Calcium-dependent phosphorylation of Plasmodium falciparum serine repeat antigen-5 triggers merozoite egress

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Keywords: Plasmodium falciparum, SERA5, CDPK1, merozoite egress

ABSTRACT

The human malaria parasite Plasmodium falciparum proliferates in red blood cells following repeated cycles of invasion, multiplication and egress. P. falciparum Serine Repeat Antigen-5 (PfSERA5), a putative serine protease, plays an important role in merozoite egress. However, regulation of its activity leading to merozoite egress is poorly understood. In the present study, we show that PfSERA5 undergoes phosphorylation prior to merozoite egress. Immunoprecipitation of parasite lysates using anti-PfSERA5 sera followed by mass spectrometry analysis identified Calcium Dependent Protein Kinase-1 (PfCDPK1) as an interacting kinase. Association of PfSERA5 with PfCDPK1 was corroborated by cosedimentation, co-immunoprecipitation and co-immunolocalization analysis. Interestingly, PfCDPK1 phosphorylated PfSERA5 in vitro in presence of calcium (Ca^{2+}) and enhanced its proteolytic activity. A PfCDPK1 inhibitor, purfalcamine, blocked the phosphorylation and activation of PfSERA5, both in vitro as well as in schizonts, which in turn blocked merozoite egress. Together, these results suggest that phosphorylation of PfSERA5 by PfCDPK1 following a rise in cytosolic Ca^{2+} levels activates its proteolytic activity to trigger merozoite egress.

Despite continued efforts to control malaria; it still remains a major public health problem in the tropical world. The development and spread of artemisinin resistant parasites in recent years has raised serious concerns with regards to the availability of effective tools to eliminate malaria. Sustained efforts are thus required to understand parasite biology, identify novel
drug targets and develop new therapeutic interventions against malaria. The clinical manifestations of malaria are largely attributed to its asexual stages, during which merozoites repeatedly invade into, multiply within and exit from red blood cells (RBCs) for their propagation. The exit of invasive merozoites from infected erythrocytes, a process referred to as parasite egress, is a critical step in blood stage parasite growth. A cascade of proteolytic activities has been implicated in the release of merozoites from mature schizonts. However, understanding the regulation of the activity of such proteases leading to egress still remains elusive\(^3\)–\(^7\).

A number of putative \textit{Plasmodium} proteases such as cysteine proteases [falcipain-2, SERA proteins, dipeptidyl aminopeptidase 3 (DPAP 3)]; aspartic protease (plasmepsin-II) and serine protease [subtilisin-like protease 1 (SUB1)] are involved in merozoite egress from \textit{P. falciparum} schizonts\(^8\)–\(^12\). Of particular interest are members of the SERA family of putative proteases that possess a central papain-like protease domain\(^13\). \textit{P. falciparum} encodes nine SERA proteins, SERA 1–9\(^14\). Among these, SERA 1–5 and SERA 9, have a serine residue in place of the conventional cysteine residue at the active site, while three SERA proteins; SERAs 6–8 have cysteine at the key catalytic position\(^15,16\). Such substitutions at the catalytic sites of enzyme homologues are well known in protozoan and metazoan proteases some of which play key regulatory roles\(^17\). SERAs are proteins of ~100-130 kDa size and are mainly localized in the parasitophorous vacuole\(^13\). Many members of the PfSERA family are proteolytically processed by PfSUB1\(^11\). Of the nine members of the PfSERA family, PfSERA5 and PfSERA6 are refractory to genetic deletion, thereby suggesting that they are essential for parasite growth and viability\(^18,19\). PfSERA5 is the best studied among PfSERA family members and is a promising vaccine candidate as well as drug target\(^20,21\). Inhibitors of PfSUB1, an enzyme involved in PfSERA5 processing, stalls merozoite egress and parasite maturation, thereby suggesting that PfSERA5 and its processing enzyme PfSUB1 are important drug targets\(^11,12\). Although PfSERA5 is highly expressed at the late schizont stage, its proteolytic activity has been an issue of debate because of low proteolytic activity displayed by recombinant PfSERA5 expressed in \textit{Escherichia coli}\(^13,22\). Few recent studies have suggested PfSERA5 serves as a pseudoprotease that regulates the kinetics/efficiency of \textit{P. falciparum} merozoite egress from mature schizonts\(^23,24\).

Post-translational modification (PTM) of proteins such as phosphorylation/dephosphorylation, palmitoylation, ubiquitination and sumoylation are fast emerging as important mechanisms for the regulation of functional activity in various organisms. Cross-talk between these PTMs may also play a role in regulating parasite development\(^25\). However, little is known about the parasite proteases that undergo PTMs and about the role of PTMs in regulating their protease activities. In most organisms including malaria parasites, of the many possible PTMs, the role of phosphorylation in regulation of the cell cycle has been studied most extensively\(^26–28\). Given that PfSERA5 is essential for parasite survival, we explored the potential role of post-translational modification, especially the role of phosphorylation of PfSERA5, in regulation of its protease and merozoite egress activities. Immuno-precipitation using anti-phosphoserine antibodies followed by mass spectrometry analysis showed that PfSERA5 is phosphorylated at schizont stage. We
identified PfCDPK1 as an interacting kinase that phosphorylates PfSERA5. Purfalcamine, a known chemical inhibitor of PfCDPK1, inhibited the native protease activity at schizont stage and also blocked merozoite egress. These results provide an insight into the regulation of PfSERA5 protease activity and its link with merozoite egress.

