), where a fraction is secreted in truncated form into the supernatant and the bulk is carried into rings. Controls are uninfected red blood cell extracts (urbc) and culture supernatant from uninfected cells (rbc sup). Bands marked with asterisks in the αRESA rbc sup track are the result of cross-reactivity with abundant red cell-derived proteins.

(D) Segmented schizonts of 3D7SUB1HA3 clone C10 dual-labeled with mAb 3F10 (αHA; green) and mAb 1E1 (αMSP1$_{19}$; red). Merged images (no DAPI) show partial colocalization of PfSUB1HA3 and MSP1.

(E) A "cloud" of PfSUB1HA3 around a bursting schizont of 3D7SUB1HA3 clone C10 dual-labeled with mAb 3F10 (αHA; green) and mAb 61.3 (αRhopH2). The right-hand panel shows the merged image. The scale bar represents 5 μm.

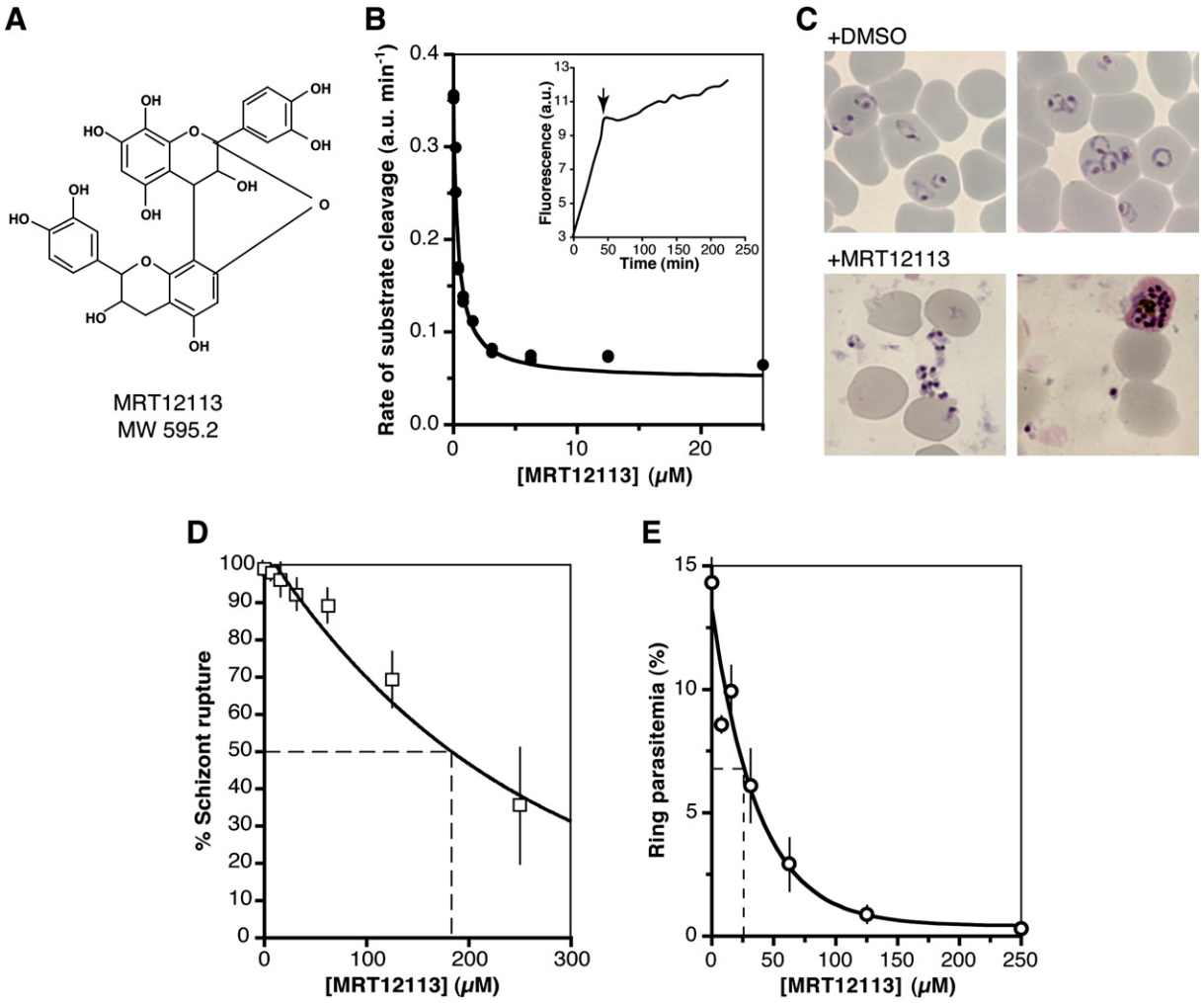

Figure 3. A Selective PfSUB1 Inhibitor Inhibits Schizont Rupture and Merozoite Invasion

(A) Molecular structure of compound MRT12113.

(B) Typical IC_{50} determination for inhibition of rPfSUB1 activity in vitro by MRT12113. Duplicate rate values were determined at each compound concentration tested. Multiple such experiments yielded an IC_{50} of 0.3 ± 0.1 μM. Inset, typical progress curve showing the effect of addition of 0.3 μM MRT12113 (point of addition arrowed) on the rate of pepF1-6R cleavage by rPfSUB1.

