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Original Article

A C2 domain containing plasma membrane protein of *Plasmodium falciparum* merozoites mediates calcium-dependent binding and invasion to host erythrocytes

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Abstract *Background:* Invasion of red blood cells by *Plasmodium falciparum* merozoites is governed by multiple receptor–ligand interactions which are critical for bridging the two cells together. The critical function of these ligands for invasion and their direct exposure to the host immune system makes them lucrative vaccine candidates. This necessitates the discovery of new adhesins with less redundancy that mediates the binding of merozoite to the red cell, and furthermore invasion into it. Here we have identified a novel membrane associated antigen (*Pf*C2DMA) that is conserved throughout the *Plasmodium* species and has a membrane targeting C2 domain at its extreme N-terminal region.
Methods: Recombinant $C2_{dom}$ was expressed heterologously in bacteria and purified to homogeneity. Mice antisera against $C2_{dom}$ was raised and used to check the expression and intraparasitic localization of the protein. RBC and Ca^{2+} ion binding activity of $C2_{dom}$ was also checked.
Results: $C2_{dom}$ exhibited specific binding to Ca^{2+} ions and not to Mg^{2+} ions. *Pf*C2DMA localized to the surface of merozoite and recombinant $C2_{dom}$ bound to the surface of human RBCs. RBC receptor modification by treatment with different enzymes showed that binding of $C2_{dom}$ to RBC surface is neuraminidase sensitive. Mice antisera raised against $C2_{dom}$ of *Pf* C2DMA showed invasion inhibitory effects.
Conclusion: Our findings suggest that $C2_{dom}$ of *Pf*C2DMA binds to surface of red cell in a Ca^{2+}-dependent manner, advocating a plausible role in invasion and can serve as a potential novel blood stage vaccine candidate.

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Introduction

The protozoan parasite *Plasmodium falciparum* that causes the most severe form of malaria interacts with a variety of host cells, during its life cycle. The clinically relevant stage of infection is the asexual blood stage during which the parasites repeatedly invade, multiplies within erythrocytes and exits from them to initiate a new round of cycle. The merozoite initially attaches to the RBC membrane and most of the invasion ligands are stored in its apical organelles: micronemes and rhoptries and are secreted at the time of invasion[1,2]. These invasion ligands are classified into two families namely: erythrocyte binding antigens (EBA family) and reticulocyte binding like (RBL family)[2–4] that engages with their cognate receptors on the surface of RBCs and forms a tight junction that bridges the two cells together.

The EBA family of invasion ligands consists of EBA-140, 175, 181, Elb1 that contains cysteine rich regions which binds to red cell glycophorins[5,6] in a neuraminidase sensitive manner. These adhesins are stored in micronemes and secreted at the time of host cell invasion. RBL superfamily consists of the *Plasmodium vivax* reticulocyte binding proteins (*Pv*RBP), and the *P. falciparum* counterpart; reticulocyte binding-like homologous proteins (*Pf*RH). *Pf*RH protein family includes the members *Pf*RH1, *Pf*RH2a, *Pf*RH2b, *Pf*RH3, 4 and 5 which localizes in the neck of rhoptries[3,7–9] and also determine the host cell specificity of *Plasmodium* species. Besides this many novel parasite molecules that are crucial for invasion are being discovered in the recent years that includes MTRAP, PTRAMP,[10] PfMA,[11] GAMA etc.

However, the polymorphic nature and functional redundancy of several merozoite invasion ligands has limited the development of a promising invasion blocking vaccine[12] against *P. falciparum*. Extensive reports of drug resistance in *P. falciparum* and absence of an effective vaccine necessitates the discovery of novel invasion ligands that can serve as novel vaccine candidates. Availability of the transcriptomics[13,14] and proteomics[15] data of *P. falciparum* has enabled us to identify novel parasite ligands that might play a role in binding and invasion of merozoite into host cell. We used these databases to identify novel membrane anchored adhesins and filtered them based on few criteria: (i) Hypothetical proteins which are conserved throughout the species; (ii) Expressed maximally during late stages of intraerythrocytic development (40—48 h post invasion); (iii) Indispensable for parasite growth, as depicted by a low mutagenesis score; and, (iv) Presence of at least one transmembrane region predicted to be localized to parasite plasma membrane.

Using this approach, we identified a gene (PlasmoDB ID: PF3D7_1110100) that encodes for a membrane antigen, hereby denoted as *Pf*C2 domain containing membrane antigen, *Pf*C2DMA. The encoded polypeptide contains a single C2 domain at the N-terminus (amino acid residues: 48 to 164) along with four transmembrane domains toward its C-terminus. We report that *Pf*C2DMA is expressed during asexual stages of intraerythrocytic development and the $C2_{dom}$ of this antigen binds to Ca^{2+} ions. Importantly, the $C2_{dom}$ exhibits binding to human erythrocytes. Furthermore, polyclonal antisera against $C2_{dom}$ of *Pf*C2DMA show potent invasion inhibitory activity. Overall, this study identifies a novel antigen from *P. falciparum* that might serve as a novel adhesin mediating the interaction between the merozoite and the erythrocyte. Thus, we propose that $C2_{dom}$ of this protein might serve as novel subunit vaccine candidate targeting blood stage of malaria.

Methods

Parasite culture

P.falciparum 3D7 was cultured in O+ erythrocytes along with RPMI-1640 medium (Gibco) supplemented with 0.5% albumax II (Gibco, CS, USA), 2 g/L sodium carbonate, 40 mg/L hypoxanthine (Sigma—Aldrich, MA, USA) under mixed gas conditions (5% CO_2, 5% O_2, 90% N_2) at 37°C. Parasites were tightly synchronised at the ring stage by treatment with 5% sorbitol in two successive cycles according to previously published methods.[16]

RT-PCR analysis of PfC2DMA

Stage specific expression of PfC2DMA was checked using Reverse-transcriptase PCR. The transcripts encoding for *Pf*C2DMA and 18S rRNA gene were amplified from cDNA derived from synchronous culture of *P. falciparum* 3D7 strain at Ring, trophozoite and schizont stages using the folllowing set of primers:

*Pf*C2DMA: 5′-ACCCTACTTACATGTGGTCTTAGT-3′
5′-TGGTACGTCTTCTTTTTCTTTCCA-3′
*Pf*18S rRNA: 5′- CCGCCCGTCGCTCCTACCG-3′
5′-CCTTGTTACGACTTCTCCTTCC-3′

The conditions used for amplification of *Pf*C2DMA and *Pf*18S were: denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and furthermore extension at 68 °C for 30 s.