RESULTS

PfSERA5 exists in the phosphorylated form at schizont stages of P. falciparum. Protein phosphorylation, in most eukaryotes, including Plasmodium spp., is one of the major regulatory processes that controls functional activities of diverse proteins to modulate cellular processes. PfSERA5 expression in P. falciparum is tightly regulated so that it is primarily expressed at the schizont stage. In order to determine if phosphorylation plays a role in the regulation of PfSERA5 activity, we investigated the phosphorylation status of PfSERA5 in P. falciparum by immunoprecipitation using anti-phosphoserine antibodies coupled to agarose. When eluates were probed using mouse antisera raised against PfSERA5 by Western blotting, a ~50 kDa band corresponding to the processed form of PfSERA5 was detected (Figure 1A). When immunoprecipitates generated from P. falciparum schizont lysates with anti-phosphoserine antibodies were digested with trypsin and analyzed by tandem mass spectrometry (LC-MS/MS), peptides corresponding to a number of parasite proteins including PfSERA5 were detected (Supplementary Table 1). A peptide corresponding to PfSERA5 sequence was identified with high confidence (Figure 1B). To identify the phosphorylation sites in PfSERA5, antibodies raised against PfSERA5 were used for immunoprecipitation from P. falciparum schizont lysates and the resulting immunoprecipitates were subjected to phosphopeptide analysis by LC-MS/MS. As shown in Figure 1C, three phosphopeptides corresponding to PfSERA5 were detected with phosphorylation on S-183, T-549 and S-866 (spectra for these peptides are shown in Supplementary Figure 1). Together, these results suggest that PfSERA5 exists in the phosphorylated state at schizont stage.

PfCDPK1 associates with PfSERA5 in the parasitophorous vacuole (PV) of P. falciparum schizonts. Given that PfSERA5 is phosphorylated in late schizonts, next we attempted to identify the kinase(s) that may associate with and phosphorylate PfSERA5. LC-MS/MS analysis of immunoprecipitates from P. falciparum schizont extract using anti-PfSERA5 sera revealed several kinases associated with PfSERA5 (Supplementary Table 2A). Among these kinases, PfCDPK1 is localized to the parasitophorous vacuole (PV) of P. falciparum-infected erythrocytes similar to the localization observed for PfSERA5. Co-localization studies using immunofluorescence assays confirmed co-localization of PfSERA5 and PfCDPK1 in the PV of schizonts (Figure 2A). Significant co-localization with a Pearson’s correlation coefficient of >0.85 was observed between PfCDPK1 and PfSERA5 (Figure 2A).

Sedimentation analysis of P. falciparum schizont lysates using glycerol density gradient centrifugation was performed to ascertain if PfSERA5 and PfCDPK1 are present together in the same fraction(s). Western blotting using antibodies raised against PfSERA5 and PfCDPK1 for sixteen fractions and LC-MS/MS analysis showed that both proteins co-sediment together in fractions.
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5 to 8 (Figure 2B and Supplementary Table 2B). Together, these results indicated a possible association between PfSER5 and PfCDPK1.

To confirm the interaction between PfSER5 and PfCDPK1, immunoprecipitation analysis was performed with *P. falciparum* schizont lysates using either anti-PfSER5 mouse sera or anti-PfCDPK1 rat sera. Western blotting of immunoprecipitates of parasite lysate generated with anti-PfSER5 mouse sera showed presence of PfCDPK1 (Figure 3A). Similarly, immunoprecipitates generated with anti-PfCDPK1 rat sera from *P. falciparum* schizont lysates showed the presence of full length and proteolytically processed fragments of PfSER5 as detected by Western blotting (Figure 3A). Analysis by mass spectrometry further confirmed the presence of PfCDPK1 and PfSER5 in the immunoprecipitates of *P. falciparum* lysate generated using anti-PfCDPK1 sera (Figure 3B). The list of unique peptides of both proteins identified by LC-MS/MS analysis is summarized in Figure 3B. A detailed list of all proteins identified in the immunoprecipitates is shown in Supplementary Table 3. These results unequivocally suggest that PfCDPK1 interacts with PfSER5 at late schizont stage. Further the recombinant PfCDPK1 and recombinant protease domain of PfSER5 (PfSER550) were examined to interact with each other in ELISA based binding assay. We found that indeed both the recombinant proteins interact with each other in vitro (Supplementary Figure 2).

**PfCDPK1 phosphorylates PfSER5 and enhances its protease activity.** Given that PfSER5 and PfCDPK1 co-localize in the PV, we tested if PfCDPK1 can phosphorylate PfSER5 in vitro. Recombinant PfCDPK1 was incubated with the recombinant protease domain of PfSER5 (PfSER550) in the presence of $^{32}$P-ATP-γ with or without Ca$^{2+}$. Recombinant PfCDPK1 phosphorylated PfSER550 in presence of Ca$^{2+}$ (Figure 4A). Phosphorylation of PfSER550 by PfCDPK1 was inhibited by purfalcamine, a known inhibitor of PfCDPK1 (Figure 4A). Subsequently, the phosphorylation site(s) were mapped on in vitro phosphorylated PfSER550 by mass spectrometry. This analysis identified seven phosphorylated peptides corresponding to PfSER5, each containing a single phosphorylated Ser/Thr residue (Figure 4B). Among these phosphorylation sites, phosphorylation at T-549 was also identified in PfSER5 immunoprecipitated from parasite extract using anti PfSER5 sera as shown in Figure 1C.

