Two *Plasmodium* Rhomboid Proteases Preferentially Cleave Different Adhesins Implicated in All Invasive Stages of Malaria

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Invasion of host cells by the malaria pathogen *Plasmodium* relies on parasite transmembrane adhesins that engage host-cell receptors. Adhesins must be released by cleavage before the parasite can enter the cell, but the processing enzymes have remained elusive. Recent work indicates that the *Toxoplasma* rhomboid intramembrane protease TgROM5 catalyzes this essential cleavage. However, *Plasmodium* does not encode a direct TgROM5 homolog. We examined processing of the 14 *Plasmodium falciparum* adhesins currently thought to be involved in invasion by both model and *Plasmodium* rhomboid proteases in a heterologous assay. While most adhesins contain aromatic transmembrane residues and could not be cleaved by nonparasite rhomboid proteins, including *Drosophila* Rhomboid-1, *Plasmodium falciparum* rhomboid protein (PfROM)4 (PFE0340c) was able to process these adhesins efficiently and displayed novel substrate specificity. Conversely, PfROM1 (PF11_0150) shared specificity with rhomboid proteases from other organisms and was the only PfROM able to cleave apical membrane antigen 1 (AMA1). PfROM 1 and/or 4 was thus able to cleave diverse adhesins including TRAP, CTRP, MTRAP, PFF0800c, EBA-175, BAEBL, JESEBL, MAEBL, AMA1, Rh1, Rh2a, Rh2b, and Rh4, but not PTRAMP, and cleavage relied on the adhesin transmembrane domains. Swapping transmembrane regions between BAEBL and AMA1 switched the relative preferences of PfROMs 1 and 4 for these two substrates. Our analysis indicates that PfROMs 1 and 4 function with different substrate specificities that together constitute the specificity of TgROM5 to cleave diverse adhesins. This is the first enzymatic analysis of *Plasmodium* rhomboid proteases and suggests an involvement of PfROMs in all invasive stages of the malaria lifecycle, in both the vertebrate host and the mosquito vector.

**Introduction**

*Plasmodium* is the etiological agent of malaria, a devastating global disease that afflicts over 10% of the world’s population, claiming between 1 and 3 million lives annually [1]. The emergence and spread of drug-resistant strains have underscored the urgency of this serious global health problem. Lack of new effective drugs with which to treat the disease has made understanding *Plasmodium* biology, with an aim of identifying new therapeutic targets, an imperative goal.

Apicomplexan parasites including *Plasmodium* are obligate intracellular pathogens that must invade host cells to survive [2,3]. This dependence has focused attention on understanding the invasion process. *Plasmodium* undergoes four invasive stages during its lifecycle [4], but invasion of erythrocytes by merozoites in iterative cycles causes the clinical manifestations of the disease, including anemia, fever, and, in severe cases, coma and death [5]. Most experimental information has been gained for merozoite invasion of red blood cells, due to the relative ease in culturing these forms. Initial contacts with the host cell can be made on any surface of the parasite, but the merozoite reorient such that its apical end contacts the host-cell membrane [6]. Specialized transmembrane adhesin proteins are secreted from internal organelles called micronemes and rhoptries to the apical surface, where they engage host-cell receptors [7]. This interaction commits the parasite to invasion and results in the formation of the moving junction, a close electron-dense juxtaposition of the parasite and host-cell membranes [8]. The moving junction is thought to link to the parasite cytoskeleton [9], allowing the adhesin–receptor complexes to be motored to the posterior of the parasite, which results in the invagination of the host-cell plasma membrane to form the parasitophorous vacuole. Once the complexes reach the posterior surface, the junctions are dissolved and the host-cell membranes seal, resulting in an internalized parasite, enclosed within a vacuole derived from the host-cell plasma membrane.

Currently four families of transmembrane adhesins have been implicated in invasion (Figure 1A), and although their

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**Abbreviations:** AMA1, apical membrane antigen 1; DmRho-1, *Drosophila* Rhomboid-1; EBL, erythrocyte binding-like; GFP, green fluorescent protein; PfROM, *Plasmodium falciparum* rhomboid; RBL, reticulocyte binding-like; TGF, transforming growth factor; TRAP, thrombospondin-related anonymous protein