Cloning, overexpression and purification of C2 domain of *Pf*C2DMA

The nucleotide sequence encoding for the $C2_{dom}$ (48—164 aa) of *Pf*C2DMA was amplified by PCR from the genomic DNA of *Pf*3D7, using the primers: Forward: 5′-CCGCGTGGATCCATGAAAGTTACATTTAATGCAAAAAAAACAAAAGG-3’; and, Reverse 5′-GCTCGAGTCGACTTAAATCATATCATATTTCGTATTAAATTTTAAATTC-3’. The gene was cloned into pET-28a(+) vector (Novagen), at BamH1 and Xho 1 (ThermoScientific) restriction sites. The positive clones were then transformed into *E. coli* BL21 (λDE3) strain for optimization of His-tagged $C2_{dom}$ expression. The bacterial cells grown in terrific broth (supplemented with 5% glycerol) were induced at an OD_{600} of 0.8 with 1 mM IPTG (Sigma) followed by growth at 18 °C for 24 h, and harvested by centrifugation at 4000*g*. The cell biomass was then lysed by sonication in lysis buffer (20 mM Tris, pH 8.0; 200 mM NaCl; 5% glycerol and 2 mM PMSF) and clarified by centrifugation. His-$C2_{dom}$ was purified by Ni-NTA affinity chromatography under native conditions, as described previously,[17] and buffer exchanged against 20 mM Tris, pH 8.0; 200 mM NaCl. Purity of the purified His-$C2_{dom}$ was assessed by SDS-PAGE analysis and western blotting.

Polyclonal antisera generation was done as described previously.[18] The specificity of the raised anti-$C2_{dom}$ sera

was checked by western blotting of bacterial lysate expressing recombinant $C2_{dom}$.

Detection of *Pf*C2DMA in parasite

Immuno-fluorescence Assay (IFA) was performed on synchronized *Pf*3D7 culture to check for expression of *Pf*C2DMA in mature stage of the parasite, as described earlier[19]. Probing was done with anti-$C2_{dom}$ antisera (1:100) and anti-MSP-1 antibody (1:200), followed by using secondary antibodies (anti-mice Alexa fluor 488 and anti-rabbit AF 546). The prepared slides were mounted with DAPI antifade (Invitrogen) and visualized under confocal microscope (Olympus Corporation). For unpermeablized conditions, parasite smears were fixed with 0.25% glutaraldehyde[20] (in PBS) at 4 °C for 15 min, followed by subsequent washing with PBS and processed furthermore, as described above. For determining the *in vivo* expression of *Pf*C2DMA in the parasite, late staged saponin released schizonts were lysed in RIPA buffer supplemented with Protease inhibitor and was probed with anti- $C2_{dom}$ antibody (dilution: 1:1000).

Ion binding activity of $C2_{dom}$

The Ca^{2+} binding affinity of recombinant $C2_{dom}$ was assessed using MST analyses, using the instrument Monolith NT.115 (Nanotemper technologies, Munich, Germany), as described previously.[21] Briefly, 10 μM of recombinant $C2_{dom}$ in HEPES-NaCl, pH 7.4 buffer was tagged with 30 μM of amine reactive dye, NT-647 NHS (Monolith Protein Labelling Kit Red-Maleimide 2nd Generation, NanoTemper). Increasing concentrations of $CaCl_2$ (3.04 μM–100 mM), diluted in HEPES-NaCl buffer supplemented with 0.01% tween-20, were titrated with the labelled $C2_{dom}$, followed by loading into standard treated capillaries (K002 Monolith NT.115) and read by the instrument. The experiments were carried out at 20% LED power and 40% MST power, respectively, and the data was analysed using Monolith software (Nanotemper, Munich, Germany). Mg^{2+} ions were used as negative control and titrated at different concentrations (3.04 μM–100 mM) with fixed concentration of the labelled protein.

RBC binding assays

The RBC binding activity of $C2_{dom}$ was confirmed by incubating 2×10^7 washed RBCs with 2 μg of the recombinant protein in PBS, under mild shaking conditions, at 37°C. The reaction mixture was then centrifuged through dibutyl phthalate (Sigma) and the supernatant was removed by aspiration. The RBC bound proteins were then eluted with 1.5 M NaCl solution and the eluted fractions were immunoblotted with anti-6X-His antibody (Invitrogen) to check for the presence of His-$C2_{dom}$ in the eluted fraction. RBC binding assays were also carried out with RBCs priorly treated with trypsin, chymotrypsin or neuraminidase, using methods described in.[11,22]

Invasion inhibition assay using anti-$C2_{dom}$ hyper-immune sera

Heat inactivated anti-$C2_{dom}$ mouse sera or pre-immune sera derived from healthy mice, diluted in incomplete RPMI in the ratio of 1:5 or 1:10 was added to schizonts adjusted to 1–2% parasitaemia and 2% haematocrit. The newly formed rings were then scored by drawing thin smears of each assay condition after 24 h. Mouse pre-immune sera served as a negative control and antisera against *Pf*AMA-1 as a positive control. At least 5000 red blood cells were counted to calculate the percent parasitaemia for each group.

Statistical analysis

Statistical analyses were performed using GraphPad 8 (GraphPad Software Inc.) and p-values were calculated by two-tailed Student's t-test wherever required.