Next, we evaluated the possible role of phosphorylation in modulation of protease activity of PfSER5. Recombinant PfSER550 has been shown to cleave LLVY-AMC, a synthetic fluorescent peptide substrate$^{13,22}$. Using LLVY-AMC as a substrate, we analyzed the activity of recombinant PfSER550 after incubation with recombinant PfCDPK1 in the presence or absence of Ca$^{2+}$. As shown in Figure 4C, phosphorylated PfSER550 showed a considerable increase in protease activity following incubation with PfCDPK1 in the presence of Ca$^{2+}$ in comparison to non-phosphorylated PfSER550. Addition of purfalcamine or EGTA inhibited the activation of PfSER5 protease activity (Figure 4C), thereby suggesting that PfCDPK1 phosphorylates PfSER5 and modulates its activity.

**PfCDPK1 activity regulates PfSER5 function in *P. falciparum* schizonts to trigger egress.** Purfalcamine, a PfCDPK1 inhibitor or a
specific PfSERA5 inhibitory peptide, SE5 P2\textsuperscript{22} were next examined to probe the possible role of PfCDPK1 and PfSERA5 in merozoites egress. We have previously shown that SE5 P2, a peptide from the C-terminal catalytic domain of PfSERA5 inhibits its activity\textsuperscript{22}. To analyze the activity of PfSERAs especially PfSERA5, \textit{P. falciparum} schizonts were incubated with the fluorogenic substrate, LLVY-AMC. These assays were performed in the presence or absence of either purfalcamine or SE5 P2 peptide. In the past, we have successfully used similar assay to analyze the activity of cysteine protease(s) in the intact parasitized erythrocytes using a fluorogenic small peptide substrate, Z-FR-AMC\textsuperscript{31}. As shown in Figure 5A, blue fluorescence resulting from the cleavage of LLVY-AMC was detected in the control schizonts. Purfalcamine or SE5 P2 treated parasites showed little or no fluorescence signal, indicating a block in serine protease(s) mediated proteolytic activity (Figure 5B). Next, the biological effect of purfalcamine on parasite egress was evaluated. \textit{P. falciparum} schizonts treated with different concentrations of PfCDPK1 inhibitor, purfalcamine, were assayed for egress ~20h post treatment using flow cytometry. A dose dependent block in merozoite egress was observed after purfalcamine treatment (Figure 6). Similar block was also observed after BAPTA-AM treatment (Figure 6). To know whether this block in merozoite egress is due to inhibition of phosphorylation of PfSERA5, we tested the phosphorylation status of PfSERA5 in purfalcamine treated parasite extracts. As shown in Figure 6 (inset), purfalcamine significantly blocked the phosphorylation of PfSERA5. Together, these results show that inhibition of PfCDPK1 activity or PfSERA5 phosphorylation produces a block in merozoite egress, suggesting that these two proteins are critical players for merozoite egress.

**DISCUSSION**

A role for the members of PfSERA family of putative proteases in merozoite egress from infected erythrocytes and sporozoite egress from oocysts has been suggested\textsuperscript{11,32}. However, relatively weak \textit{in vitro} catalytic activity and differences in crystal structure of PfSERA5 compared to papain-like cysteine proteases has raised questions about the role of PfSERA5 as an actual protease during the egress process\textsuperscript{13,23}. A couple of genetic studies have recently described PfSERA5 as a pseudoprotease that is essential for merozoites egress from human erythrocytes although its functional role is unclear\textsuperscript{23,24}. However, role of post translational modifications in regulating protease activity of SERA5 has never been addressed. It is well known that phosphorylation by protein kinases can regulate protease activities; a number of human metallo-proteases, cysteine proteases and threonine proteases have been shown to be activated/deactivated by kinases\textsuperscript{33}. To find out whether post translation modifications of proteases or kinases in apicomplexan parasites play a role in regulating biological processes, we investigated the phosphorylation status of PfSERA5 and its role in \textit{Plasmodium} merozoite egress from infected erythrocytes.

To determine whether PfSERA5 gets phosphorylated, \textit{P. falciparum} schizont extracts were immunoprecipitated using anti-phosphoserine antibodies. Western blotting was performed using anti-PfSERA5 sera, which identified a specific band of PfSERA5 in immunoprecipitates. Mass spectrometry
analysis of these immunoprecipitates and phosho-peptide analysis of immunoprecipitates made from *P. falciparum* extracts using anti-PfSERA5 sera identified phosphorylation sites within PfSERA5, thus confirming the phosphorylation status of PfSERA5 at schizont stage. These results are in line with previous reports where 7 phosphorylation sites have been described within PfSERA5 at asexual blood stages. We next looked for the kinase(s) that interact with and phosphorylate PfSERA5. Since most of the SERAs are localized in PV, we focused on PfCDPK1, a kinase identified in the co-immunoprecipitates that is localized in the PV. A number of experimental strategies including density gradient sedimentation, co-localization by immunofluorescence assays and co-immuno-precipitation studies with *P. falciparum* schizont extracts unequivocally suggested an interaction between PfSERA5 and PfCDPK1. The ability of PfCDPK1 to phosphorylate PfSERA5 was tested *in vitro* in the presence of Ca$^{2+}$. One of the phosphorylation sites on PfSERA5 following *in vitro* phosphorylation by PfCDPK1 was common to the phosphorylation sites found on PfSERA5 in the parasite at late schizont stage. Purvalcamine, a known PfCDPK1 inhibitor, blocked the phosphorylation of recombinant PfSERA5 by recombinant PfCDPK1 *in vitro* as well as phosphorylation of PfSERA5 *in vivo* in schizonts.