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precise molecular roles remain incompletely understood, recent experimental evidence suggests that they may function in separate phases of the invasion program. Apical membrane antigen 1 (AMA1) is an essential gene, and anti-AMA1 antibodies inhibit invasion [10]. A recent analysis has shown that anti-AMA1 antibody–treated parasites fail to reorient, suggesting an early role of AMA1 in invasion [11]. AMA1 is also likely to be directly part of the moving junction [12,13]. Two families of adhesins are thought to bind host-cell receptors directly. The erythrocyte binding-like (EBL) family was identified by the ability to bind the Duffy antigen, a chemokine receptor [14]. Since invasion of Duffy negative reticulocytes by *Plasmodium knowlesi* resulted in binding and reorientation but not tight junction formation, the EBLs may directly form the moving junction. *Plasmodium vivax* invades reticulocytes exclusively, and this observation has led to the identification of the reticulocyte binding-like family (RBL) of adhesins [15], which are also conserved in *Plasmodium falciparum*, despite the fact that this species invades erythrocytes [16]. EBL and RBL adhesins may have similar functions, since disruption of one EBL adhesin resulted in a compensating switch in the invasion pathway by upregulation of an RBL adhesin [17,18]. Last, the thrombospondin-related anonymous protein (TRAP) family contains thrombospondin and von Willebrand domains [19]. TRAP was first identified in sporozoites, but subsequently homologs have been identified that are expressed in ookinetes [20] and merozoites [21]. Importantly, this is the only class of adhesins that have been shown to bind the cytoskeleton-bridging protein aldolase, suggesting that TRAP adhesins may be the proteins that link the moving junction to the parasite cytoskeleton directly [9,22].

The ectodomains of various adhesins are shed from parasites during invasion [23,24], and processing of trans-
membrane adhesins at the posterior of the parasite is thought to be essential for dissolving the moving junctions at the end of invasion. Inhibition of these proteolytic processing events using panels of protease inhibitors can block host-cell invasion [25,26], suggesting that interfering with adhesion-cleaving proteases may be a viable therapeutic strategy. Despite these encouraging observations, the enzymes responsible for this processing have eluded identification.

Recent work has identified two classes of proteases as potential enzymes involved in adhesion processing. The membrane-bound subtilisin enzyme PISUB2 has been implicated in the processing of both surface antigens and now AMA1 in *Plasmodium* merozoites [27], but the enzymes responsible for cleavage of other transmembrane adhesins have not been identified. Studies in the related parasite *Toxoplasma* have suggested a role for a rhomboid intramembrane protease in adhesion cleavage during invasion [28–30]. Rhomboid intramembrane proteases were first discovered by studying cleavage of the epidermal growth factor receptor ligand Spitz during *Drosophila* development but are conserved in many branches of life [31,32]. A mechanistic analysis revealed that cleavage requires helix-relaxing residues within the top of the substrate transmembrane domain [33,34], and we discovered that similar residues are found in certain apicomplexan adhesins, including some from *Toxoplasma* and *Plasmodium*. An analysis of *Toxoplasma* rhomboid enzymes indicates that one of its six rhomboid proteases, TgROM5, is localized to the posterior surface of the tachyzoite and cleaves several different adhesins essential for host-cell invasion, including TgMIC2, the TRAP homolog [28]. Importantly, several of these adhesins have been found to be cleaved within their transmembrane anchors in tachyzoites [35,36].

Nothing is known about rhomboid enzyme function in *Plasmodium*, but *Plasmodium* lacks a TgROM5 homolog and contains adhesins with divergent transmembrane segments that are not substrates for rhomboid enzymes. We therefore sought to study the enzymatic properties of *Plasmodium falciparum* rhomboid proteins (PIROMs) directly and test their ability to process transmembrane adhesins implicated in invasion, as a first step in assessing the potential involvement of PIROMs in host-cell invasion. We discovered that the specificity of TgROM5 appears to be covered by two distinct PIROMs: PIROM1 and PIROM4 display strong substrate specificity and preferentially cleave different adhesin families. Together, these two PIROMs are able to cleave all four families of transmembrane adhesins implicated in invasion. Since rhomboid enzymes are highly specific proteases and are expressed during invasion, these observations suggest that two distinct PIROM proteases are likely to process many diverse adhesins during all invasive stages of the *Plasmodium* lifecycle.

**Results**

**EBL and RBL Adhesins Are Not Substrates for *Drosophila* Rhomboid-1**

Since invasion of red blood cells by *Plasmodium* merozoites has been studied intensively and is the basis of the disease, we first focused on examining the cleavage of adhesins implicated in merozoite invasion. Three families of adhesins have been implicated directly in this process: AMA1, the EBL adhesins EBA-175, BAEBL/EBA-140, and JSEBL/EBA-181 and the RBL adhesins Rh1, Rh2a, Rh2b, and Rh4. Analysis of transmembrane sequences from these adhesins revealed that AMA1 contains residues that have been found to allow
disordered structures composed of tandem asparagine regions starting at various points before their transmembrane domains [23,37]; we therefore cloned the C-terminal–most extracellular regions of RBL proteins contain no identifiable signal peptidase cleavage site. Since the EBL and RBL adhesins contain very large ectodomains of approximately 140 to 300 kDa, we generated truncated forms of EBLs that started from region VI and proceeded to their natural C-domain (white region in diagram) ASSAA to VLVVV strongly reduced cleavage by endogenous cellular metalloproteases in all lanes in (A) and (B) except those denoted MP, which served as positive controls. 