Results

Sequence analysis of *Pf*C2DMA and expression of recombinant $C2_{dom}$

Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/) was used to identify conserved domains in *Pf*C2DMA, which revealed that it encodes for an 850 aa long polypeptide with $C2_{dom}$ (residue 48 to 164) at its N-terminus and four transmembrane helices: 1 between the N & C-termini and other 3 at the extreme C terminus. A pictorial representation depicting domain architecture of *Pf*C2DMA is represented in Fig. 1A. Sequence data of PF3D7_1110100 and orthologues from other *Plasmodium* species were retrieved from PlasmoDB (www.plasmoDB.org.in). The protein was found to be conserved throughout the *Plasmodium* species with orthologues present in *P. vivax* (PVX_091,155), *Plasmodium berghei* (PBANKA_0937,500), *Plasmodium knowlsei* (PKNH_0907,400), *Plasmodium yoelii* (PY03705) and *Plasmodium malariae* (PmUG01_09019600). Multi-sequence alignment of putative C2 domains from different *Plasmodium* species, *Mus musculus* and *Homo sapiens* is presented in Fig. 1B.

Three-dimensional structure of $C2_{dom}$ was modelled by I-TASSER[23] and furthermore refined using ModRefiner,[24] and quality of the modelled structure was checked using Procheck.[25] Ramachandran plot of the modelled structure of $C2_{dom}$ showed that 87% of the residues lie in the most favoured region. The modelled structure depicted that $C2_{dom}$ consists of six β-sheets that are connected to each other by variable loops. $C2_{dom}$ of *Pf*C2DMA showed no sequence similarity with the well characterized C2 domains present in humans, but forms the characteristic β-sandwich scaffold characteristic of C2 domain[26] (Fig. 1C i). The tertiary structure of *Pf*$C2_{dom}$ was also superimposed with $C2_{dom}$ of cytosolic Phospholipase A2 from *H. sapiens*. Root-Mean-Square Deviation (RMSD) score was found to be 1.28 Å, suggestive of a close relatedness between tertiary structure of both the polypeptides, despite of no sequence similarity between them [Fig. 1C ii)].

Phylogenetic tree of $C2_{dom}$ from genus *Plasmodium* and its orthologues from other organisms was generated using Molecular Evolutionary Genetics Analysis (MEGA11)[27] software via Neighbour adjoining method. Phylogenetic analysis revealed that the $C2_{dom}$ of genus *Plasmodium* are evolutionary conserved and formed a separate cluster,

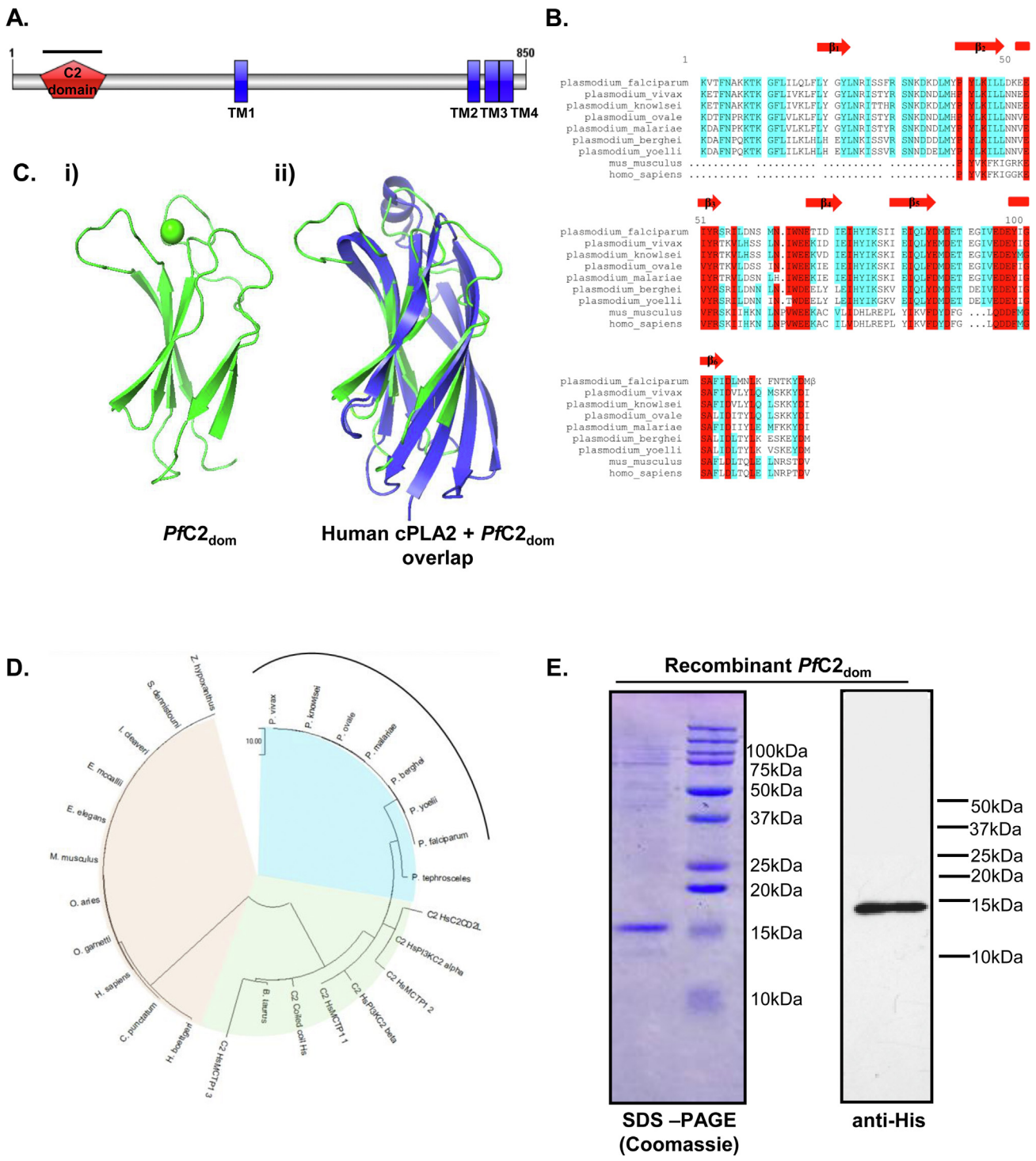

Figure 1. **A. Domain organization, structural analysis and over-expression of recombinant $C2_{dom}$ in *E. coli*. A.** Schematic representation of *Pf*C2DMA. Predicted C2 domain is represented with red pentagon and blue rectangular boxes represent trans-membrane (TM) helices. **B.** Multi sequence alignment of $C2_{dom}$ from *Plasmodium* genus, mouse and human counterparts. Residues with high consensus values (>90%) are marked in red color whereas with low consensus values (>50%, <90%) are marked in blue color. β-sheets are indicated as arrowheads. **C. i)** Homology modeling based predicted structure of $C2_{dom}$ of *Pf*C2DMA, bound Ca^{2+} ion is represented by green sphere. **ii)** Superimposed modelled structure of *Pf*$C2_{dom}$ (green) with crystal structure of *H. sapiens* cytosolic Phospholipase A2 (blue). **D.** Phylogenetic analysis of $C2_{dom}$ from *Plasmodium*, *H. sapiens* and other eukaryotes. **E.** SDS-PAGE showing purified recombinant $C2_{dom}$ at 15 kDa and recognition of protein by anti-6X-His monoclonal antibody.