Having demonstrated that PfSERA5 exists in phosphorylated form at late schizont stage and that PfCDPK1 is possibly involved in PfSERA5 phosphorylation, we next examined the significance of PfSERA5 phosphorylation and consequence of blocking this phosphorylation using PfCDPK1 inhibitor. Phosphorylation of PfSERA5 by PfCDPK1 *in vitro* greatly enhanced proteolytic activity of PfSERA5 as measured using the fluorescent peptide substrate LLVY-AMC (Figure 4). Purvalcamine blocked phosphorylation of PfSERA5 by PfCDPK1 and inhibited rise in proteolytic activity of PfSERA5 (Figure 4). We also examined these activities *in vivo* in presence of purvalcamine and a PfSERA5 specific peptide that has previously been shown to inhibit PfSERA5 activity *in vitro* as well as in cultured parasites. Fluorescence measurements of parasites incubated with LLVY-AMC substrate and treated with purvalcamine or PfSERA5 inhibitory peptide SE5 P2, showed considerable drop in fluorescence signal from AMC release in comparison to untreated parasites. These results thus demonstrated that purvalcamine or SE5 P2 peptide blocked the PfSERA5 activity *in vivo*, thereby confirming the need for PfSERA5 and its phosphorylation by PfCDPK1 for activation of its protease activity. We further analyzed the effect of purvalcamine on parasite development especially in merozoite egress, as a role for PfSERAs, in particular PfSERA5 has earlier been suggested in merozoite egress. Incubation of schizont stage parasites with purvalcamine blocked merozite egress, thereby demonstrating that both PfCDPK1 and PfSERA5 activities are required for merozoite egress from infected erythrocytes. Further we found that phosphorylation of PfSERA5 was blocked in purvalcamine treated schizont extracts (Figure 6, inset). Overall these results demonstrate that PfCDPK1 phosphorylates PfSERA5 following rise in Ca$^{2+}$ in mature schizonts and this phosphorylation step activates proteolytic activity of PfSERA5 to trigger merozoite egress. Since Collins et al have proposed a role of PfSERA5 in timely rupture of invasive merozoites, we speculate that its phosphorylation is a key regulator in the later stages of egress, which needs to be explored further. The process of egress being a complicated and multiproteolytic cascade of
SERA5 phosphorylation and merozoite egress events; multiple phosphorylation events are expected to be taking place involving multiple kinases and their substrates. A role for PfCDPK1 and PfCDPK5 in merozoite egress has been suggested earlier. PfCDPK1 has also been shown to be essential for merozoite invasion and plays a role in the phosphorylation of PfMTIP and PfGAP45, components of the glideosome complex, which provides gliding motility to the merozoite during invasion. These data sets suggest that multiple kinases and proteases participate in the processes of egress and invasion during blood stage growth of malaria parasites.

In conclusion, results of the present study demonstrate that PfSERA5 is phosphorylated in *P. falciparum* schizonts by PfCDPK1 following a rise in intracellular Ca\(^{2+}\), which has been previously shown to trigger egress. Phosphorylation enhances the protease activity of PfSERA5. Although this is one of the first reports to show that the activity of a parasite protease can be regulated by a kinase, many human proteases have been shown to be regulated by different kinases. Evidence discussed above thus clearly provides data about a link between PfSERA5 protease activity and its phosphorylation status, which is essential for the timely egress of merozoites from infected erythrocytes. Understanding the kinase-protease crosstalk may lead to the introduction of combined therapies targeting specific components of both families as part of the new generation of anti-malarial therapies.

**EXPERIMENTAL PROCEDURES**

*In vitro parasite culture.* *P. falciparum* 3D7 was cultured in 4% hematocrit with RPMI 1640 with L-glutamine (Invitrogen) supplemented with 0.5% Albumax I (Invitrogen), 2.1mg ml\(^{-1}\) sodium bicarbonate (Sigma-Aldrich) and 25µg ml\(^{-1}\) gentamycin reagent solution (Invitrogen) according to previously described protocol. Parasite cultures were maintained in a mixed gas environment (5% O\(_2\), 5% CO\(_2\), 90% N\(_2\)) at 37°C. Parasites were synchronized by two consecutive sorbitol treatments 4 hours apart at early ring stage and allowed to mature till schizonts [44-46 hours post invasion (hpi)] for further experiments.

Cloning, expression and purification of PfSERA5 and generation of PfSERA5 antiserum. Plasmid expressing the catalytically active region of PfSERA5 (~50 kDa domain) was obtained from Anthony N Hodder, WEHI, Australia. Protein was expressed and purified by a previously described protocol. Polyclonal antibodies against recombinant PfSERA5 were generated in mice and used for further experiments.