(C) Giemsa-stained samples showing the effect of 125 μM MRT12113 (bottom panels) or solvent (DMSO) alone (top panels) on schizont rupture and erythrocyte invasion.

(D and E) Dose-response curves of MRT12113-mediated inhibition of schizont rupture and erythrocyte invasion. Dotted lines indicate the calculated ED_{50} value for egress of 180 ± 26 μM (D) and for invasion of 25 ± 7 μM (E). Data points are shown as mean ± SEM (error bars).

(see Supplemental Data for details of the screen). This resulted in identification of a potent PfSUB1 inhibitor called MRT12113 (Figure 3A). Secondary assays confirmed that MRT12113 was a fast-acting inhibitor of PfSUB1 with an IC_{50} of 0.3 μM (Figure 3B). In contrast, at concentrations of up to 50 μM MRT12113 displayed no inhibition of any other protease tested, including the related bacterial subtilisins BPN' and Carlsberg, the mammalian serine proteases trypsin, chymotrypsin, and elastase, the cysteine protease papain, human caspase-3, the *P. falciparum* cysteine protease falcipain 2, the *P. falciparum* aspartic protease plasmepsin II, and the *P. falciparum* sheddase PfSUB2 (data not shown; see Supplemental Experimental Procedures for protocols). These data indicated that MRT12113 is a highly selective inhibitor of PfSUB1. Consistent with this, MRT12113 showed no toxicity against mammalian COS-7 cells or against an organism related to the malaria parasite, the ciliate *Tetrahymena thermophila*, even when present in cultures for extended periods (up to 7 days) at a concentration of 125 μM (data not shown). On the basis of its high selectivity for PfSUB1, MRT12113 was deemed suitable for studies of PfSUB1 function in the parasite.

Addition of MRT12113 to synchronous *P. falciparum* cultures at up to 250 μM had no effect on intracellular parasite growth over the majority of the erythrocytic life cycle

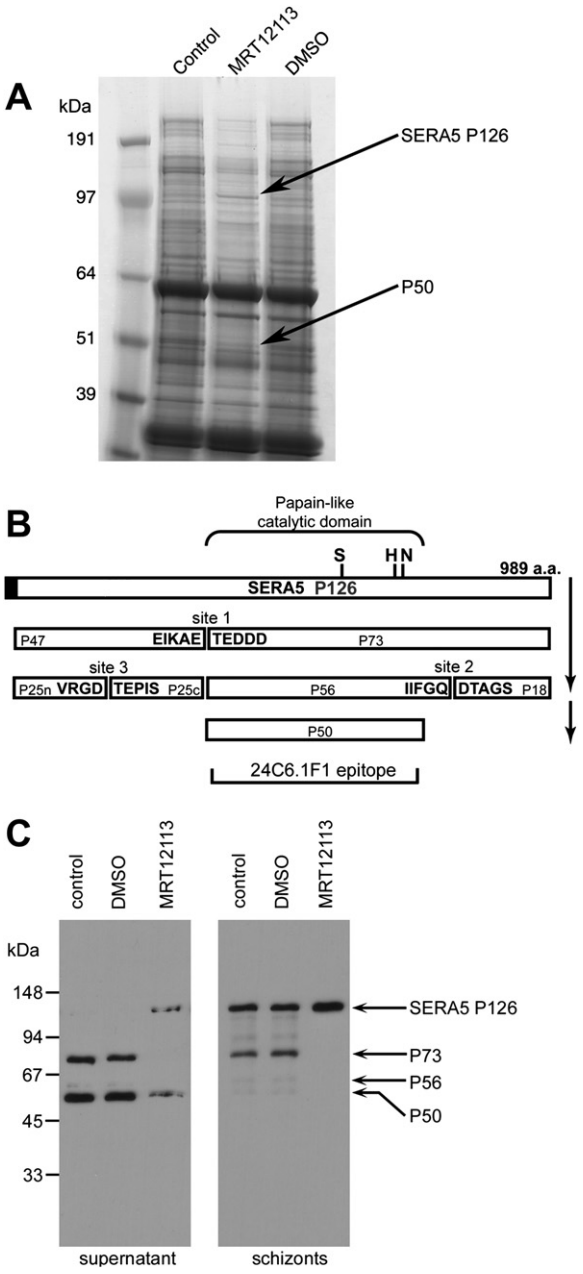

Figure 4. Inhibition of PfSUB1 Activity Prevents Proteolytic Processing of SERA5

(A) Supernatants from schizonts incubated for 12 hr in Albumax-free medium supplemented with no additions (control), 125 μM MRT12113 or 1% (v/v) DMSO only, analyzed by SDS PAGE and Coomassie blue staining. A ~120 kDa and a ~50 kDa species, which were respectively increased or decreased in relative abundance in the presence of MRT12113, were identified by tryptic peptide mapping as SERA5 P126 and P50.

(B) Proteolytic processing of SERA5. Amino acid sequences flanking two known cleavage sites (Debrabant et al., 1992) are named sites 1 and 2. A third is called site 3, previously described (Li et al., 2002b) and mapped in this study. The secretory signal sequence is shaded and the papain-like central domain is indicated, as are the putative catalytic Ser, His, and Asn residues and the location of the mAb 24C6.1F1 epitope. Arrows indicate the direction of processing. The site at which P56 is cleaved to convert it to P50 is unknown.