suggestive of its divergence from human and other eukaryotic counterparts (Fig. 1D).

Purified recombinant $C2_{dom}$ of *Pf*C2DMA migrated as a single band corresponding to ~15 kDa on SDS-PAGE depicting high purity of protein, and its identity was confirmed by immunoblotting with anti-6X His tagged antibody (Fig. 1E).

C2 domain of *Pf*C2DMA binds Ca^{2+} ions with high affinity

To assess the ability of $C2_{dom}$ to bind Ca^{2+} ions, Microscale thermophoresis (MST) was employed. It is a biophysical technique that measures binding affinities between biomolecules in free solution with high sensitivity. It is based on the principle of movement of molecules in aqueous form on application of a thermal gradient (induced by an infrared laser in MST) and this phenomenon is termed as thermophoresis. This temperature induced molecular movement of molecules is very sensitive to alterations in size, charge and solvation shell of a molecule. The thermophoretic properties of a fluorescently labelled protein are altered when it is bound by a peptide, small molecule or even ions.[28,29] Thermophoresis is a measure of the state of protein, and binding induced change in thermophoretic parameters allow MST to determine binding affinities of protein ligand pair.

Upon titration of labelled $C2_{dom}$ with $CaCl_2$, change in thermophoretic mobility of the labelled protein was observed, suggestive of an effective binding with Ca^{2+} ions (Fig. 2A i). A dose response curve was constructed using Monolith software (Nanotemper, Munich, Germany) which represented a decrease in fluorescence of the bound labeled protein, as compared with its unbound form. The equilibrium dissociation constant, K_d came out to be 35 μM (Fig. 2A ii). Our observation corroborated with a previous study which reported that $C2_{dom}$ from tomato phospholipase Dα has a K_d for Ca^{2+} ions in the micromolar range (59.73 μM).[30] As a control, MST of $C2_{dom}$ was carried out with $MgCl_2$ (Fig. 2B i) and K_d of 61.4 mM was observed (Fig. 2B ii), which is suggestive of a very weak or negligible affinity of $C2_{dom}$ for Mg^{2+} ions. Thus, our results demonstrate that $C2_{dom}$ of *Pf*C2DMA folds into a functional module that is capable of binding to Ca^{2+} ions both with high affinity and specificity.

C2 domain of *Pf*C2DMA binds to human erythrocytes

Because many invasion ligands show erythrocyte binding activity, we assessed the ability of $C2_{dom}$ of *Pf*C2DMA for binding to erythrocytes. $C2_{dom}$ binds specifically to surface of human RBCs and this binding was abrogated in the presence of EGTA, suggestive of Ca^{2+} mediated binding of the protein to RBC surface (Fig. 3A). To furthermore delineate the nature of RBC receptor bound by $C2_{dom}$, its ability to bind untreated, chymotrypsin, trypsin and neuraminidase treated