Glycerol density gradient centrifugation. Late schizonts (44-46 hpi) from a *P. falciparum* 3D7 culture were lysed in 0.5% NP-40 in HEPES-buffered saline (10 mM HEPES, 2 mM MgCl\(_2\), 10 mM KCl, 0.5 mM EDTA, 150 mM NaCl, pH 7.0) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics Corporation) for 15-20 min at 4°C. Lysates were cleared by centrifugation at 13,000 rpm for 10 min and ~500 µl of lysate was layered on top of an 11-ml ultracentrifuge tube containing 45-50% glycerol density gradient. Tubes were centrifuged at 38,000 rpm for 19 h at 4°C in an SW41 rotor (Beckman Coulter Life Sciences). The molecular mass standards bovine serum albumin (66 kDa), aldolase, catalase (220 kDa), ferritin (440 kDa) and thyroglobulin (660 kDa) were also prepared in the same
buffer and run in parallel. Twenty fractions of 500 μl were collected from each gradient, and equal volumes of each fraction were mixed with Laemmli buffer and analyzed by Western Blotting with antibodies against PfSERA5 and PfCDPK1.

Indirect immunofluorescence assay. Synchronized *P. falciparum* 3D7 cultures at late schizont stage (44-46 hpi) were smeared on glass slides, air-dried and fixed in chilled absolute methanol for 30 min. Non-specific binding sites on the cells were blocked in 3% BSA prepared in PBS. Smears were probed with anti-PfSERA5 mouse sera and anti-PfCDPK1 rat sera at dilutions of 1:50 and 1:100 respectively for 1 hour at 37°C. Slides were then washed once with PBST (PBS + 0.02% Tween-20) and three times with PBS. Smears were probed with Alexa Fluor 594-conjugated goat anti-mouse IgG antibody (Invitrogen) and Alexa Fluor 488-conjugated donkey anti-rat IgG antibody (Invitrogen) at dilutions of 1:500 and 1:200 respectively for 1 hour at 37°C. All antibodies were diluted in 1% BSA prepared in PBS. Slides were washed, mounted and sealed. Nuclear DNA was visualized with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were acquired using a Nikon A1R Confocal microscope using the NIS Elements software.

Co-immunoprecipitation. Immuno-precipitation experiments were performed using the Pierce Crosslink Immunoprecipitation Kit (Product # 26147; Pierce Biotechnology Inc., Rockford, IL) as per the instructions provided by the manufacturer. Briefly, erythrocytes infected with synchronized *P. falciparum* 3D7 late schizonts (44-46 hpi) were pelleted at 1000g for 5 min, treated with 0.15% saponin prepared in PBS for 10 min at 40°C and centrifuged at 2500xg for 20 min. The parasite pellet obtained was washed 2-3 times with PBS by centrifugation at 15000xg for 1 min. The parasites were subsequently lysed using Lysis buffer (250 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol; pH 7.4) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics) for 15-20 min at 40°C with intermittent mixing. Lysates were clarified by centrifugation at 15000g for 30 min. The protein concentration of the supernatants was determined by the BCA protein estimation assay kit (Pierce Biotechnology Inc., Rockford, IL). Approximately 1mg of total protein was incubated overnight at 4°C with about 10μg of anti-PfSERA5 and/or anti-PfCDPK1 sera cross-linked to 10μl of Protein A/G-agarose resin using disuccinimidyl suberate (DSS) as cross-linker with constant mixing. An equal amount of protein was allowed to bind to the resin conjugated to normal mouse or rat sera as a control. Following binding, resins were washed with Wash Buffer and bound proteins were eluted using the Elution Buffer (Tris-Glycine pH 2.8). The eluates were separated on SDS-PAGE and analyzed by Western blotting using antisera against PfSERA5 and PfCDPK1.

Immunoprecipitation assay for the identification of phosphorylated PfSERA5 in schizont extracts. For the detection of native phosphorylated PfSERA5, cell lysates prepared from late schizonts (44-46 hpi) of *P. falciparum* 3D7 strain (as mentioned earlier) were incubated with mouse monoclonal anti-phosphoserine antibodies coupled to agarose (Product # A8076; Sigma-Aldrich) for 12-15 hours (or overnight) at 4°C to allow binding. About 2 mg of total cellular protein was used for binding to either anti-phosphoserine agarose or control agarose beads. The bound phospho-proteins were eluted with Tris-Glycine buffer, pH 2.8 and analyzed by
Western blotting using anti-PfSERA5 mouse sera. The eluates were also digested with trypsin for identification of proteins by mass spectrometry as described below.

Immunoprecipitation using anti-phosphoserine agarose was also performed using schizont (44-46 hpi) lysates treated with DMSO or pafalcamine. The eluates were separated by SDS-PAGE and transferred on to PVDF membrane. The membranes were probed with anti-PfSERA5 sera.

Protein digestion and identification by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Proteins in the immunoprecipitated samples were proteolysed by in-solution trypsin digestion. Samples were brought to a final volume of 100 μl in 50 mM ammonium bicarbonate buffer to adjust the pH to 8.0, reduced with 10 mM DTT (final concentration) for 1h at RT and alkylated with 40 mM iodoacetamide (Sigma-Aldrich) for 1h at RT in the dark. Proteins were digested by the addition of trypsin-Gold (Promega) at a ratio 1:50 (w/w) of trypsin:protein. For complete digestion, samples were placed in a water-bath at 37°C for 18 hours. The digestion was stopped by acidification using formic acid (0.1% final concentration). The peptide mixtures were concentrated in a speed-vac and analyzed by LC-MS/MS.