(A) Region of AMA1 important for substrate recognition by rhomboid proteases. Mutation of the top region of the AMA1 transmembrane domain (white region in diagram) ASSAA to VLVVV strongly reduced AMA1 cleavage by TgROM5 and PfROM1, but not by cellular metalloproteases (MP). Molecular weight standards in kDa are denoted to the right of each panel. 

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Expression of PfROMs in Heterologous Mammalian Cells 

Since nothing is known about the function of PfROMs, it was important to study their enzymatic properties directly and to assess their ability to process any Plasmodium adhesins directly. The genome of P. falciparum encodes eight rhomboid-like genes, nomenclature of which follows from their discovery in Toxoplasma; the six TgROMs are numbered 1 through 6, and those Plasmodium ROMs that are homologous in sequence are numbered equivalently: PfROM1 (PF11_0150), PfROM3 (MAL8P1.16), PfROM4 (PFE0340c), and PfROM6 (PF13_0241) (gene designations in parentheses are from the published Plasmodium genome; www.plasmodb.org) [28,38]. The remaining four PfROMs that do not have homologs in Toxoplasma are sequentially numbered PfROM7 through PfROM10.

Based on a sequence analysis, several PfROMs are unlikely to cleave adhesins during host-cell invasion. PfROM6 (PF13_0241) contains a mitochondrial targeting signal and is predicted to be part of a conserved subclass of rhomboid proteases localized to mitochondria in eukaryotes [38,39]. PfROM10 (MAL6P1.241) lacks conserved residues predicted to be essential for catalysis and thus also is not likely to function as a protease during invasion [31]. We examined the expression of the remaining six rhomboid-like genes encoded in the malaria genome by analyzing microarray data. Four PfROMs are expressed in the merozoite stage of the lifecycle, while PfROM3 (MAL8P1.16) is highly expressed in gametocytes and PfROM9 (PFE0755c) is expressed very weakly. We thus chose to focus our analysis on PfROMs 1, 4, 7, and 8. Notably, PfROMs 1 and 4 are conserved in other Apicomplexa including in Toxoplasma, while PfROMs 7 (PF13_0312) and 8 (PF14_0110) have atypical topologies and are not conserved among Apicomplexa.

In order to test the ability of PfROMs to cleave adhesins, we sought to adapt the transfection-based proteolysis assay. The ability of transfected mammalian cells to express Plasmodium rhomboid-like genes was first tested by cloning the open reading frame of ROM4 genes from P. falciparum, Plasmodium berghei, and Plasmodium yoelli and tagging them at their extreme N-termini with a triple HA tag (which we determined previously does not interfere with rhomboid activity). Unfortunately, none of these ROM4s could be expressed to any detectable level in mammalian cells (Figure 2A).

The approximately 80% AT-rich Plasmodium genome can result in nucleic acid sequences that limit protein expression in mammalian cells by introducing rare codons as well as...
sequences that are perceived as transcription stop sites. To overcome this limitation, we recoded the four ROM genes including PfROM4 such that the same proteins are encoded by synthetic genes where the codons have been optimized for expression in mammalian cells. This resulted in highly efficient expression of PfROM1, PfROM4, and PfROM8 and efficient expression of PfROM7, as detected by anti-HA Western analysis (Figure 2A).

Activity and Specificity of PfROMs against Drosophila Spitz Protein

Rhomboid proteins from many diverse organisms, including from bacteria, protozoa, plants, and animals, are able to cleave the Drosophila substrate Spitz, a transforming growth factor a (TGFα)-like ligand of the EGF receptor [33,40]. We first used this assay to test for proteolytic activity of PfROMs (Figure 2). Mammalian cells were co-transfected with GFP-tagged Spitz and PfROMs, and cleavage of Spitz was assessed by Western analysis of cell culture media for the cleaved form of GFP-Spitz, which is secreted by the cells. To further this assay to test for proteolytic activity of PfROMs (Figure 2). Mammalian cells were co-transfected with GFP-tagged Spitz and PfROMs, and cleavage of Spitz was assessed by Western analysis of cell culture media for the cleaved form of GFP-Spitz, which is secreted by the cells.