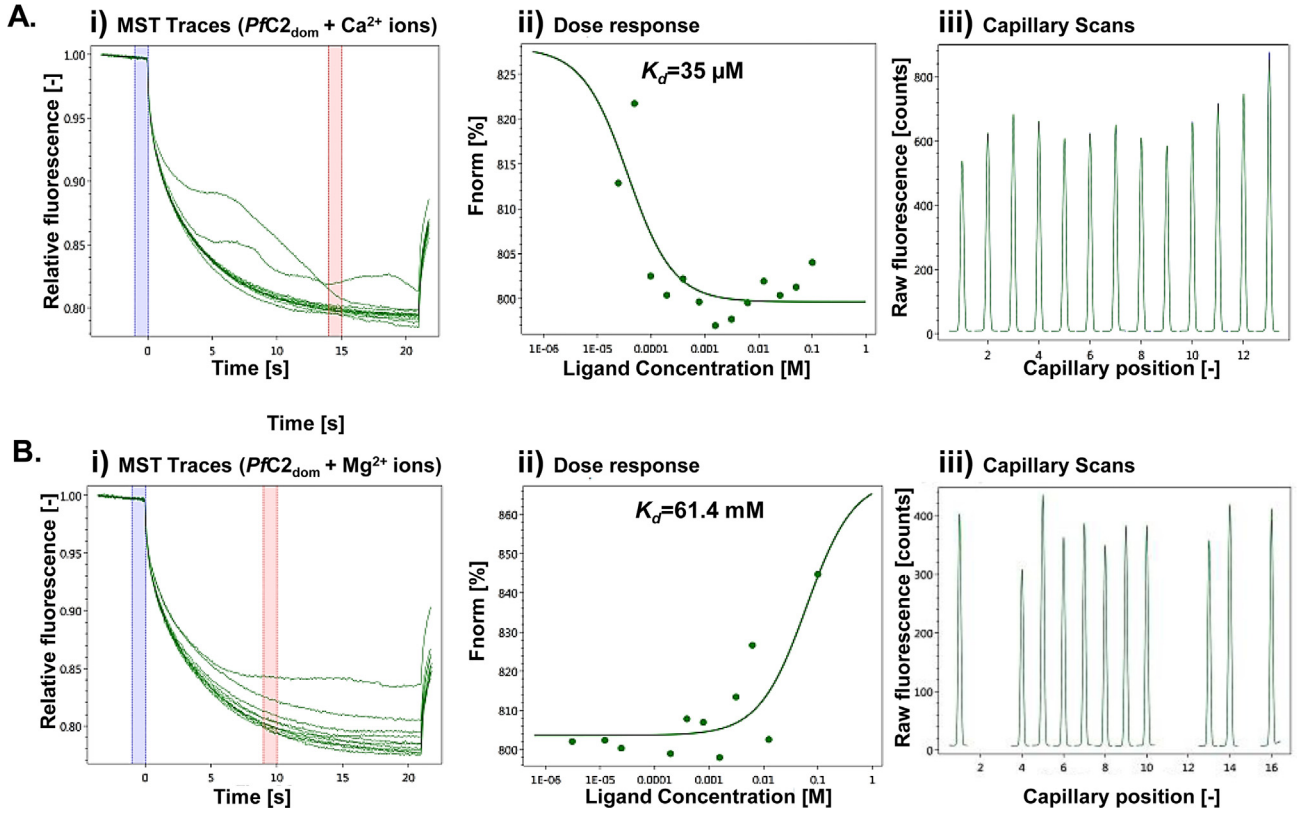

Figure 2. $C2_{dom}$ binds specifically to a crucial divalent ion A. i) Graph showing MST signal of labelled-$C2_{dom}$ with different concentrations of $CaCl_2$. **ii)** Dose response curve plotted between normalized fluorescence and ligand (Ca^{2+}) concentration. **iii)** Capillary scan data of labelled $C2_{dom}$ with varying concentrations of Ca^{2+} ions. **B. i)** MST analysis of labelled $C2_{dom}$ with different concentrations of $MgCl_2$. **ii)** Dose response curve between normalized fluorescence of protein and ligand (Mg^{2+}) concentration. **iii)** Capillary scan data of labelled $C2_{dom}$ with varying concentrations of Mg^{2+}ions.

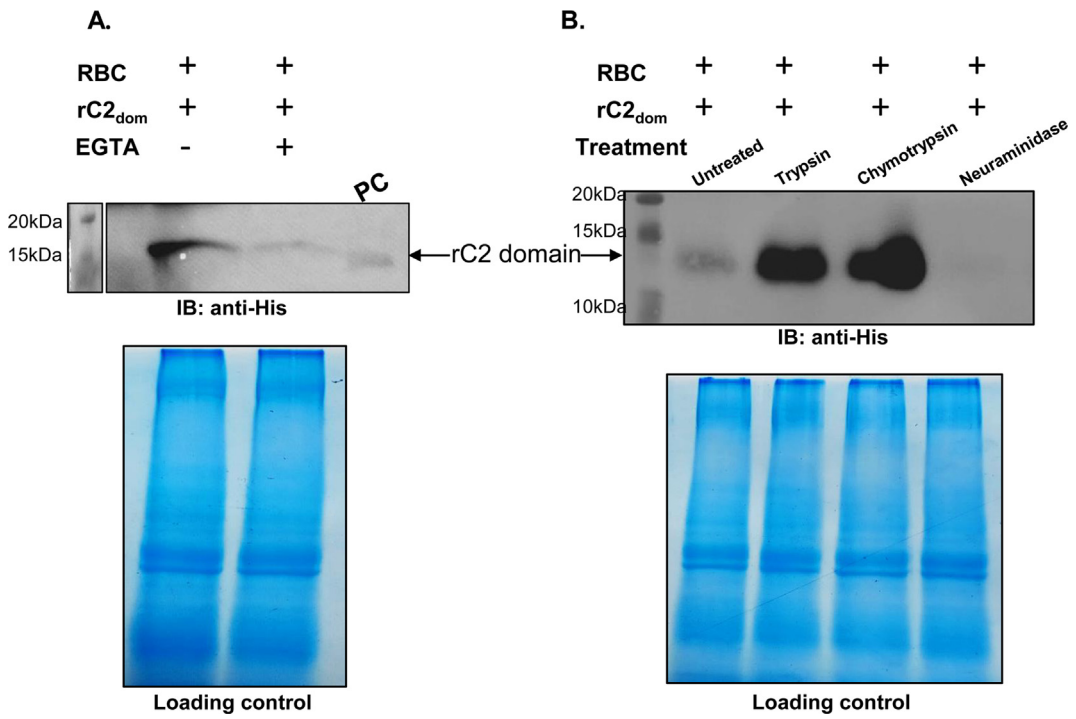

Figure 3. Recombinant $C2_{dom}$ binds to surface of human erythrocytes A. Erythrocyte binding activity of $rC2_{dom}$ was assessed in the presence of extracellular Ca^{2+} ion chelator, EGTA (2.5mM). SDS-PAGE gel represents RBC pellet boiled in laemmli buffer and run to serve as a loading control in order to ensure that equal number of RBCs are used for the assay. PC represents positive control. **B.** The binding of $rC2_{dom}$ to different enzymatically treated erythrocytes was also assessed.

erythrocytes was also analysed. The $C2_{dom}$ of *Pf*C2DMA showed binding to untreated, trypsin and chymotrypsin treated RBCs, however, it fails to bind neuraminidase treated RBCs (Fig. 3B). This suggests that $C2_{dom}$ binds to the erythrocyte in a sialic acid dependent manner and it binds to a heavily sialiated receptor on the erythrocyte surface.

*Pf*C2DMA is expressed during intraerythrocytic blood stages of parasite

After functionally characterizing the $C2_{dom}$ of *Pf*C2DMA, we checked the expression of this antigen, both at RNA and protein levels. Transcript levels of *Pf*C2DMA were detected by Reverse transcriptase PCR and qPCR using cDNA derived from synchronous cultures of Rings, trophozoites and schizonts infected RBCs. The transcripts encoding for *Pf*C2DMA was amplified using specific primers which revealed that it is expressed maximally during late stages of intraerythrocytic development (Fig. 4A). 18S was used as a housekeeping gene to confirm that equal amounts of RNA from each stage was used for RT-PCR analysis.

For checking the protein expression of full-length *Pf*C2DMA in parasite, lysate from schizont staged parasites was probed with anti-$C2_{dom}$ antisera. Desired band of ~101 kDa corresponding to the expected size of protein was observed (Fig. 4B), in addition with two bands of lower molecular weight, below the 101 kDa band. A prominent band was observed at ~55 kDa that might correspond to the processed form or degradation product of the protein or some unrelated protein.