Enrichment of phosphopeptides. For phosphorylation studies of PfSERA5 by LC-MS/MS (with both recombinant PfSERA5 and PfSERA5 from parasite lysates), the trypsin-digested peptide mixtures were first enriched for phospho-peptides before mass spectrometric analysis. Phosphopeptides from the digested peptide pool were enriched using immobilized metal affinity chromatography (IMAC) as described by Villen and Gygi. Briefly, vacuum-dried peptides were redissolved in 120 μl of IMAC binding buffer (40% acetonitrile and 25 mM formic acid) and allowed to bind to 10 μl of pre-equilibrated Fe-NTA IMAC beads (Sigma-Aldrich). The peptides were incubated for 1 hour at room temperature with constant mixing. Following the incubation, the flow-through containing non-phosphorylated peptides was collected separately. The resin was washed three times with 120 μl IMAC binding buffer. The bound phosphopeptides were eluted three times with 40 μl IMAC elution buffer (50 mM K2HPO4 in NH4OH, pH 10.0). The eluates were pooled and the pH was neutralized with 40 μl 10% formic Acid. The flow through and eluates were vacuum dried, desalted and analyzed by LC-MS/MS.

Mass spectrometry analysis was performed on an Orbitrap Velos Pro MS coupled to Easy n-LC 1000 (Thermo Fisher Scientific). Tryptic digested peptide mixtures were loaded on to a reverse phase C-18 pre-column (Acclaim PepMap, 75 μm x 2 cm, 3μm, 100A°, Thermo Fisher Scientific), which was in line with an analytical column (Acclaim PepMap, 50μm x 15 cm, 2μm, 100A°). The peptides were separated using a gradient of 5% solvent B (0.1% formic acid in 95/5 acetonitrile/water) to 50% solvent B in 60 min. For recombinant proteins, a relatively smaller gradient was used. The eluted peptides were injected into the Orbitrap Velos Pro MS and MS1 data were acquired using mass range from 350-2000 Da in Full scan mode at 60000 resolution. Top 20 precursors were allowed to fragment using CID (collision induced dissociation) in Ion trap with collision energy of 35. For phosphopeptide analysis, precursors were fragmented using high-energy collision dissociation (HCD) and detected in Orbitrap at a resolution of 7500. The raw data was analyzed using Proteome Discoverer 1.4 (PD 1.4, Thermo Fisher Scientific) with SEQUEST algorithm against P. falciparum database.
downloaded from PlasmoDB and Uniprot. The identification of peptides was validated using Percolator. The sites of phosphorylation were scored using PhosphoRS 3.0.

**In vitro kinase assay.** Phosphorylation assays were performed as previously described. Briefly, recombinant PfCDPK1 (40 nM) was allowed to phosphorylate 1 μg of recombinant PfSERA5 in a kinase assay buffer (50 mM MgCl₂, 50 mM Tris, pH 8.0, 20 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM dithiothreitol (DTT), and 2.5 mM CaCl₂) for 1 hour at 30ºC using radioactive [γ-³²P]ATP as the source of phosphate group. Syntide-2 was used as a control substrate for PfCDPK1. The reaction was stopped by the addition 4X SDS-PAGE Laemmli buffer. The reaction mixture was separated on a polyacrylamide gel and phosphate incorporation was detected using Storage Phosphor Screen (GE Healthcare, USA). Purfalcamine, a known inhibitor of PfCDPK1, was used to confirm the role of PfCDPK1 in phosphorylating PfSERA5.

**Protease activity assay with recombinant PfSERA5 following phosphorylation by recombinant PfCDPK1.** To evaluate the effect of PfCDPK1-mediated phosphorylation on the proteolytic activity of recombinant PfSERA5, 1 μg of PfSERA5 was incubated with recombinant PfCDPK1 (40 nM) in a kinase assay buffer in 50 μl reaction volume, as described previously. Here, the radioactive [γ-³²P] ATP was replaced with non-radioactive ATP as the source of phosphate group. Reaction was allowed to proceed for 1 hour at 30ºC. The reaction mixture was diluted to 400 μl in PfSERA5 activity assay buffer (0.1M NaHCO₃, pH 7.5, 5 mM CaCl₂, and 0.1% Tween-20) and incubated with 20 μM LLVY-AMC [7-amino-4-methyl coumarin] (Sigma-Alrich) at room temperature. Released AMC was detected and measured using a LS50B Perkin-Elmer fluorimeter (excitation 355nm; emission 460 nm) after incubation of SE5 P2 with mature schizonts for 2 hours at 37ºC.

**Merozoite Egress Inhibition Assay.** To determine the effect of inhibition of PfSERA5 phosphorylation by inhibiting PfCDPK1 on *P. falciparum* merozoite egress, egress inhibition assays were performed as previously described. Briefly, late stage schizonts (42-44 hpi) were treated with different concentrations of purfalcamine (0.5–10 μM),
50 μM of BAPTA-AM and appropriate solvent controls for 8-10 hours in duplicate wells. Following incubation, parasites from treated and control wells were collected, washed, stained with ethidium bromide (10 mg/ml) and EtBr positive cells were scored by flow cytometry on FACS-Calibur (Becton Dickinson & Co) using CellQuest software. Fluorescence signal (FL-2) was detected with 90 nM band pass filter using an excitation laser of 488 nm collecting typically 100,000 cells per sample. Post-acquisition data were analyzed using FlowJo software (Tree Star, Inc.) by determining the proportion of FL-2-positive cells representing schizonts. Inhibition in egress of merozoites was calculated as, % Inhibition of merozoite egress = 100 [(T – C)/(I – C)], where I: frequency of schizonts (%) at the start of the assay; T: frequency of schizonts (%) after treatment; C: frequency of schiz(%) in the control.

