Plasmapins IX and X are essential and druggable mediators of malaria parasite egress and invasion

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Proteases of the malaria parasite Plasmodium falciparum have long been investigated as drug targets. The P. falciparum genome encodes 10 aspartic proteases called plasmapins, which are involved in diverse cellular processes. Most have been studied extensively but the functions of plasmapins IX and X (PMIX and PMX) were unknown. Here we show that PMIX is essential for erythrocyte invasion, acting on rhoptry secretory organelle biogenesis. In contrast, PMX is essential for both egress and invasion, controlling maturation of the subtilisin-like serine protease SUB1 in exoneosome secretory vesicles. We have identified compounds with potent antimalarial activity targeting PMX, including a compound known to have oral efficacy in a mouse model of malaria.

Considerable work has gone into the synthesis of plasmapin inhibitors as antimalarials (1–4). Most efforts have been directed at the digestive vacuole plasmapin I to IV (PMI to PMIV) because of the availability of crystal structures and recombinant proteins. However, genetic knockouts reveal that these are not essential for parasite survival (5). Thus, rationally designed inhibitors of PMI to PMIV likely exert their antimalarial effects through outer targets (1, 3). PMV, PMIX, and PMX are the only other plasmapins expressed in asexual blood-stage parasites (6). PMV is an essential protease that processes proteins for export into the host erythrocyte and is a focus of ongoing drug development efforts (7–9). PMV is, however, quite divergent from the other plasmapins (10), and most digested vacuole plasmapin inhibitors are not potent against this enzyme (7). PMI to PMIV have more sequence homology to PMIX and PMX (10). PMIX and PMX could be the targets of digestive vacuole plasmapin inhibitors that have antimalarial activity.

To characterize the functions of PMIX and PMX in the biology of blood-stage Plasmodium falciparum parasites, we used new TetR-aptamer conditional knockout (KD) technology (12) (fig. S1), enabling translational repression of the target gene when anhydrotetracycline (aTc) is removed from the culture. Using CRISPR-Cas9 editing, we installed the TetR-aptamer regulatory system at the PMIX and PMX loci to create PMIXaTc and PMXaTc lines (fig. S1). When aTc levels were lowered in synchronous, early ring-stage parasites, we observed a major decrease in target protein levels in late-stage schizonts, in both PMIXaTc and PMXaTc (Fig. 1A). This led to decreased replication, revealing a critical role for both of these enzymes in parasite survival (Fig. 1B).

To determine the stage at which the defect occurred, cell cycle progression was monitored using highly synchronous ring-stage parasites cultured under KD (−aTc) or induced (+aTc) conditions. Flow cytometry revealed that both PMIXaTc and PMXaTc developed normally until they reached segmented schizonts (~44 hours). At the end of the cycle (between 46 to 52 hours), similar numbers of PMIXaTc schizonts had egressed, irrespective of PMX expression status. However, +aTc cultures featured about one-fourth as many new rings (Fig. 1C). A similar fourfold decrease was seen in −aTc PMXaTc cultures (Fig. 1D). Unlike for PMIXaTc, at 52 hours, 80% of PMXaTc parasites had egressed in +aTc cultures, whereas only 56% had egressed in −aTc cultures. Even taking the egress defect into account, however, we observed fewer rings than expected, indicating an additional infection phenotype. We further characterized this by live microscopy of individual cells (13) (Fig. 1E) and observed similar egress and invasion impairment (Fig. 1F). Unruptured schizonts accumulated in the −aTc culture as merozoite clusters and some distorted schizonts; occasionally, these partially ruptured or displayed defective merozoite dispersal (Fig. 1G). Of the parasites that could egress in a normal time frame under −aTc conditions, the merozoite invasion rate was ~50% of that observed in the presence of aTc (Fig. 1F). These data implicate PMIX in erythrocyte invasion and PMX in both egress and invasion. A PMIXaTc−PMXaTc double aptamer-tagged line displayed a similar defect in egress and a greater block in invasion, yielding a sevenfold decrease in new rings (fig. S2). The data imply independent contributions of PMIX and PMX to these processes.

To evaluate the subcellular localization of these proteins, we engineered epitope tags on the 3′ end of the endogenous genes (fig. S3) and performed immuno-electron microscopy. PMIX was found largely in the bulbs of rhoptry secretory organelles that are involved in invasion (14) (Fig. 2, A and B). PMX was found in exosomes—small, oviod secretory vesicles that discharge during egress into the parasitophorous vacuole surrounding the parasite (15) (Fig. 2, C to E).