(C) Mature schizonts were cultured for 12 hr in Albumax-free medium in the presence of no additions (control), DMSO alone, or 100 μM MRT12113, then parasite extracts and culture supernatants were analyzed by Western blot with mAb 24C6.1F1. SERA5 P126 and its processed forms are indicated. The small amounts of unprocessed SERA5 P126 released into supernatants of MRT12113-treated schizonts probably derive from physical breakage of the fragile schizonts during manipulation or egress from parasites in which SERA5 processing was only partially blocked.

(data not shown). However at the end of the cycle partial inhibition of schizont rupture was observed and invasion by those merozoites that were released was reduced, as evidenced by an accumulation of unruptured schizonts, a decrease in ring formation and the presence of numerous free extracellular merozoites (Figure 3C). These dual effects of MRT12113 were dose-dependent, with an ED_{50} against schizont rupture of ~180 μM (Figure 3D), and a much lower ED_{50} against invasion of ~25 μM (Figure 3E). To dissect the mechanistic basis of these effects, schizonts were cultured in the presence of high concentrations of MRT12113 and culture supernatants analyzed by SDS PAGE. MRT12113 produced an overall decrease in protein content compared to control supernatants, due to the inhibition of schizont rupture. However, close examination of the gels revealed changes in the relative abundance of two prominent species; a ~120 kDa protein, levels of which were increased, and a ~50 kDa protein, which almost disappeared in the presence of MRT12113 (Figure 4A). Tryptic peptide mapping (Tables S1 and S2) identified both polypeptides as being derived from the *P. falciparum* protein SERA5.

SERA5 is an abundant, soluble protein of the *P. falciparum* PV. Expressed as a precursor of ~126 kDa (SERA5 P126), it undergoes extensive proteolytic processing (Figure 4B) (Debrabant et al., 1992, 1988, 1985; Li et al., 2002a). SERA5 P126 is initially converted to a ~47 kDa N-terminal and an ~73 kDa C-terminal fragment (P47 and P73). P73 is further cleaved to produce P56, which derives from the central region of the precursor, plus P18. P47 can also undergo further cleavage, although this only occurs in some allelic forms of SERA5 (Li et al., 2002b). P56 is finally truncated at its C terminus to produce P50. Importantly, P50 is abundant in culture supernatants following schizont rupture, but processed forms of SERA5 are not prominent in schizonts, suggesting that processing occurs rapidly just prior to or at the point of egress. Previous studies have indicated that a parasite serine protease plus at least one other enzyme are involved in SERA5 processing, but their identities are unknown (Debrabant and Delplace, 1989; Li et al., 2002a). Interest has focused on SERA5 for a number of reasons, including the presence of a papain-like central domain containing a Ser residue in place of the putative catalytic Cys. SERA5 therefore resembles a protease, and indeed a recombinant form of SERA5 displays chymotrypsin-like activity (Hodder et al.,

2003). Attempts to disrupt the SERA5 gene have been unsuccessful, suggesting it is important for blood-stage growth (Miller et al., 2002; McCoubrie et al., 2007).

Our observations indicated that blockade of PfSUB1 activity with MRT12113 inhibits SERA5 processing. We confirmed this using a SERA5-specific mAb to probe extracts and culture supernatants from schizonts cultured with MRT12113 (Figure 4C); the compound blocked the small amount of SERA5 processing detectable in the parasite, and resulted in accumulation of SERA5 P126 in culture supernatants. These results suggested that PfSUB1 plays a role in SERA5 processing. They also implied that PfSUB1 can access SERA5 intracellularly (presumably in the PV) just prior to its release into culture supernatants at egress. Furthermore, given the inhibitory effects of MRT12113 on schizont rupture and merozoite invasion, they suggested that SERA5 processing by PfSUB1 is important for release of invasive merozoites.

SERA5 Is a Physiological Substrate for PfSUB1

To test directly whether SERA5 can be correctly processed by PfSUB1, SERA5 P126 was purified from schizont extracts (Figure S4) and incubated with recombinant PfSUB1 (rPfSUB1). This resulted in rapid conversion to the P56 form via the P73 intermediate (Figure 5A). No conversion to P50 occurred, consistent with previous reports that this last step of SERA5 processing is mediated by a distinct, leupeptin-sensitive activity (Debrabant and Delplace, 1989; Li et al., 2002a) and that PfSUB1 is insensitive to leupeptin (Withers-Martinez et al., 2002). An identical profile of processing was seen using SERA5 P126 that had been treated during purification with a cocktail of protease inhibitors (Figure 5A). Also, processing was completely sensitive to MRT12113 even if added after processing had commenced (Figure 5B), demonstrating that all the observed processing was mediated directly by PfSUB1 and was not the result of autocatalytic maturation of SERA5 triggered by the presence of PfSUB1. To establish whether the cleavage mimicked authentic SERA5 processing, we examined larger amounts of rPfSUB1-processed SERA5 P126 by silver-staining. This allowed us to detect all the products of cleavage (Figure 5C). N-terminal sequencing confirmed correct cleavage at the known sites 1 and 2, and mapped a third, allele-specific processing site (site 3) described by Li et al. (2002b) but not previously precisely defined.