Sequences of SE5 P2 and P3 peptides

SE5 P2: Ac-KASPEFYHNLYFKNF-NH2
SE5 P3: Ac-NVGKKNLFSEKEDN-NH2

Acknowledgments: We thank Dr. Brendan Crabb & Dr Anthony N. Hodder for kindly providing the PfSERA5P50 expression clone. We also thank Rotary Blood Bank, for providing hRBCs, and Surbhi Dabral for help with confocal microscopy. This work was supported by financial support from Department of Biotechnology (DBT), Govt. of India (BT/01/CEIB/11/V/01). ES is supported by DBT Junior Research Fellowship and AB was supported by CSIR research fellowship. AM and IK are recipients of National Bioscience Award for Career Development [BT/HRD/NBA/34/01/2011(v)] and Innovative Young Biotechnologist Award [BT/010/IYBA/2016/03] respectively from DBT, Govt. of India.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: PM conceptualized and designed the study and wrote the manuscript. CC helped design experiments, contributed reagents and helped write the manuscript. AM critically evaluated the study throughout and also helped in designing the experiments. GRI, IK and SS performed most of the experiments and also contributed in writing. SA and GRI performed the merozoite egress assay; GK generated the recombinant PfSERA5 protein, AB and MAS produced recombinant PfCDPK1, while ES helped in proteomic studies. GP performed the interaction assay.

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FIGURE LEGENDS

Figure 1: PfSERA5 is phosphorylated at schizont stage in *P. falciparum*. (A) Representative immunoblot showing a band of ~50 kDa in eluates of affinity pull down from *P. falciparum* schizont extracts using anti-phosphoserine antibody when probed with anti-PfSERA5 mouse sera. (B) Identification of PfSERA5 from the LC-MS/MS analysis of above mentioned immunoprecipitates. A list of other proteins identified by this pull down is summarized in Supplementary Table 1. (C) Table describes the sites of phosphorylation in PfSERA5 as identified by LC-MS/MS analysis after immunoprecipitation using anti-PfSERA5 sera from *P. falciparum* schizonts extracts.

Figure 2: PfSERA5 and PfCDPK1 co-localize in parasitophorous vacuole (PV) of *P. falciparum* schizonts. (A) Confocal microscopy analysis of *P. falciparum* schizonts demonstrates that PfSERA5 and PfCDPK1 co-localize in the PV. (B) Western blotting shows that PfSERA5 and PfCDPK1 are concentrated in fractions 6-8 when the protein extract of schizont stage parasites was subjected to ultra-centrifugation on a glycerol density gradient. This indicates that both these proteins may be a part of a complex within the parasite.

Figure 3: PfSERA5 is associated with PfCDPK1 at schizont stage of *P. falciparum*. (A) Representative immunoblots show an interaction between PfSERA5 and PfCDPK1 in the parasite. Protein extracts of mature schizonts were independently used for immune-precipitation using anti-PfSERA5 sera and anti-PfCDPK1 sera, eluates were separated on SDS-PAGE and used for Western blotting with anti-CDPK1 and anti-PfSERA5 sera respectively. (B) The same eluates were trypsin digested and analyzed by LC-MS/MS. Table shows the sequences of the unique peptides corresponding to PfCDPK1 and PfSERA5 identified by mass spectrometric analysis of the immuno-precipitate using anti-PfCDPK1 sera. Detailed list of all the proteins identified in this analysis is summarized in Supplementary Table 3.

Figure 4: PfCDPK1 phosphorylates and enhances the enzymatic activity of recombinant PfSERA5\textsubscript{50}. Recombinant PfCDPK1 phosphorylates PfSERA5\textsubscript{50} in vitro. (A) An autoradiograph showing the incorporation of radio-labeled phosphate group in recombinant PfSERA5\textsubscript{50}. (B) Table describes the sites of phosphorylation in PfSERA5\textsubscript{50} as identified by LC-MS/MS analysis. The phosphorylation of T-549 is the common site, which was also detected in LC-MS/MS analysis following immunoprecipitation with anti-PfSERA5 sera from schizont-stage protein extracts (Figure 1C). (C) Pre-incubation of recombinant PfCDPK1 with recombinant PfSERA5\textsubscript{50} significantly amplifies its protease activity as measured by the hydrolysis of fluorogenic synthetic peptide substrate; LLVY-AMC. The fluorescence signal was completely abrogated by the addition of inhibitors of PfCDPK1, pufalcamine or EGTA.

Figure 5: Pufalcamine and SE5 P2 peptide block protease activity. (A, i) A synthetic fluorescent peptide substrate LLVY-AMC was added to *P. falciparum* cultures at early schizonts
stage and its hydrolysis was visualized by blue fluorescence. (A, ii) The fluorescence signal was inhibited by the addition of PfSERA5 inhibitory peptide, SE5 P2 (500 µM) and this change was quantified spectrophotometrically. (B, i & ii) The hydrolysis of the synthetic substrate peptide was also inhibited by purfalcamine, a known inhibitor of PfCDPK1. This inhibition was captured and quantified using confocal microscopy. Together these results indicate a possible regulation of PfSERA5 activity by phosphorylation.