The localization studies guided us to examine organellar proteins whose processing could be affected by PMX action. RAPI is a rhoptry bulb protein that is processed from an 84-kDa precursor to 82- and 67-kDa forms. The PMIXaTc line failed to process the precursor efficiently when aTc was withdrawn (Fig. 3A). In contrast, the rhoptry neck protein RON4 was processed despite PMIX KD. By electron microscopy, a rhoptry biogenesis defect was evident under KD conditions (Fig. 2, F and G).

The subtilisin-like serine protease SUB1 is an exonemal protein that plays a critical role in egress and invasion (15, 16). SUB1 is synthesized as an 82-kDa zymogen that rapidly self-processes into a 54-kDa semi-proenzyme in the endoplasmic reticulum (ER). The cleaved promdomain remains bound to the 54-kDa protein and inhibits activity (17). A second processing step converts the 54-kDa form into a 47-kDa mature protein. This step can occur autocatalytically in vitro but is slow and partial (18, 19). A processing enzyme has been postulated for this step, but) its identity is unclear. Notably, a major defect in SUB1 processing was observed in PMIXaTc (Fig. 3B) but not PMXaTc (Fig. 3D) under aTc withdrawal, indicating that PMX is important for the final SUB1 processing step. Consistent with this, PMX is synthesized and processed shortly before SUB1 synthesis and processing occurs (Fig. 3C). Similar to the second processing step of SUB1, PMX maturation is blocked by brefeldin A (fig. S5), suggesting a post-ER event.

During egress, SUB1 processes a family of cystein proteases (SERAs) and a family of merozoite surface proteins (MSPs) (15, 16). SERAs is synthesized as a 126-kDa protein and is processed sequentially by SUB1 into 73- and 56-kDa forms. The latter is further processed into a 50-kDa fragment in a SUB1-independent process (21). We assessed SERA5 in PMIXaTc parasites. In the absence of aTc, SERA5 accumulated in the 126-kDa form with very little processing to other intermediates (Fig. 3D). Similarly, MSP1 accumulated as its 193-kDa precursor (fig. S6). Thus, PMX KD impairs downstream egress events.
We tested whether PMX is an active protease by introducing an ectopic gene copy (Fig. 3, E and F, and fig. S7). PMX<sup>apt</sup> parasites constitutively expressing a second-copy PMX gene with an active site aspartate mutation [Asp<sup>266</sup>→Gly<sup>266</sup>] had reduced growth in the absence of aTc and were unable to restore processing of SUB1. In contrast, those expressing a wild-type second-copy gene were rescued. These data show that PMX enzymatic activity in vivo is crucial to parasite egress and invasion defects. PMX KD results in both egress and invasion defects. Synchronized ring-stage cultures were grown with or without aTc. Schizonts and rings were counted by flow cytometry at 44 and 52 hours postinvasion (hpi). Shaded bars, schizonts; open bars, rings. (C) 52-hour rings were fewer in the −aTc condition [P < 0.0001 (t test)]. Number of schizonts was not significantly different. (D) 52-hour rings were fewer in the −aTc condition (P < 0.001). Number of remaining schizonts was greater (P < 0.001). (E to G) Live-cell microscopy of PMX parasites with or without aTc. (E) Individual schizonts were scored for egress and subsequent invasion (arrows in image of control parasitemia) Scale bar, 5 μm. (F) Quantification of PMX<sup>apt</sup> egress and invasion defects in the −aTc condition (**P < 0.01). (G) Abnormal schizont classes observed after PMX KD: 1, distorted schizont; 2, unruptured merozoite cluster; 3, defective egress or merozoite dispersal. 4, Normal schizont for comparison. Scale bars, 5 μm. All experiments in this and subsequent figures were replicated at least three times. +aTc: 1 μM. Error bars indicate SEM.