These results suggested that SERA5 is an authentic physiological substrate for PfSUB1. By analogy with other processing proteases we predicted that peptides based on the SERA5 processing sites would be substrates for PfSUB1. To explore this, peptides incorporating SERA5 processing sites 1, 2, and 3 were assessed for susceptibility to PfSUB1 cleavage. Synthetic decapeptides Ac-TVRGDTEPIS, Ac-EIKAETEDDD and Ac-IIFGQDTAGS—but not similar peptides with a P1 or P2 Leu substitution—were rapidly and specifically cleaved by rPfSUB1 at the expected Asp-Thr, Glu-Thr and Gln-Asp bonds respectively (data not shown). To quantify this, two fluorogenic derivatives were produced by labeling the site 1 and site 2-related peptides Ac-CIKAETEDDC and Ac-CIFGQDTAGC with 6 iodoacetamidotetramethylrhodamine (6-IATR). These peptides, named SERAst1F-6R and SERAst2F-6R respectively, were also cleaved only at the expected bonds by rPfSUB1 (Figure S5A). They were then compared in kinetic assays with pepF1-6R (6-IATR-labeled Ac-CLVSADNIDIC), a substrate based on the PfSUB1 autocatalytic processing site (Blackman et al., 2002). Because of internal quenching and intermolecular dimerization effects observed at concentrations $\geq$ 5 μM (a feature of many fluorogenic peptides) it was not possible experimentally to determine K_m values for cleavage of these peptides (data not shown). However, at concentrations well below the K_m (i.e., under conditions where cleavage rate is proportional to substrate concentration and to k_{cat}/K_m, a measure of substrate specificity) SERAst1F-6R and SERAst2F-6R were hydrolyzed respectively 7.1-fold and 2.6-fold faster than pepF1-6R (Figures 5D and S5B). This confirms that both are much better substrates than pepF1-6R, supporting our evidence that SERA5 is a physiological substrate for PfSUB1. Our results definitively identify PfSUB1 as the protease responsible for conversion of SERA5 to P56 and its associated fragments, and demonstrate that this does not require the participation of any other protease.

PfSUB1 Mediates Maturation of Other Blood-Stage SERA Family Members

Earlier work using a small panel of synthetic peptides had indicated that PfSUB1 has a broad preference for polar or small residues at the P1 position, an inability to cleave after a Leu residue, and a requirement for hydrophobic residues at P4, consistent with the predicted polar nature of the PfSUB1 S1 substrate-binding pocket and its hydrophobic S4 pocket (Withers-Martinez et al., 2002). Despite this apparent relaxed specificity, PfSUB1 is not a promiscuous protease and prior to the present study we had identified only one protein—in vitro-translated PfSUB1 itself—that is cleaved by rPfSUB1 (Withers-Martinez et al., 2002). Mapping of the SERA5 processing sites provided further evidence for a relaxed P1 specificity (except for Leu), a preference for Ile, Leu, or Val at P4, and a restriction to Gly or Ala at P2. This suggested a putative consensus recognition motif of Ile/Leu/Val-Xaa-Gly/Ala-Paa(not Leu)↓Xaa (where Xaa is any amino acid residue and Paa tends to be a polar residue). The sequences just downstream of the three SERA5 processing sites also include at least one acidic residue. SERA5 belongs to a family of nine genes in *P. falciparum*. Whereas SERA1, 2, 3, 4, 5 and 9 ("serine-type") all have a Ser residue at the position analogous to the canonical catalytic Cys in papain, SERA6, 7 and 8 ("cysteine-type") possess a Cys at this position and form a phylogenetically distinct group (Miller et al., 2002). In blood stage schizonts, SERA5 is particularly abundant (Lasonder et al., 2002) but SERA3, 4 and 6 are also readily detectable and are located in the PV (Aoki et al., 2002; Knapp et al., 1991; Miller et al., 2002).