**Figure 6: Effect of PfCDPK1 inhibitor purfalcamine on merozoites egress.** Schizont stage parasites (40-42 hpi) were treated with DMSO control, purfalcamine (500 nm, 1 µM, 10 µM) or, BAPTA-AM at 50 µM in duplicates and assayed for egress by flow cytometer. Data from two independent experiments (mean SE) showed dose-dependent effect of purfalcamine on parasite egress. Egress in DMSO control was considered 100% for all experiments. The representative FACS raw data file shows loss of schizonts in DMSO control whereas presence of stalled schizonts is seen in presence of purfalcamine at 10 µM concentration. Inset: Purfalcamine treatment abrogated the phosphorylation of PfSERA5 in schizonts stage parasites. Western blot using anti-PfSERA5 sera shows a band at ~50 kDa in DMSO treated schizonts extracts after pull down using anti-phosphoserine agarose while no such band was visible in purfalcamine treated parasite extracts.
FIGURE 1

(A) - Phosphorylation levels of SERA5 protein. 
(B) - Table showing phosphorylation scores and unique peptides. 
(C) - Peptide sequences and modifications identified in SERA5 phosphorylation.

Table:

| Peptide Sequence       | Modification | Phosphosite Identified | PhosphoRS Binomial Peptide Score |
|------------------------|--------------|------------------------|---------------------------------|
| LP8NGTTGEQGSSTGTVR     | S3 (Phospho) | S183                   | 222                             |
| NLFSEKEDNENNKK          | S4 (Phospho) | S866                   | 139                             |
| GKGVHVHVDTKE           | T9 (Phospho) | T 549*                 | 32                              |
FIGURE 2

(A)

(B)

Fraction 2 4 6 8 10 12 14 16 WB:

PfCDPK1 (~61 kDa)
PfSER-A5 (~50 kDa)
FIGURE 3

(A) IP: Pre-Bled α-PiCDPK1

(B) IP: Pre-Bled α-PiSERA5

WB: α-PiSERA5

| Accession No.  | Name of the Protein                              | Score | Sequence Coverage (%) | Unique peptides | PSMs | Sequences of Peptides Identified                                                                 |
|----------------|--------------------------------------------------|-------|-----------------------|-----------------|------|------------------------------------------------------------------------------------------------|
| PF3D7_0217500  | Calcium-dependent protein kinase 1 (CDPK1)       | 40.46 | 24.81                 | 11              | 12   | -YFYLVTEFYEGGELFEQIQeR -NSETDLAINPGMYVR -VDGFGLSSFFSK -LG8QAYOEVLLcR -LGTAYYIAPEVLR -LAQAAIHF6G3K -ELIEQYNLR -HSSLNKH -NVREEVNDNILK -TEcGALSNMRE -n8EDLAINPGMYVR -rGSEYFK |
| PF3D7_0207600  | Serine repeat antigen 5 (SERA5)                  | 23.96 | 9.43                  | 06              | 08   | -ESNTALESATSNVNER -TNNAI5FSNSOSLEK -LPSnGTTGEQGSSTGTVR -LPSNGTTGEQGSSTGTVR -cDTLA5mcFLSGmFNEK -ETPTNHLHAYK -KmYIDTQDVNKK |
FIGURE 4

(A) Purvalacmine 

(B) Ca²⁺  

(C) 50 kDa

| Peptide Sequence | Modification | Phosphosite Identified | PhosphoRS Binomial Peptide Score |
|------------------|--------------|------------------------|---------------------------------|
| TNENNDKSELIK     | S8 (Phospho)| S425                   | 237                             |
| GKGVVHDTTLEK     | T9 (Phospho)| T549*                  | 184                             |
| LTESIDNILVK      | S4 (Phospho)| S407                   | 132                             |
| ILHKNPNSLDGK     | S10 (Phospho)| S697                  | 165                             |
| GVHVHDTTLEK      | T7 (Phospho)| T549*                  | 203                             |
| GYTAYSER         | T3 (Phospho)| T704                   | 142                             |
| GSVIAYIK         | S2 (Phospho)| S731                   | 121                             |

PI SERA5: + + + + +  
P1CDPK1: - + + + +  
Ca⁺²: + + - +  
Purvalacmine: - - - +
FIGURE 5

(A) (i) DIC  LLVY-AMC  Merge

(ii)

LLVY-AMC:  -  +  +
SERA5 P2:   -  -  +

(B) (i) Control  Purvalacine

(ii)

Mean Fluorescence Intensity

Control  Purvalacine treated
FIGURE 6

SERA5 phosphorylation and merozoite egress
Calcium-dependent phosphorylation of Plasmodium falciparum serine repeat antigen-5 triggers merozoite egress
Gayatri R. Iyer, Shailja Singh, Inderjeet Kaur, Shalini Agarwal, Mansoor A Siddiqui, Abhisheka Bansal, Gautam Kumar, Ekta Saini, Gourab Paul, Asif Mohmmed, Chetan E. Chitnis and Pawan Malhotra

J. Biol. Chem. published online May 1, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA117.001540

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