While Toxoplasma ROM1 and ROM5 have robust activity against Spitz, only PfROM1 of the four PfROMs proved to be active against Spitz (Figure 2B). Mutation of the PfROM1 putative active site serine to alanine abolished activity against Spitz (Figure 2D), confirming that the proteolytic activity was from PfROM1. Rhomboid proteases display strong substrate specificity and rely on the top seven amino acids of the Spitz transmembrane domain for cleavage [33]. Similarly, a mutant form of Spitz where these seven transmembrane residues were mutated could not be cleaved by PfROM1, as well as TgROMs 1 and 5 that were analyzed in parallel (Figure 2C), suggesting that PfROM1 shares substrate specificity with this subclass of Spitz-cleaving rhomboid proteases.

Cleavage of Plasmodium falciparum AMA1

Since we discovered one PfROM to be active, we next tested the ability of PfROMs to process GFP-tagged Plasmodium adhesins directly. AMA1 is an efficient substrate for DmRho-1 (Figure 1C), and its co-transfection with TgROM5 and PfROM1, but not TgROM1 or PfROMs 4, 7, and 8, resulted in AMA1 cleavage (Figure 3A). Cleavage by PfROM1 was much less than that observed with TgROM5, but both relied on their putative active site serine residues, since their mutation to alanine abolished cleavage. Finally, mutation of residues in the AMA1 transmembrane domain in the full-length adhesin strongly reduced cleavage by TgROM5 and by PfROM1, suggesting that PfROM-1 recognizes AMA1 by this region (Figure 3B). Note that this is a somewhat more conservative mutant, since we only changed five residues of the transmembrane segment, which might account for the reduction, rather than abolishment, of cleavage. This mutant AMA1 was still shed efficiently by cellular metalloproteases, arguing that the mutant form of the protein was expressed and trafficked correctly. Overall, these observations are consistent with the specific cleavage of Spitz by PfROM1 and TgROM5, which was also dependent on the substrate motif in the Spitz transmembrane domain. Therefore, PfROM-1 uses a similar specificity mechanism to cleave AMA1.

Cleavage of EBL Adhesins by PfROMs

Despite not being substrates for the model rhomboid protease DmRho-1, we also examined the ability of parasite rhomboid enzymes to cleave GFP-tagged EBL adhesins directly. Surprisingly, both TgROM5 and PfROM4 were able to cleave each EBL adhesin robustly, while PfROM1 was able to cleave them weakly (Figure 4A to 4C). TgROM1, PfROM7, and PfROM8 failed to show any activity against these adhesins. Note that PfROM4, which failed to show any activity against Spitz or AMA1, displayed very strong activity against all EBLs tested and is the first rhomboid enzyme found to be active but cannot process Spitz. Moreover, mutating the predicted active site serine of PfROM4 to alanine abolished cleavage. Finally, mutating of residues in the AMA1 transmembrane domain in the full-length adhesin strongly reduced cleavage by TgROM5 and by PfROM1, suggesting that PfROM-1 recognizes AMA1 by this region (Figure 3B). Note that this is a somewhat more conservative mutant, since we only changed five residues of the transmembrane segment, which might account for the reduction, rather than abolishment, of cleavage. This mutant AMA1 was still shed efficiently by cellular metalloproteases, arguing that the mutant form of the protein was expressed and trafficked correctly. Overall, these observations are consistent with the specific cleavage of Spitz by PfROM1 and TgROM5, which was also dependent on the substrate motif in the Spitz transmembrane domain. Therefore, PfROM-1 uses a similar specificity mechanism to cleave AMA1.
parasite rhomboid proteins, we mutated the top half of both BAEBL and EBA-175 transmembrane domains to that of TGFα. Neither mutant adhesin could be cleaved by PIROM1, PIROM4, or TgROM5 (Figure 4E and 4F), suggesting that these substrates are recognized at least in part by the same region as other rhomboid substrates.

RBL Proteins Are Substrates for PIROMs 1 and 4

Since parasite rhomboid enzymes were able to cleave EBL adhesins that contain sequences within their transmembrane domains that are not conducive for Drosophila rhomboid cleavage [33], we also examined the ability of parasite rhomboid proteins to cleave Rh adhesins.

Co-transfection of GFP-tagged Rh adhesins with TgROM5 and PIROM4 resulted in cleavage of all Rh proteins, with Rh1, Rh2a, and Rh4 being cleaved better than Rh2b (Figure 5A to 5D). PIROM1 was also able to cleave Rh1, Rh2b, and Rh4, while PIROMs 7 and 8 were unable to cleave any Rh protein.

Interestingly, Rh2a and Rh2b are distinct but paralogous genes arranged head-to-tail that contain the same N termini but different C termini (including transmembrane domains) [37]. Thus, the different transmembrane domains may confer a functional difference, since Rh2a was cleaved by PIROM4 but not at all by PIROM1, while Rh2b was cleaved less well by PIROM4 but more efficiently by PIROM1.