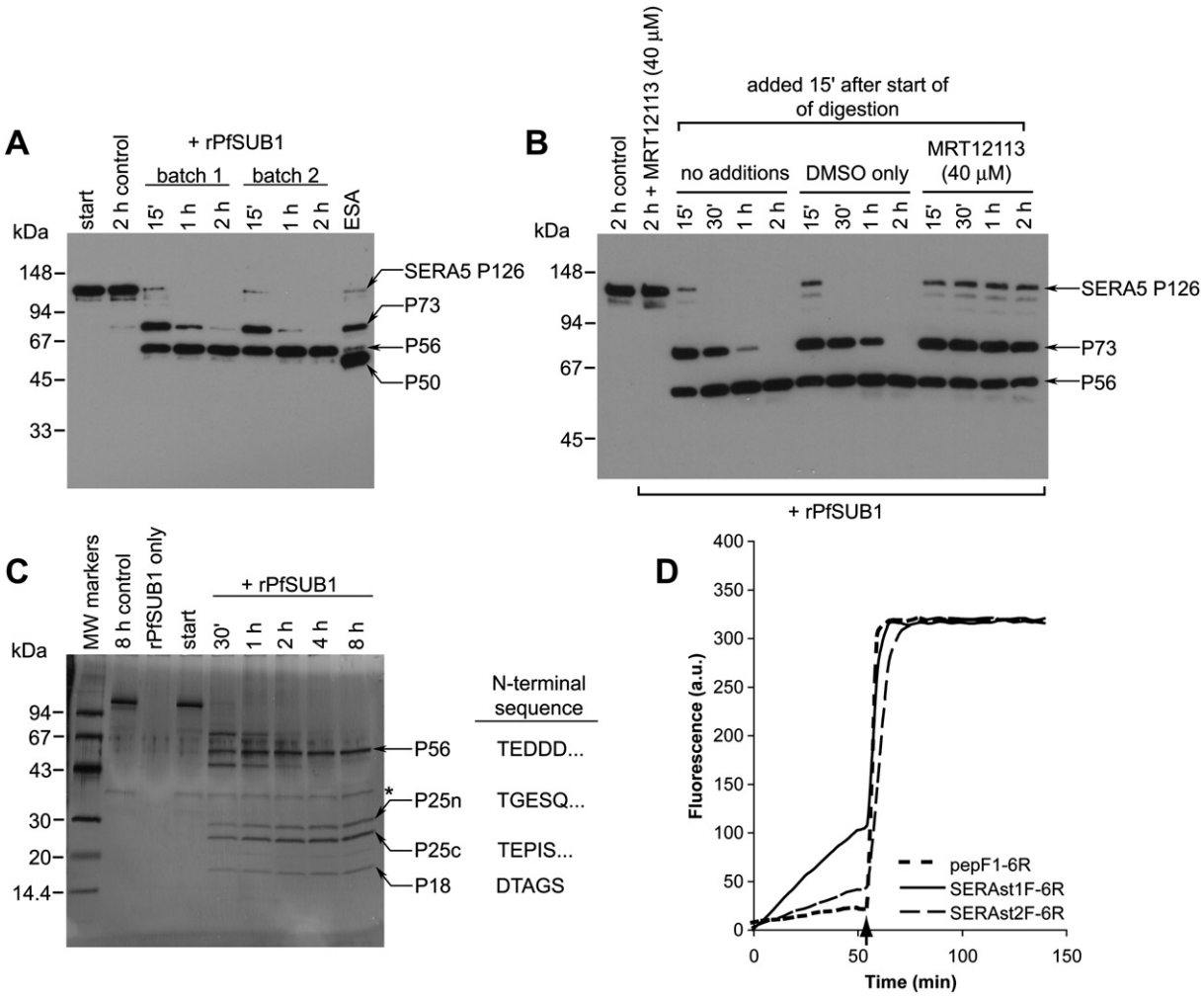

Figure 5. SERA5 Is a Physiological Substrate for PfSUB1

(A) Western blot probed with anti-SERA5 mAb 24C6.1F1, showing a time course of incubating two different batches of purified SERA5 P126 with rPfSUB1; batch 1 was from schizont lysate treated with a cocktail of protease inhibitors, while batch 2 was not exposed to protease inhibitors during purification. The "start" sample was from batch 2 taken immediately after addition of rPfSUB1, while the "2 hr control" sample was batch 2 incubated for 2 hr with control buffer only (no rPfSUB1). Identities of the processed forms of SERA5 in a sample of ESA (supernatant from normal schizonts allowed to rupture into Albumax-free medium) are indicated. Whereas P50 is the dominant form of SERA5 in ESA, rPfSUB1-mediated processing of SERA5 P126 resulted in conversion only to P56.

(B) Conversion to both P73 and P56 is inhibited by MRT12113 and is directly mediated by PfSUB1. Western blot probed as in (A) showing the effects of MRT12113 on the time course of processing of purified SERA5 P126 (batch 2 as described above) by rPfSUB1. The presence of MRT12113 from the beginning of the reaction (second track from left) prevented all processing, while addition of MRT12113 after 15 min stopped all subsequent processing.

(C) Silver-stained gel showing a time course of SERA5 P126 digestion by rPfSUB1. Each SERA5-containing track contains ~200 ng SERA5. Major processing products are arrowed, alongside their determined N-terminal amino acid sequences; only the first five residues are shown. Sequence from the P25n fragment matches that of the N-terminus of SERA5 P126 following secretory signal peptide removal (Debrabant et al., 1992), whereas the P25c N-terminal sequence lies within a polymorphic region of SERA5 as predicted by Li et al. (2002b). Start and 8 hr control samples are as above. The asterisk indicates a contaminant of the SERA5 P126 preparation, which was not modified by rPfSUB1.

(D) Progress curve experiment comparing initial hydrolysis rates by rPfSUB1 of pepF1-6R, SERAst1F-6R and SERAst2F-6R. Substrates were used at a concentration of 0.2 μM ($< < K_m$). The arrow indicates the point at which pronase was added to obtain the cleavage endpoint (100% hydrolysis).

Little is known about their proteolytic processing, but they are found in schizonts in predominantly full-length form. Given their structural similarity to SERA5 we predicted that they undergo maturation in a similar manner. An alignment of the *P. falciparum* SERA primary sequences (Figures S6 and 6A) showed that sequences accommodated by the consensus motif summarized above are evident in a position similar to the SERA5 site 1 and site 2 processing sites in all sequences except SERA8, which is upregulated in insect stages (Aly and Matuschewski, 2005). This suggested that, in addition to SERA5, other blood stage SERA family members might also be substrates