Fig. 1. PMIX is essential for invasion of erythrocytes; PMX is essential for egress and invasion. (A) Immunoblot with aFlag antibody showing knockdown (KD) of PMIX and PMX after anhydrotetracycline (aTc) withdrawal for one cycle. Haloacid dehalogenase–like hydrolase (HAD1) was used as a loading control. (B) Expansion of PMIX<sup>apt</sup> and PMX<sup>apt</sup> parasites is impaired in −aTc medium (P < 0.0001 for each by two-tailed t test). Triangles, PMIX<sup>apt</sup>; circles, PMX<sup>apt</sup>. Open symbols, −aTc; closed symbols, +aTc. (C and D) PMIX KD results in an invasion defect. PMX KD results in both egress and invasion defects. Synchronized ring-stage cultures were grown with or without aTc. Schizonts and rings were counted by flow cytometry at 44 and 52 hours postinvasion (hpi). Shaded bars, schizonts; open bars, rings. (C) 52-hour rings were fewer in the −aTc condition [P < 0.0001 (t test)]. Number of schizonts was not significantly different. (D) 52-hour rings were fewer in the −aTc condition (P < 0.001). Number of remaining schizonts was greater (P < 0.001). (E to G) Live-cell microscopy of PMX parasites with or without aTc. (E) Individual schizonts were scored for egress and subsequent invasion (arrows in image of control parasitemia). Scale bar, 5 μm. (F) Quantification of PMX<sup>apt</sup> egress and invasion defects in the −aTc condition (**P < 0.01). (G) Abnormal schizont classes observed after PMX KD: 1, distorted schizont; 2, unruptured merozoite cluster; 3, defective egress or merozoite dispersal. 4, Normal schizont for comparison. Scale bars, 5 μm. All experiments in this and subsequent figures were replicated at least three times. +aTc: 1 μM. Error bars indicate SEM.
Fig. 2. PMIX localizes to rhoptries, whereas PMX localizes to exonemes. (A and B) The PMIX gene was tagged with 3X hemagglutinin (HA) sequence at the 3′ end of the endogenous open reading frame. Tagged parasites grew normally. Segmented schizonts were prepared for immuno–electron microscopy, and PMIX was visualized with an anti-HA antibody and an 18-nm colloidal gold-labeled secondary antibody. Colocalization with an anti-RAP1 antibody, a marker for rhoptries, was performed using a 12-nm colloidal gold-labeled secondary antibody. The arrowhead points to an 18-nm particle; the arrow points to a 12-nm particle. (A) schizont; (B) merozoite. (C and D) The PMX gene was tagged with HA sequence. (C) Schizont; (D) enlarged view of the boxed area in (C). (E) The PMX gene was tagged with green fluorescent protein (GFP) sequence, and the exoneme marker SUB1 was tagged with 3X HA. Segmented schizonts were prepared for immuno–electron microscopy, PMX was visualized with an anti-GFP antibody and an 18-nm colloidal gold-labeled secondary antibody, and SUB1 was visualized with an anti-HA antibody and a 12-nm colloidal gold-labeled secondary antibody. Two examples are shown. Controls omitting the primary antibody were negative in all cases. (F and G) Electron micrographs of PMIXAPT cultured with (F) or without (G) aTc. Note the apical granularity and discoid morphology (G). See fig. S1 for statistics. Scale bars, 500 nm [(A) to (C), (F), and (G)]; 100 nm (D); 200 nm (E). R, rhoptry; N, nucleus.

Fig. 3. Plasmepsin knockdowns impair schizont protein maturation. (A) RAP1 maturation is impaired when PMIX is knocked down, but RON4 maturation is not. PMIXAPT parasites were cultured with and without aTc to 44 hpi, and extracts were blotted for RAP1 (left) and RON4 (right). Arrows mark precursor and processed RAPI forms. (B) The second maturation step in SUB1 processing is defective when PMX is knocked down. PMXAPT parasites were cultured as in (A). The rabbit anti-SUB1 immunoblot shows that processing of the SUB1 substrate SERA5 is impaired by PMX KD. PMXAPT parasites were cultured for 46 hpi (with and without aTc) and processed for immunoblot. (C) Immunoblot shows that processing of the SUB1 substrate SERA5 is impaired by PMX KD. PMXAPT parasites were cultured for 46 hpi (with and without aTc) and processed for immunoblot. (D and E) A catalytically dead mutant cannot rescue PMX KD. PMXAPT parasites were complemented with a second copy of the PMX gene, wild-type (WT) or mutant (D266G). Cultures were maintained with and without aTc; parasitemia (E) and SUB1 processing (F) were assessed. ****P < 0.0001 by two-tailed t test. KD of PMX and expression of second-copy genes were confirmed by immunoblot (fig. S6). ns, not significant. Error bars indicate SEM.
with a common scaffold that are specific inhibitors of PMX and that recapitulate the actions of PMX KD phenotypically. Our PMIX and PMX lines should allow high-throughput screening of aspartic protease inhibitor collections and may inform efforts to improve on the promising CWHM-117 lead compound.

REFERENCES AND NOTES


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SUPPLEMENTARY MATERIALS
www.sciencemag.org/content/358/6362/518/suppl/DC1
Materials and Methods
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Plasmodium parasite entrance and exit
Sweats and fever are the hallmarks of malaria. Red blood cells are the replication factories for malaria parasites. Fever occurs when the parasites' merozoite stages burst en masse from red blood cells into the circulation. Nasamu et al. and Pino et al. discovered that two parasite proteases, plasmepsin IX and X, are essential for mass cell exit (see the Perspective by Boddey). Plasmepsin X is also used by the merozoites to enter a fresh red blood cell to continue the replicative cycle. These two plasmepsins act by regulating the maturation of enzymes required to disrupt host cell membranes. Because these functions are essential for the parasite, the authors used protease inhibitors to show that plasmepsins provide potential drug targets.

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