PIROMs 1 and 4 Display Distinct Substrate Specificity

One surprising outcome of these analyses is that PIROM1, like TgROM5, is similar to the Spitz-cleaving class of rhomboid proteases, while PIROM4 is unable to cleave Spitz but is able to cleave EBL adhesins. Thus PIROM4 appears to have different substrate specificity than the Spitz-cleaving subclass of rhomboid proteases currently defined.

To determine which regions of the different adhesins confer specificity for cleavage by PIROM1 compared to PIROM4, we generated chimeric molecules between AMA1 and BAEBL in which their transmembrane regions were interchanged. TgROM5, which can cleave both AMA1 and BAEBL, cleaved both chimeras well, indicating that the chimeric proteins are correctly folded and trafficked in cells (Figure 6A). PIROM1 can cleave BAEBL weakly, but substituting the AMA1 transmembrane domain converted this form of BAEBL into an efficient substrate for PIROM1. Conversely, while PIROM4 can cleave BAEBL very efficiently but cannot cleave AMA1, it was no longer able to cleave BAEBL harboring the transmembrane domain from AMA1. The reciprocal chimeras also yielded consistent results (Figure 6B). While PIROM4 cannot cleave AMA1, substituting the BAEBL transmembrane domain into AMA1 converted AMA1 into a strong substrate for PIROM4. Conversely, while PIROM1 could cleave AMA1 (and BAEBL only weakly), substituting the transmembrane domain from BAEBL into AMA1 reduced the ability of PIROM1 to cleave this AMA1 chimera. Again, TgROM5, which cleaves both AMA1 and BAEBL efficiently, cleaved BAEBL and BAEBL with the AMA1 transmembrane domain.

Importantly, a similar pattern as observed with PIROM1 was also observed with several other Spitz-cleaving rhomboid proteases from diverse organisms including Drosophila, humans, and bacteria. All of these rhomboid enzymes could not cleave BAEBL efficiently or at all, while replacing the BAEBL transmembrane domain with that from AMA1 converted BAEBL into an efficient substrate for these rhomboid proteases (Figure 6C).

These results suggest that all of the enzymes tested rely on the substrate transmembrane domain region for cleavage. However, PIROM1 shares substrate specificity with other Spitz-cleaving rhomboid enzymes, while PIROM4 has a distinct substrate specificity that is unlike Spitz-cleaving enzymes. TgROM5 thus appears to be a “dual specificity” rhomboid protease, in that it cleaves both Spitz-like and EBL-like substrates.

Processing of TRAP Adhesins by PIROMs

In addition to the AMA1, EBL, and RBL adhesins that are involved in merozoite invasion of red blood cells, the TRAP family of adhesins are also thought to be essential for invasion, because only their cytoplasmic tails are known to bind the parasite cytoskeleton through the bridging protein aldolase, thus linking the moving junction to the motor...
Rhomboid Proteases May Function during Mosquito Cell Invasion: Cleavage of CTRP and MAEBL

Only a few adhesins have been identified that are involved in host-cell invasion in the mosquito vector. Genetic evidence has implicated CTRP, a large circumsporozoite and TRAP-related protein expressed in ookinetes, as essential for penetration of the midgut epithelium [44–46]. In addition to TRAP, MAEBL, a chimeric protein with the adhesive domains M1 and M2 homologous to AMA1 and its C-terminal region to EBLs, was found to be essential for invasion of salivary glands by sporozoites [47]. Both contain transmembrane sequences that would not be expected to be efficient rhomboid substrates (Figure 1B), and neither could be cleaved by DmRho-1 (unpublished data) but may be substrates for the newly discovered specificity of PIROM4.

Since CTRP is a large protein with an approximately 3,000 amino acid extracellular domain, we tagged a truncated version of CTRP with GFP as for other large adhesins, starting with its first of six thrombospondin domains and proceeding to its natural end. A truncated form of MAEBL, starting from region VI and proceeding to its natural end like for the EBLs, was tagged with GFP at its N terminus. Both proteins were expressed at high levels in mammalian cells and were shed by endogenous cellular metalloproteases, suggesting that both were trafficked efficiently to the cell surface (Figure 8).

Co-transfection of CTRP and MAEBL with TgROM5 and PIROM4 resulted in strong cleavage of both proteins, while co-transfection with PIROM1 resulted in less cleavage (Figure 8). These observations indicate that both CTRP and MAEBL are substrates for PIROM-catalyzed intramembrane proteolysis, and since both PIROMs 1 and 4 are expressed in these invasive stages, raise the possibility that PIROMs could also function during host-cell invasion in the mosquito vector.