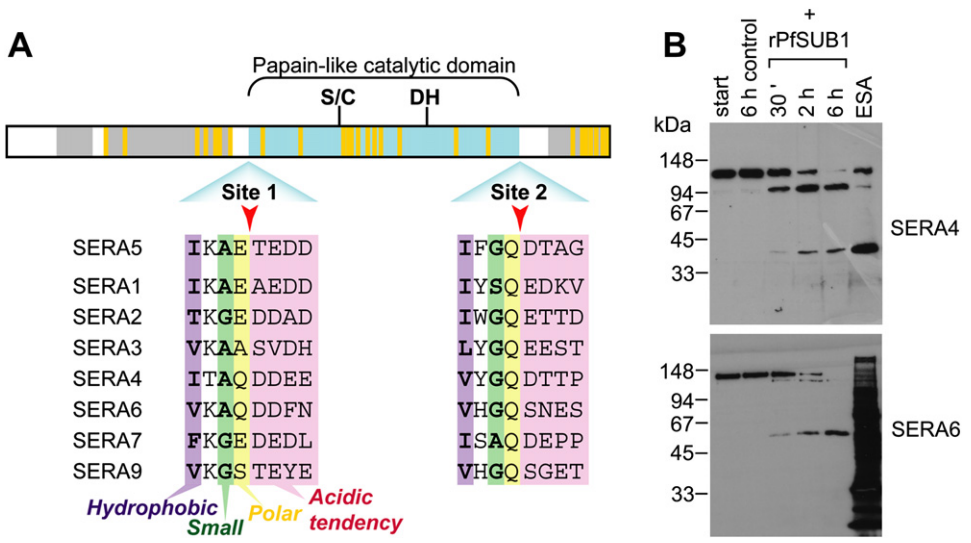

Figure 6. PfSUB1 Processes Other Members of the SERA Family

(A) The top schematic represents a consensus SERA structure. Conserved cysteines (yellow bars), regions of high sequence homology across the family (gray), and the papain-like central domain (light blue) with putative catalytic triad residues are highlighted. SERA5 cleavage sites 1 and 2 are indicated with red arrows. Proposed processing sites for other SERA proteins are aligned below, and consensus features of the flanking sequences are highlighted. SERA8 is excluded from the alignment because homologous sequences at either site could not be identified. Data were derived from a multiple Clustal alignment of the predicted *P. falciparum* SERA protein sequences (Miller et al., 2002).

(B) Processing of purified SERA4 and SERA6 by rPfSUB1. Start and "6 hr control" tracks are as in Figure 5A. The remaining tracks show a time course of digestion by rPfSUB1, comparing the products to those in ESA. The antibodies used here recognize the variable N-terminal end of SERA4 and SERA6 (Miller et al., 2002), so the fragmentation pattern is consistent with cleavage at the two predicted sites. The anti-SERA4 antibody is highly specific (see Figure S4), but the anti-SERA6 antibody cross-reacts with SERA4 and the abundant SERA5 (and perhaps other SERAs) probably explaining the complexity of the ESA signal in this case.

for PfSUB1. To test this, full-length SERA4 and SERA6 were isolated from schizont extracts (Figure S4) then incubated with rPfSUB1 as described above for SERA5. Both were digested in a manner consistent with cleavage at the predicted sites (Figure 6B). Importantly, in the case of SERA4 the digestion products were similar to those present in culture supernatants, suggesting that, like SERA5, SERA4 is processed upon schizont rupture. To obtain further evidence that processing occurred at the predicted sites, peptides based on the predicted SERA4 and SERA6 processing sites were incubated with rPfSUB1. All were specifically cleaved only at the expected positions (Figure S7).

Our findings suggest that, upon its release into the PV, PfSUB1 mediates maturation of the entire family of PV-located SERA proteins, including representatives of both the serine-type and cysteine-type subgroups. Collectively, our results suggest that discharge of PfSUB1 into the PV, SERA maturation, and egress of invasive merozoites are regulated, consecutive, and causally related processes that take place within a very short time-scale right at the end of schizogony.

DISCUSSION

Regulation of the final stages of malaria parasite maturation and escape from its host red blood cell is poorly understood. Numerous reports have shown that protease activity is required but prior to this study none of the proteases involved had been identified. We have now shown that a parasite subtilase, PfSUB1, plays a key role in this process.

Two major conclusions may be drawn from this work. The first of these is that egress involves—and may even be triggered by—discharge of PfSUB1 from exonemes into the PV. The notion that the malaria parasite possesses a class of secretory organelle that functions in egress provides an intuitively appealing and conceptually simple mechanistic model for how egress may be temporally controlled (Figure 7). In addition, although our study does not address the issue directly, our findings lend indirect support to the "inside-out" model of schizont rupture in which the primary event in egress is breakdown of the PV followed by rupture of the host cell membrane (Wickham et al., 2003). This is because we can now conceive mechanisms by which release of PfSUB1 into the vacuolar space could directly lead to degradation of the PV membrane (see below); in contrast, it is difficult to reconcile our observations with the alternative model of Salmon et al. (2001) which proposes that breakdown of the erythrocyte membrane precedes PV rupture. The identification of other exoneme components will aid in further dissecting exoneme function and biogenesis, so it will be important to include PfSUB1-specific markers in future subcellular localization studies on parasite proteins.