*Pf*C2DMA localizes to the merozoite surface and colocalizes with MSP-1

To check the intracellular localization of *Pf*C2DMA within the parasite, fixed smears of late staged parasites were probed with $C2_{dom}$ antisera along with MSP-1 antisera, which serves as a marker for merozoite surface. *Pf*C2DMA was found to localize to the merozoite surface in punctated schizonts and individual merozoites (Fig. 4C). It colocalized with the GPI-anchored membrane protein marker, Merozoite Surface Protein-1 (MSP-1), suggesting that it localizes to parasite plasma membrane.

Also, the spatial arrangement of *Pf*C2DMA on the merozoite surface was detected by staining parasites at merozoite stage, under unpermeabilized conditions, so that the antibodies can only recognize and bind to extracellularly exposed antigens. Both staining of *Pf*C2DMA and MSP-1 were observed in unpermeablized merozoites (Fig. 4D), suggesting that $C2_{dom}$ region of *Pf*C2DMA localizes on the outer leaflet of parasite plasma membrane, facing toward the extracellular space.

Antisera against *Pf*C2DMA inhibits merozoite invasion into erythrocyte

Because like other invasion ligands, $C2_{dom}$ also showed erythrocyte binding activity, we wanted to assess whether antibodies against it have any effect on merozoite invasion. For this, late schizonts were allowed to infect fresh RBCs in the presence of heat inactivated mice *Pf*$C2_{dom}$ antisera at a

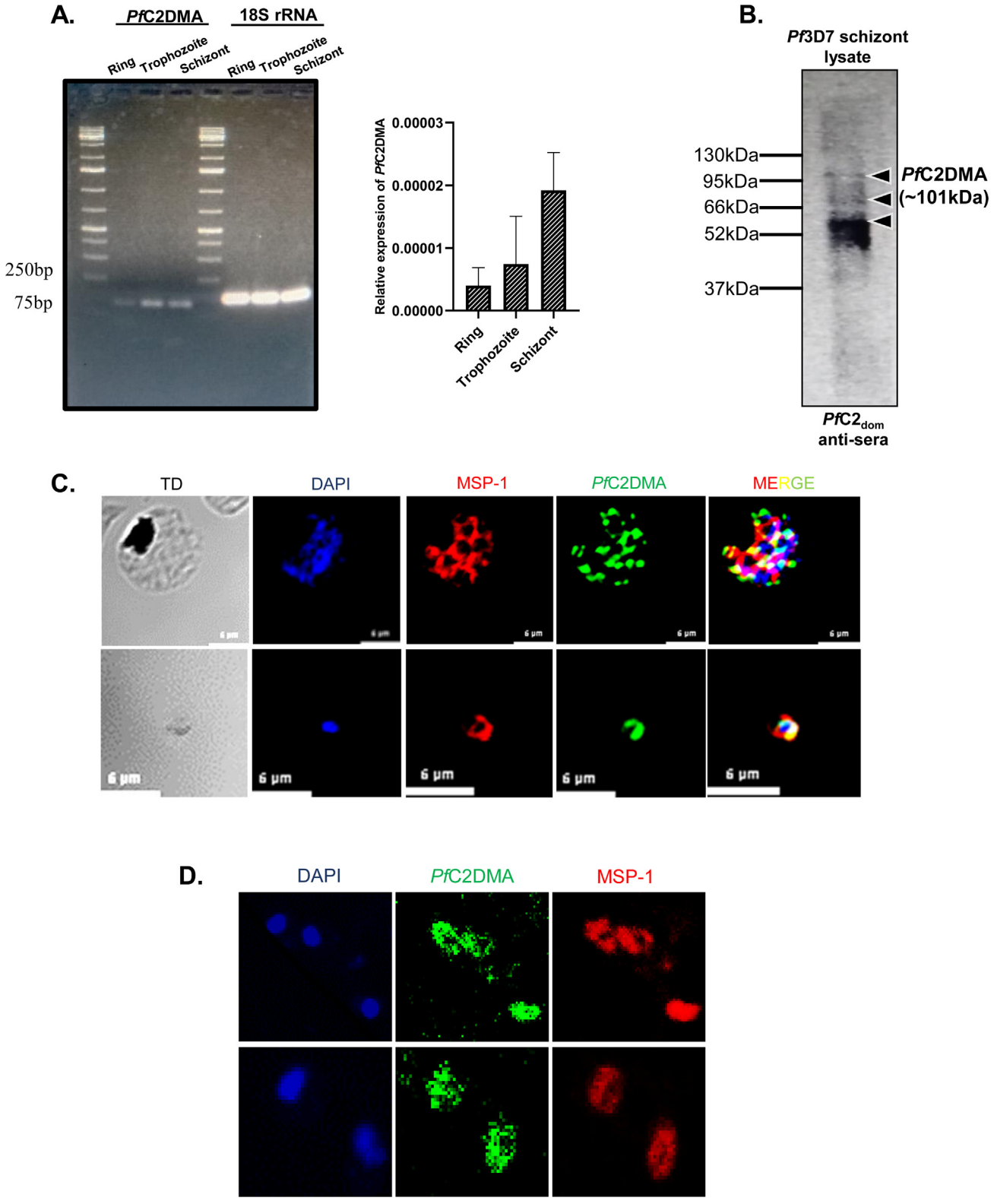

Figure 4. ***Pf*C2DMA is expressed during asexual blood stages of *P. falciparum*.** **A.** Semiquantitative and quantitative RT-PCR analysis of *Pf*C2DMA in cDNA derived from Ring, trophozoites and schizonts stages of *P. falciparum*. 18S rRNA transcripts were taken as a loading control. **B.** Immunoblot using $C2_{dom}$ antisera showing *Pf*C2DMA (~101 kDa) in schizont lysate. **C.** Localization of *Pf*C2DMA within mature schizont and free merozoite; green represents *Pf*C2DMA, red represents MSP-1. TD represents transmitted light channel. Parasite nuclei were stained with DAPI. **D.** The spatial organization of $C2_{dom}$ of *Pf*C2DMA on parasite plasma membrane was also analyzed in merozoites under unpermeablized conditions.