Discussion

Rhomboid Enzymes in Plasmodium

The ectodomains of various adhesins are shed from parasites during invasion, but the enzymes responsible for this processing have largely eluded identification [23,24,26,37]. We previously discovered that Toxoplasma transmembrane adhesins are processed by a rhomboid protease [33], but nothing is known about rhomboid function in other parasites including Plasmodium. We have now directly analyzed the properties of four Plasmodium rhomboid enzymes as well as the ability of 14 Plasmodium adhesins to be processed by intramembrane proteolysis.

Interestingly, most Plasmodium adhesins contain aromatic residues at the top region of their transmembrane domains and cannot be cleaved by canonical rhomboid enzymes studied previously. Conversely, we discovered that parasite
rhomboid enzymes are indeed able to process all *Plasmodium* adhesins tested except PTRAMP, which has recently been shown to be cleaved by the membrane-tethered subtilisin-like enzyme PfSUB2 [48]. Mutagenesis of the transmembrane domains of several different *Plasmodium* adhesins abrogated cleavage, further supporting the notion that cleavage is specific and relies on the substrate transmembrane domain. Since rhomboid enzymes are highly specific and are expressed during invasion, these observations imply that rhomboid proteases may be a major class of sheddases for diverse adhesin cleavage during invasion.

Surprisingly, the main adhesin-cleaving rhomboid from *Toxoplasma*, TgROM5, is not conserved in *Plasmodium* [28,29], but our analysis now indicates that two separate PfROMs take its place. The Spitz-cleaving rhomboid protease encoded by *Plasmodium* is PfROM1, which relies on the same substrate motif in the transmembrane domain to cleave Spitz and AMA1 as most other canonical rhomboid proteases [28,33]. Conversely, PfROM4 contains a novel specificity that is the predominant activity for cleaving EBL, RBL, and TRAP adhesins. Thus, while TgROM5 appears to be a “dual specificity” ROM, in that it is able to cleave both Spitz-like substrates as well as all other adhesins tested (except PTRAMP), these two activities are split among PIROM1 and PIROM4, respectively, in *Plasmodium*. PIROM1 and PIROM4 may thus function with distinct but partially overlapping roles in processing most adhesins that TgROM5 can process directly. One implication of these findings is the potential redundancy of PIROMs 1 and 4 in EBL/RBL adhesin processing, and this should be noted when designing genetic experiments aimed at deciphering the role of these enzymes in parasite invasion.

Thus, current experimental evidence indicates that PIROM4 may function more like TgROM5 than its closer sequence homolog TgROM4; TgROM4 is not able to cleave any substrate tested thus far [28,29] (unpublished data), indicating that it may have lost its activity in favour of the dominant TgROM5 enzyme, while in the absence of a PIROM5 in *Plasmodium*, PIROM4 has a prominent role. However, we cannot exclude the possibility that other PIROMs can cleave adhesins in parasites but rely on other unidentified cofactors or conditions not recapitulated in our transfection-based assay. This unprecedented caveat notwithstanding, one prediction of our model is that both TgROM5 and PIROM4, but not TgROM4, would be localized to the posterior of invading parasites, the expected site of moving junction cleavage during invasion. Indeed, TgROM4 is localized uniformly on the parasite surface while TgROM5 is concentrated predominantly at the posterior end, but the localization of PIROM4 has not yet been reported [28,29].

**Adhesin Processing in the Vertebrate and Mosquito Hosts**

AMA1, EBL, RBL, and MTRAP adhesins are thought to play essential roles during erythrocyte invasion by merozoites, and most are experimentally known to be cleaved during invasion [14,23,24,37]. However, processing of most of these adhesins has not been studied directly, although AMA1 has been shown to be a substrate for both intramembrane and juxtamembrane proteolysis in merozoites under physiological conditions [27]. We have now determined that all four families of adhesins are specific substrates for either PIROM1, PIROM4, or both. PIROM4 is likely to be the major activity for EBL, RBL, and MTRAP cleavage; while contrary to previous proposals, our current analysis indicates that the intramembrane proteolytic activity for AMA1 is likely provided by PIROM1, not PIROM4, because PIROM1 is unique among PIROMs expressed in merozoites in being able to cleave AMA1 directly. Taken together, our cleavage data thus predict a prominent role for rhomboid proteases in cleavage of different transmembrane adhesin families during merozoite invasion of erythrocytes.