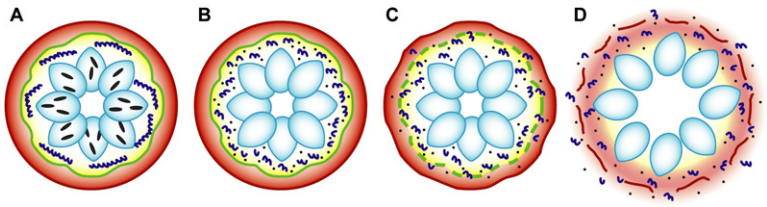

Figure 7. Model for PfSUB1-Mediated Regulation of Schizont Rupture

(A) PfSUB1 (black) accumulates in exonemes in daughter merozoites (light blue), while enzymatically inactive SERA precursor proteins (dark blue) reside in the PV (yellow).

(B) PfSUB1 (small black granules) is discharged into the PV, where it rapidly mediates activation of SERA4, 5, 6, and other PV-resident SERA family members by proteolytic cleavage.

(C and D) The PV membrane (green) ruptures as a result of SERA activation, followed by or concurrent with rupture of the erythrocyte plasma membrane (dark red). Merozoites, PfSUB1, and SERA5 fragments are released into the extracellular milieu.

The second major conclusion from our study is that PfSUB1—directly or through its action on SERA proteins—plays a dual role in both development of invasive merozoites and their release. Most proteases are synthesized as zymogens that are activated by proteolytic cleavage. It therefore seems reasonable to predict that PfSUB1-mediated processing of SERA4, 5 and 6 is an activation event; indeed, the timing of SERA5 processing has been a major factor in prompting suggestions that it plays a role in egress, perhaps by degrading membrane proteins of the PV leading to its destabilization. The SERA proteins—in particular SERA5—are abundant, and their activation by PfSUB1 would result in a rapid amplification cascade in the PV. As a corollary, however, their very abundance may require very efficient PfSUB1 inhibition to completely block SERA processing. This factor, together with the requirement for MRT12113 to cross several membranes in order to access intracellular PfSUB1, may account for the substantial difference between the IC_{50} of MRT12113 against rPfSUB1 in vitro (~0.3 μM) and its ED_{50} against egress in culture (~180 μM). Our observation that MRT12113 was much more potent in blocking the invasive capacity of released merozoites (ED_{50} ~25 μM) than in preventing egress is intriguing. One plausible explanation for this is that some or all activated SERA proteins may function not only in egress per se but also in "priming" merozoites for invasion, perhaps by binding to the merozoite surface as has been suggested by Li et al. (2002b) for SERA5, or even by mediating some of the well-described proteolytic modifications of merozoite surface proteins that occur in the final stages of schizogony (see Blackman, 2000 for a review). This latter hypothesis is consistent with the observed effects of MRT12113, since whereas partial inhibition of SERA processing might be insufficient to prevent schizont rupture, even partial inhibition of parasite surface protein maturation is likely to lead to a complete block in invasion.

An alternative or additional explanation for the potent inhibitory effects of MRT12113 on invasion is that PfSUB1 itself may mediate additional functions in the PV besides maturation of the SERA family. The consensus PfSUB1 recognition motif identified here is strikingly similar to known processing sites in a number of merozoite surface proteins, raising the possibility that these too may be substrates for PfSUB1 following its discharge into the PV. Work is underway to establish the significance of this.

The malarial life cycle in the human host involves passage through the liver, and maturation and egress of liver-stage merozoites share features with asexual blood stages. Since orthologs of PfSUB1 and SERA proteins are present in the genomes of all *Plasmodium* species examined to date, our findings suggest the possibility of a common involvement of SUB1 in blood-stage and liver-stage egress in all malarial species. All other apicomplexan pathogens are also intracellular parasites and several, including *Toxoplasma*, possess subtilases (Kim, 2004). Our work may shed new light on how these organisms escape from their host cell and how this process may be targeted in new approaches to disease control.

EXPERIMENTAL PROCEDURES

Parasite Culture, Transfection, and Growth Assays

P. falciparum clone 3D7 was cultivated in medium containing the serum substitute Albumax using standard procedures (Blackman, 1994). For transfection, ring-stage parasites were electroporated with plasmid DNA as described previously (Harris et al., 2005). After initial selection using 2.5 nM WR99210 (Jacobus Pharmaceuticals, New Jersey, USA) for ~4 weeks, parasites were subjected to repeated cycles of drug selection for 3 weeks followed by removal of the drug for 3 weeks ("drug cycling"). Clonal populations were obtained by limiting dilution.

To assess its effects on parasite growth, MRT12113 (Molecularnature, Reading, UK) was added to synchronous 3D7 cultures from stock solutions in DMSO, and parasite growth and morphology over the course of an entire cycle monitored by microscopic examination of Giemsa-stained thin films. Control cultures contained similar concentrations (up to 1% v/v) of DMSO alone. For investigating effects on egress and invasion specifically, synchronous cultures of schizonts (2%–8% parasitaemia) were supplemented with MRT12113 or DMSO only, cultured for 4–8 hr to allow schizont rupture and merozoite invasion, then examined microscopically by counting the number of unruptured schizonts, ring-stage parasites, and free merozoites visible per 20,000 erythrocytes.

Parasite Culture Supernatant Analysis and Tryptic Peptide Mapping

To obtain preparations of parasite proteins released upon schizont rupture, referred to as excretory/secretory antigens (ESA), mature 3D7 schizonts were allowed to rupture for 4 hr in Albumax-free medium. Clarified supernatants were concentrated by ultrafiltration. To examine the effects of MRT12113 on release of parasite proteins, similar cultures in Albumax-free medium supplemented with MRT12113, DMSO only, or no additions were incubated for ~12 hr. Culture supernatants were concentrated as above, subjected to SDS PAGE and stained with Coomassie blue. Bands of interest were

analyzed by tryptic peptide fingerprinting (see Supplemental Experimental Procedures).