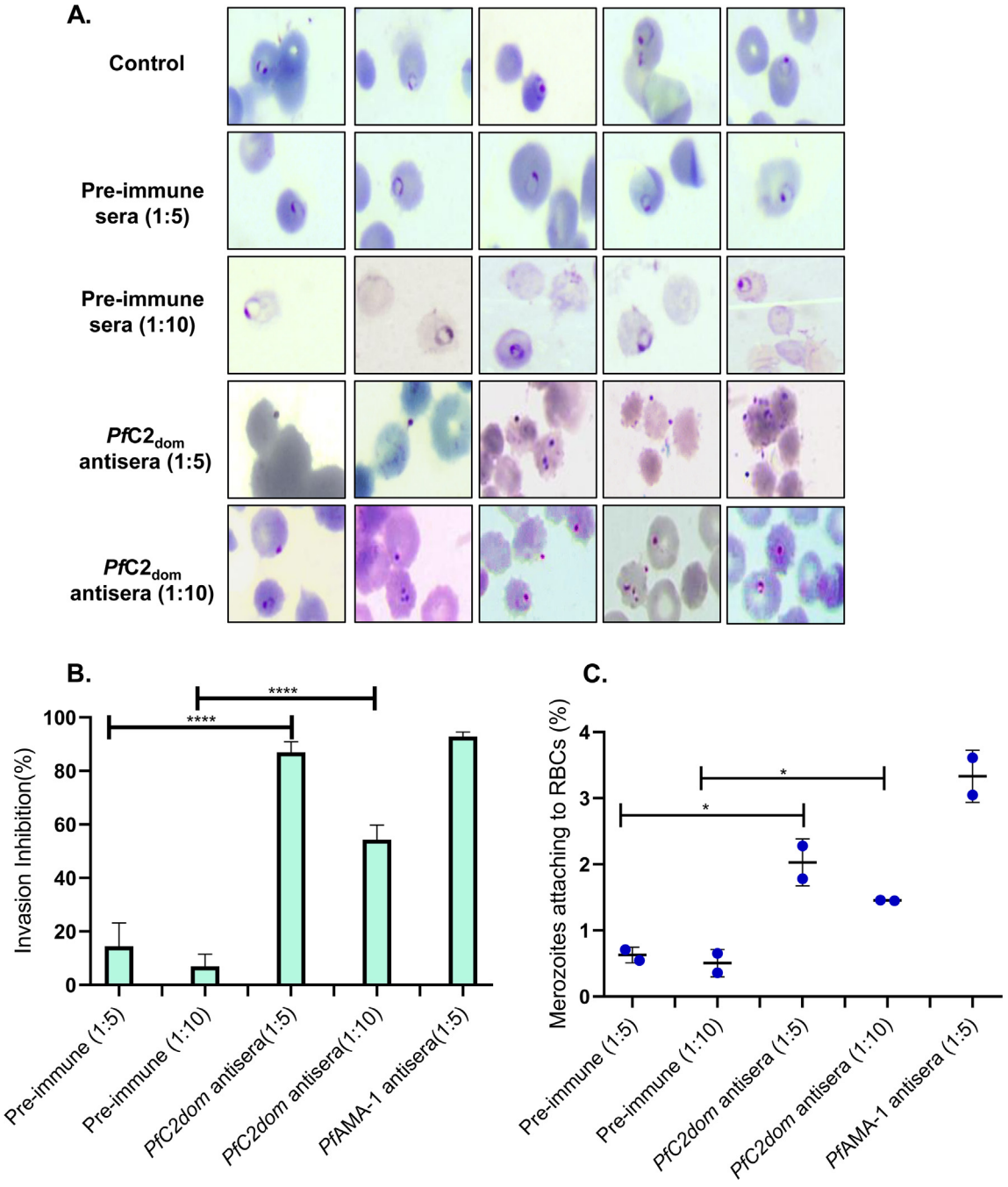

Figure 5. Hyperimmune sera against $C2_{dom}$ inhibit merozoite invasion. A. Representative images of giemsa stained smears showing successful formation of rings in untreated and Pre-immune sera groups. However, merozoites failed to invade RBCs in the presence of *Pf*$C2_{dom}$ antisera and found attached to the RBCs. **B.** Graph showing percent inhibition in invasion under different dilutions of pre-immune and immune antisera ($p < 0.0001$). Results are represented as mean ± s.d. **C.** Graph representing percentage of merozoites attaching to erythrocytes in the presence of mice pre-immune or *Pf*$C2_{dom}$ antisera.

dilution of 1:5 and 1:10. The number of new rings formed were scored by observing thin Giemsa-stained smears of the assay wells. Mice pre-immune sera served as negative control. Healthy rings were seen in control and pre-immune control groups at both the dilutions. The merozoites were found bound to the RBCs in the presence of *Pf*$C2_{dom}$ anti-sera and found stuck at mid and/or later stages of invasion, unable to form rings (Fig. 5A). Antiserum against *Pf*$C2_{dom}$ was able to inhibit ∼87% of parasite invasion at dilution of 1:5, and ∼54% at dilution of 1:10. A significant inhibition of merozoite invasion was observed in the presence of *Pf*$C2_{dom}$ hyperimmune sera when compared with pre-immune control groups ($^{*}***p < 0.0001$) (Fig. 5B). Mice Pre-immune sera at dilutions of 1:5 and 1:10, served as a negative control which showed negligible invasion inhibitions of 15% and 7% respectively. Apical Membrane Antigen-1 (AMA-1) anti-sera (1:5 dilution) was used as a positive control which showed potent invasion inhibitory

effect of ~93%. The experiment was done twice in duplicates, and each group consists of sera derived from three individual mice. The merozoites attached to erythrocytes were also quantified and represented as a percentage of merozoites attached to total erythrocytes in Fig. 5C. A high number of merozoites were found attaching to RBCs which failed to invade erythrocytes in the presence of *Pf*C2$_{dom}$ antisera as compared with pre-immune controls ($p < 0.05$) pointing toward a defect in invasion event.