Our current data also suggest the involvement of *Plasmodium* rhomboid proteases in all other invasive stages of the parasite’s lifecycle. Invasion of hepatocytes, which establishes the infection following a bite of the female mosquito, is currently known to rely only on two adhesins: TRAP and AMA1 [49,50]. TRAP is the *Plasmodium* homolog of TgMIC2 and is known to be proteolytically cleaved, although little information is available regarding the type of cleavage
involved [35]. We have determined that TRAP is a potent substrate for PIROM4, suggesting that PIROM4 might be the TRAP sheddase during invasion of hepatocytes. Importantly, AMA1 has recently been found to be expressed in sporozoites, where its proteolytic cleavage was insensitive to PMSF [50], a compound that potently inhibited AMA1 cleavage by PfSUB2 in merozoites [27,51]. This observation raises the possibility that other proteases may be involved in AMA1 processing in sporozoites. Since AMA1 can be cleaved by PIROM1, which is expressed in sporozoites, we suggest that PIROM1 may process AMA1 during sporozoite invasion of host cells.

Although much less is known about host-cell invasion and the adhesins involved in the mosquito host, both PIROMs 1 and 4 are expressed in both gametocytes and sporozoites. Moreover, PIROM3 has the sequence elements required for proteolytic activity and is preferentially expressed in the gametocyte stage, although its activity has not been tested. Nevertheless, our data show that PIROM4 and, to a weaker extent, PIROM1 are able to cleave both the CTRP and MAEBL adhesins, the only two adhesins that are known to be essential for mosquito midgut [44–46] and salivary gland invasion [47], respectively. These observations provide evidence that rhomboid proteases may also be involved in invasion in the mosquito host, but this needs to be evaluated directly, preferably using genetic approaches.

Functional Rationale for Intramembrane Proteolysis

Mechanistically, it is not clear why intramembrane proteolysis, rather than the more conventional juxtamembrane proteolysis—cleavage of adhesins in their stalk regions that lie next to the membrane—would be used for shedding adhesins during invasion. Although there may be no reason per se, the function of adhesins in merozoite invasion may suggest a rationale for the different modes of cleavage. Various adhesins are thought to form the moving junction directly; EBL/RBL adhesins engage the host-cell receptor for invasion [14,17,18,23,24,37], while TRAP adhesins such as MTRAP in merozoites are thought to link the moving junction to the parasite cytoskeleton (since EBLs/RBLs are not thought to be able to bind cytoskeletal components directly) [21,22]. Thus, intramembrane proteolysis may be important for disengaging the moving junction specifically; this electron-dense structure might sterically occlude juxtamembrane cleavage of those adhesins in the moving junction that complex to other components. In contrast, adhesin transmembrane domains are thought to remain free as monomers and could thus be approached by a predominantly membrane-embedded enzyme such as a rhomboid protease. But it should be noted that further analysis is required to test this speculative model.

Therapeutic Potential of Rhomboid Proteases

While invasion of hepatocytes by sporozoites establishes infection of the vertebrate host, the clinical manifestations of malaria are not felt until invasion of erythrocytes by merozoites ensues [5]. Therefore, treating malaria the disease would be dependent on the ability to block red blood cell invasion by merozoites. Analysis of field-isolated parasites has revealed a surprising level of diversity in the expression of different adhesins and the invasion pathways used by various strains to invade erythrocytes [17,29,37,52]. Moreover, switching between the adhesin-receptor pathways is one way in which merozoites compensate for blocks in individual pathways for invasion. Strikingly, genetic disruption of EBA-175 resulted in a switch from a sialic acid–dependent receptor for invasion, to a sialic acid–independent mode of invasion [24]. Subsequent transcriptional profiling identified Rh4 as the adhesin that was upregulated in the EBA-175–disrupted strain and was responsible for conferring the new mode of invasion [18]. But the potential to evade blocks in different invasion pathways is not endless; it was not possible to disrupt both EBA-175 and Rh4 together [18].

Importantly, our current work suggests that both the EBL and RBL adhesins, and other adhesins, go through the same proteolytic processing pathway, mediated by PIROMs 1 and 4. This unexpected commonality could provide a convergence point that could be used to therapeutic advantage; while blocking one adhesin-receptor pathway for invasion often results in a compensating switch to a new pathway [18,24,52], most of these pathways appear to require rhomboid-mediated processing for the successful completion of the invasion program. In this way, a drug that could inhibit or reduce rhomboid activity could block multiple different invasion pathways used by merozoites. Moreover, cleavage of adhesins implicated in invasion by sporozoites and ookineties by PIROMs suggests that rhomboid enzymes could also be targets in other parts of the parasite lifecycle. With this in mind, PIROMs warrant further cell biological and genetic study to complement our biochemical analysis, which together should reveal the full biological roles of these membrane enzymes.