Antibodies and Indirect Immunofluorescence Assay

A rabbit anti-PfSUB1 serum has been described previously (Withers-Martinez et al., 2002). Rabbit antibodies to SERA4 and SERA6 were kind gifts of Brendan Crabb (WEHI, Melbourne, Australia). The SERA5-specific mAb 24C6.1F1 (Delplace et al., 1985), which recognizes an epitope within the P50 processing product, was a gift from Jean-François Dubremetz (University Montpellier 2, France). The RESA-specific mAb 28/2 (Anders et al., 1987) was a gift of Robin Anders (LaTrobe University, Victoria, Australia). Acetone-fixed thin films of the *P. falciparum* 3D7SUB1HA3 clones C10 and F7 were incubated with a 1:500 dilution of the anti-HA mAb 3F10 (Roche), then with biotinylated goat anti-rat IgG (Chemicon) diluted 1:500, followed by FITC streptavidin (Vector Laboratories) diluted 1:500. For dual labeling, samples were additionally probed with mAb 4G2 (anti-PfAMA1), mAb 61.3 (anti-RhopH2), or mAb 1E1 (anti-$MSP1_{19}$; Harris et al., 2005) conjugated directly to Alexa Fluor 594 (Molecular Probes), or rabbit anti-PfSUB1, mAb 28/2 (anti-RESA), mAb H5 (anti-RAP2; Harris et al., 2005) or mouse anti-EBA-175 region VI (O'Donnell et al., 2006) followed by Alexa Fluor 594-conjugated anti-mouse IgG or anti-rabbit IgG (Molecular Probes) diluted 1:500. Slides were stained with DAPI and mounted in Citifluor (Citifluor Ltd., Canterbury, UK). Images were collected using AxioVision 3.1 software on an Axioplan 2 Imaging system (Zeiss) using a Plan-APOCHROMAT 100×/1.4 oil immersion objective, and annotated using Adobe PhotoShop.

Immunoelectron Microscopy

Schizonts were fixed on ice in 0.075% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in RPMI 1640 (pH 7.2), then washed in ice-cold RPMI, dehydrated through a progressively low temperature ethanol series, infiltrated with LR White resin (Agar Scientific, UK) and polymerized by ultraviolet light. Sections mounted on 400 mesh nickel grids without a supporting film were incubated with rabbit anti-PfSUB1 (diluted 1:50) followed by Protein A conjugated to 10 nm gold particles diluted 1:70 in PBS 1% (w/v) BSA (PBS/BSA). Sections were then turned over and stained on the other side with the anti-RESA mAb 28/2 ascites diluted 1:50 in PBS/BSA followed by goat anti-mouse antibody conjugated to 5 nm gold particles, diluted 1:50 in PBS/BSA. Control samples were treated with irrelevant primary antibodies. Sections were counter-stained for 4 min with 2% (w/v) aqueous uranyl acetate. Images were captured digitally with a Hitachi 7600 electron microscope.

Purification and Processing of SERA5 P126, SERA4, and SERA6

Purification of full-length SERA proteins from schizont extracts is described in Supplemental Experimental Procedures. Purified proteins in 25 mM HEPES, 12 mM $CaCl_2$ (pH 6.5) were supplemented with purified rPfSUB1 (Withers-Martinez et al., 2002) at an estimated protease:substrate molar ratio of 1:100, or control buffer (20 mM Tris-HCl, 150 mM NaCl, 50% v/v glycerol, [pH 8.2]). After incubation at 37°C for the required time, the reaction was stopped by boiling in SDS sample buffer. Samples were subjected to SDS PAGE and either analyzed by Western blot or stained. For N-terminal amino acid sequencing, samples were transferred to polyvinylidene difluoride membrane and Edman degradation performed at the Protein and Nucleic Acid Chemistry Facility (University of Cambridge, UK).

IC_{50} Determinations and Evaluation of SERA-Derived Peptide Substrates

IC_{50} values for MRT12113 inhibition of rPfSUB1 were determined using a previously-described assay (Harris et al., 2005) in which hydrolysis of pepF1-6R in the presence of compound was continuously monitored using a Cary Eclipse fluorescence spectrophotometer (Varian) equipped with a microplate reader.

Peptides based on SERA processing sites were synthesized by standard Fmoc chemistry. Analysis of intact and rPfSUB1-digested peptides by RP-HPLC and electrospray mass spectrometry was as described previously (Withers-Martinez et al., 2002). Cysteine-containing derivatives were labeled with 6-IATR, purified and quantified as described previously (Blackman et al., 2002). Kinetic assays comparing hydrolysis of SERAst1F-6R and SERAst2F-6R with pepF1-6R were as described (Blackman et al., 2002; Withers-Martinez et al., 2002). Briefly, solutions of substrate (0.1–2.0 μM) were supplemented with rPfSUB1 and the increase in fluorescence intensity measured until a steady state initial rate was determined. Pronase was then added to obtain complete substrate hydrolysis, and fluorescence values converted to moles of substrate hydrolyzed using the known starting substrate concentration.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, seven figures, two tables, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/131/6/1072/DC1/.

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