Discussion

C2 domains, originally discovered in Protein Kinase C conserved 2 are ubiquitous structural modules that function as membrane tethering domains of 80–160 aa residues, which independently folds into a functional module that binds to phospholipids in a Ca^{2+}- dependent manner.[26,31] These domains are usually a part of eukaryotic proteins involved in signal transduction or membrane trafficking, recruiting them to biological membranes. Proteins harbouring these domains includes kinases such as PKC, phospholipases (PLA_2, PLC, PLD)[32] and membrane trafficking proteins including synaptotagmin[33] and rabphilin 3A. C2 domain from various organisms share a compact eight stranded anti parallel β-sandwich, connected by variable loops that coordinates multiple Ca^{2+} ions and binds to biological membranes in a Ca^{2+} dependent manner. However, some C2 domains have diverged evolutionarily and have lost Ca^{2+} binding activity,[34,35] majority of them still possess it. The Ca^{2+} binding sites are formed by aspartate side chains that serves as bidentate ligands for the metal ion.[35] This activity enables the domain to bind to biological membranes in a Ca^{2+} -dependent manner thus serving as a Ca^{2+} effector domain.

Recent studies point toward the importance of C2 domain containing proteins in micronemal secretion and subsequent invasion process of *Pf* merozoite. Conditional knockout of double C2 (Doc 2) protein compromised the invasion efficiency of parasites[36]. The cytoadhering protein *Pf*EMP1 also harbours C2 domain which along with DBL2β domain binds to host ICAM-1.[37] A recent study illustrates a C2 domain containing rhoptry protein *Pf*CERLI2 which is essential for invasion and rhoptry morphogenesis. Ribozyme based knockdown of *Pf*CERLI2 in parasites leads to a defect in merozoite invasion and alters the overall morphology of rhoptries, characterized by elongated rhoptries in the knockdown parasites.[38,39] These evidences are suggestive of involvement of C2 domain containing proteins in the growth and pathogenesis of the parasite, particularly during the event of invasion.

The genome for *P. falciparum* 3D7 encodes for four C2 domain containing proteins namely: Ferlin-like protein putative (PF3D7_0806,300), double C2 like domain containing protein (PF3D7_1243900), Ferlin putative (PF3D7_1455600) and a conserved *Plasmodium* protein of unknown function (PF3D7_1110100, PfC2DMA). All other proteins except for *Pf*C2DMA contains multiple C2 domains that is five in ferlin-like protein, two in DOC2 and four in ferlin putative protein. On the contrary, *Pf*C2DMA is the only protein that contains a single orphan C2 domain (rather than multiple tandem C2 domains in other proteins) at its extreme N-terminal and whose function remained uncharacterized till date.

The conservation of this novel protein across the *Plasmodium* species and its essentiality as pointed by a low Mutagenesis Index score, intrigued us to study this protein in detail and furthermore characterize its role in the growth of parasite. We found that C2$_{dom}$ of this protein can independently fold into a structural module that is capable of binding to Ca^{2+} ions and also to the surface of erythrocytes. Transcripts of *Pf*C2DMA were found in abundance during trophozoite and schizont stages of intraerythrocytic development. This is in support with other proteins involved in invasion whose expression peaks up during the schizont stage.[40] IFA analysis in segmenters and individual merozoites revealed a surface localization of the protein, colocalizing with the well-established merozoite surface marker MSP-1.[41] This confirms the membrane association of *Pf*C2DMA as predicted by the presence of four transmembrane domain in the polypeptide. Full length *Pf*C2DMA was also detected at proteomic level in schizont staged parasite lysate in addition with smaller fragments of low molecular weight, that might correspond to the processed forms of the protein. This has also been reported for many invasion ligands that are proteolytically processed during invasion.[10,42–45] Similar processing is observed for well characterized invasion associated proteins such as MSP-1,[46] AMA-1[47] and this processing is thought to enable removal of receptor ligand interactions so that the merozoite can gain entry into the host cell.[48] Like other adhesins, C2$_{dom}$ of *Pf*C2DMA alone could bind to surface of RBCs which was reduced upon chelation of extracellular Ca^{2+} ions by EGTA. Binding studies of recombinant C2$_{dom}$ with enzyme-treated erythrocytes revealed that it binds to erythrocytes in a sialic acid-dependent manner. This is also observed with invasion ligands belonging to the erythrocyte binding family (EBL) that binds to heavily sialiated glycophorins[49–51] on the erythrocyte surface thus making the binding sensitive to neuraminidase treatment. Interestingly, C2$_{dom}$ exhibits higher level of binding to trypsin and chymotrypsin treated erythrocytes which can be because of the exposure of specific receptor as a result of removal of other hindering receptors by the respective enzyme treatments. This is in line with another study which reported increased binding of *Pf*TRAMP to enzyme treated erythrocytes.[10]

Furthermore, mice antisera were potent in inhibiting merozoite invasion to about 87% and 54% at a dilution of 1:5 and 1:10 respectively. The merozoites were found binding to erythrocytes in the presence of *Pf*C2$_{dom}$ antisera, suggesting that *Pf*C2DMA is not involved in the initial attachment to the red cell. Moreover, it is involved in later stages of invasion events, probably during the formation of tight junction between the two cells. Furthermore, immunolocalization of *Pf*C2DMA under unpermeablized conditions depicted that C2$_{dom}$ is accessible to the antibodies and interacts directly with the erythrocyte at the time of merozoite invasion. This point toward the involvement of *Pf*C2DMA; present on the surface of merozoite in directly binding to erythrocytes and mediating invasion into it.

Conclusively, our study reports a novel membrane localized *Pf*C2DMA protein that is expressed during late

intraerythrocytic stages of parasite development and its $C2_{dom}$ binds with erythrocytes and Ca^{2+} ions. Antisera against the protein show invasion inhibitory activity supporting its role as an invasion ligand. It would be intriguing to access the effect of purified monoclonal antibodies against *Pf*C2DMA on merozoite invasion in combination with antibodies against known key invasion ligands to provide an additive invasion inhibitory effect, as reported in previous studies.[43,52,53]

Declaration of competing interest

We declare no conflict of interest.

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