Materials and Methods

DNA constructs. All rhomboid proteins were tagged at their extreme N-terminus with a triple hemagglutinin (3xHA) tag, and expressed from a CMV promoter in the pcDNA3.1 vector (Invitrogen,
Carlsbad, California, United States). Recoded PIROM genes were synthesized by GeneArt AG (Regensburg, Germany). Sequences of the synthetic DNAs encoding PIROMs were deposited in GenBank. **Toxoplasma** **ROMs**, DmRom-1, Bacillus subtilis YqgP, and human RHBDL2 were described previously [28,33,34].

P. falciparum adhesins were expressed from the CMV promoter of pcDNSA1 (Plasmodium) as N-terminal GFP-tagged fusions and preceded by an N-terminal mammalian signal peptide (from TGFp residues 1 to 32) for efficient insertion into the secretory pathway. Full-length adhesins AMA1, TRAP, MTRAP (PF10_0281), and PTRAAP were cloned immediately downstream of the GFP tag from the first residue after their predicted endogenous signal peptide cleavage site. Due to their large size, the remaining adhesins were truncated by deleting portions of their extracellular domains starting from their N-terminus. Several Rh constructs cloned in this way could not be efficiently expressed in COS-7 cells. We therefore tried several different N-terminal starting points and discovered that expression of GFP-tagged Rh2a and Rh2b with longer ectodomain of approximately 500 residues and Rh1 and Rh4 bearing smaller ectodomains of approximately 200 residues could be achieved. Cloned constructs contained residues 1294 to 1402 of EBA-175, residues 1401 to 1210 of BAEBL, residues 1399 to 1566 of JESEBL, residues 2768 to 2931 of Rh1, residues 2659 to 3130 of Rh2a, residues 2585 to 3179 Rh2b, residues 1454 to 1716 of Rh4, residues 1003 to 1370 of PF08090c, residues 1495 to 2038 of CTRP, and residues 1882 to 2055 of MAEBL.

Point mutants and AMA1 transmembrane mutation (from AASSA to VLVVV) were generated using QuikChange site-specific PCR mutagenesis (Stratagene, La Jolla, California, United States) with a Stratagene Robocycler. Chimeras between AMA1 and BAEBL, and mutation of the top ten residues of AMA1 to EBA-175 and BAEBL transmembrane domains to those of TGFp (AITALVVVIS), were generated by inverse PCR using primers that encoded the new sequences at their 5’ ends (residues 1128 to 1154 of BAEBL and residues 458 to 467 of AMA1). Following the inverse PCR, DNA products were purified using the QiAQuick PCR purification method (Qiagen, Valencia, California, United States), and the ends were phosphorylated by T4 polynucleotide kinase (New England Biolabs, Beverly, Massachusetts, United States) and circularized by ligation with T4 DNA ligase (Roche, Basel, Switzerland). All constructs were verified by DNA sequencing.

**Cleavage assay.** COS cells were maintained in DMEM with high glucose, t-glutamine, 50 mg/ml gentamicin (Invitrogen), and 10% fetal bovine serum, in humidified incubators at 37°C. Cells were seeded in six-well plates (Costar), and transiently transfected at 50% to 75% confluency the following day with FuGene6 (Roche) and incubated at 37°C in humidified incubators. For the following 24 h. COS cells were maintained in DMEM approximately 18 h post-transfection, and serum-free DMEM was conditioned in the presence of metalloprotease inhibitor BB1101 for the following 24 h.

Media and cell lysate samples were prepared in Laemmli buffer and proteins were separated by 4% to 20% gradient Tris-glycine SDS-PAGE under denaturing conditions, using prestained molecular weight standards (Invitrogen). The separated proteins were transferred onto nitrocellulose membranes, and probed with anti-GFP or anti-HA (Santa Cruz Biotechnology, Santa Cruz, California, United States) in phosphate-buffered saline with 0.05% Tween-20 and 1% fat-free milk. The resulting immunocomplexes were probed with anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology), revealed with enhanced chemiluminescence (GE Health Sciences, Amersham, Little Chalfont, United Kingdom), and imaged with X-ray film (Marsh), and the images were digitized with a UMAG scanner (UMAX Technologies, Dallas, Texas, United States) operating in transmissive mode.

**Supporting Information**

**Accession Numbers**

The GenBank ([http://www.ncbi.nlm.nih.gov/Genbank]) accession numbers of synthetic PIROM DNAs used in this paper are PIROM/1_F10_0281 (DQ868368), PIROM/1_F10_0284 (DQ868570), PIROM/1_F10_0312 (DQ986369), and PIROM/8_F10_0110 (DQ986771).

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**Competing interests.** The authors have declared that no competing interests exist.

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