Structure of Plasmodium falciparum TRAP (thrombospondin-related anonymous protein) A domain highlights distinct features in apicomplexan von Willebrand factor A homologues

INTRODUCTION

Plasmodium parasite species, causative of approximately 400 million human malaria infections and one million deaths annually, is one of the biggest challenges in health care and economy in the developing world. Although more than a dozen Plasmodium species can cause malaria in humans, P. falciparum is the most virulent [1].

The Plasmodium parasite life cycle involves many stages of host cell invasion and differentiation. First, parasites penetrate as ookinetes into the Anopheles mosquito midgut and develop into sporozoites, which invade mosquito salivary glands. The sporozoites are transferred into the mammalian host during the blood meal of mosquito. Within minutes or hours, they leave the bite site on the skin and travel via the bloodstream to liver hepatocytes, passing through various cell layers in the process. In hepatocytes, sporozoites develop into merozoites that are released into the blood, infect erythrocytes, develop further, and cause symptoms of malaria [4].

Mechanism of host cell invasion common to all Apicomplexa parasites, e.g. Plasmodium, involves a gliding motion that requires a transmembrane linker between the cytoskeleton of a parasite and the surface of the target cell. The process has been studied most extensively on TRAP (thrombospondin-related anonymous protein), a type-1 transmembrane protein that is crucial for cell invasion and in vivo infectivity [5,6]. TRAP is the founding member of the TRAP family of parasitic invasion and motility proteins, containing structurally related attachment assays were used to investigate the functional roles of the surface epitopes discovered. The reported structures are the first determined for a complete vWA domain of parasitic origin, highlighting unique features among homologous domains from other proteins characterized hitherto. Some of these are conserved among Plasmodiae exclusively, whereas others may be common to apicomplexan organisms in general.

Key words: cell binding, malaria, nuclear magnetic resonance (NMR), thrombospondin-related anonymous protein (TRAP), von Willebrand factor A (vWA), X-ray crystallography.
of the A domain by addition of a chelating agent or by site-directed mutagenesis of the MIDAS (metal-ion-dependent adhesion site) consensus site has been shown to reduce cell binding and decrease infectivity [18,23,24]. These findings suggest that TRAP also recruits another, non-HSPG, receptor upon hepatocyte invasion [18].

Many aspects of the molecular mechanisms of hepatocyte invasion by Plasmodium remain elusive, in part owing to a lack of atomic-resolution structural data on some of the key components. In the present study, we have solved the crystal structures of Mg\(^{2+}\)-loaded and Mg\(^{2+}\)-free forms of PITRAP-A (PITRAP vWA) domain. Our structures reveal new features among the Plasmodium family of vWA domains that differ, or are completely absent from, vWA domains known to date. The functional roles of the newly discovered surface epitopes were studied further by site-directed mutagenesis, followed by cell-attachment assays.

### EXPERIMENTAL

#### Production and crystallization of recombinant PITRAP-A

A DNA clone coding for full-length PF TRAP extracellular domain [19] was used to amplify the entire vWA module (residues 41–242). For recombinant expression, the amplicon was ligated to a bacterial expression vector and transferred to Escherichia coli BL21(DE3) cells. The cells were grown to a D\(_{600}\) of \(\sim 0.6\) and induced with 0.5 mM IPTG (isopropyl \(\beta\)-D-thiogalactopyranoside) for 4 h at 37\(^\circ\)C. Cells were collected, suspended in ice-cold 50 mM Tris/HCl buffer (pH 7.5), supplemented with 1 M urea, 100 mM NaCl, 10 mM EDTA, 10 mM DTT (dithiothreitol), 1% (w/v) Triton X-100, 150 \(\mu\)g/ml lysozyme and 0.15% (v/v) P8340 protease inhibitor cocktail (Sigma), and disrupted by sonication. The inclusion bodies were collected by centrifugation at 21000 \(g\) for 30 min and washed twice in the same buffer without EDTA and DTT, followed by a final wash with 50 mM Tris/HCl buffer (pH 7.5) containing 100 mM NaCl. The purified inclusion bodies were dissolved in a buffer containing 6 M guanidinium chloride, and refolded by rapid dilution method [19]. In detail, the solubilized A domain was clarified by centrifugation and the supernatant was quickly diluted 28-fold in 50 mM Tris/HCl buffer (pH 7.2), supplemented with 1 M urea, 0.5 M L-arginine, 2 mM MgCl\(_2\), 2 mM reduced glutathione, 0.2 mM oxidized glutathione and 0.4 g/l Triton X-100, and stirred for 15 min. The sample was diluted slowly with 0.41 vol. of 50 mM Tris/HCl buffer (pH 7.2) and 16 mM \(\beta\)-cyclodextrin cooled to 12\(^\circ\)C, and stirred for an additional 15 min at 12\(^\circ\)C. The bottle was filled with inert gas and refolding was allowed to proceed at 12\(^\circ\)C for 36 h. After an extensive dialysis, refolded A domain was purified by heparin-affinity chromatography, eluting with a linear NaCl gradient in the presence of 1 M urea. The combined peak fractions were concentrated and washed with PBS in an ultrafiltration device (Millipore) to reduce urea concentration to 0.1 M. After SEC (size-exclusion chromatography) on a Superdex 75 column calibrated with commercial standards (GE Healthcare), the fractions containing monomeric PITRAP-A domain were combined and concentrated.

For crystallization trials, monomeric PITRAP-A domain in PBS was concentrated to 3 mg/ml, and subjected to automated screening using the sitting-drop vapour-diffusion method. After manual optimization screens, the Mg\(^{2+}\)-free crystals were grown over a reservoir solution of 0.1 M Hepes buffer (pH 7.0), 0.2 M Li\(_2\)SO\(_4\) and 25% (w/v) PEG [poly(ethylene glycol)]] 3350, and the Mg\(^{2+}\)-loaded crystals were grown over 0.1 M Bis-Tris buffer (pH 5.5), 0.2 M MgSO\(_4\) and 25% (w/v) PEG3350. Mixing 0.5 \(\mu\)l of reservoir solution with 0.5 \(\mu\)l of protein solution yielded crystals within a few days at room temperature (20\(^\circ\)C). For data collection, the crystals were briefly soaked in reservoir solution supplemented with 20% (v/v) ethylene glycol, and flash-frozen in liquid nitrogen. For heavy-atom derivative data collection, the Mg\(^{2+}\)-free crystals were soaked in reservoir solution supplemented with 1 mM K\(_2\)PtCl\(_4\), for 2 h, followed by flash-freezing with Paratone-N (Hampton Research) as a cryoprotectant.

#### Structure determination

Complete datasets to 1.73 and 1.87 Å (1 Å \(= 0.1\) nm) resolution were collected from single Mg\(^{2+}\)-loaded and Mg\(^{2+}\)-free crystals respectively at the Swiss Light Source (SLS) (Table 1). For phasing, a platinum-derivative dataset from the Mg\(^{2+}\)-free crystal was collected to 2.13 Å resolution at beamline X06DA (Table 1). Data were processed with XDS [25]. Platinum sites were located with SHELXC/D [26] and phasing and phase extension was carried out using SHARP [27] and SOLOMON/DM. After this, the initial model was built with ARP/wARP [28], and further refinement cycles were carried out using REFMAC5 [29] and building with Coot [30]. The Mg\(^{2+}\)-loaded crystal form was solved from the Mg\(^{2+}\)-free structure by rigid body refinement, and refinement of the structure was finalized as above. The structures had R-factors (R/\(R\_\text{free}\)) of 18.8%/23.7% (Mg\(^{2+}\)-free) and 17.7%/21.8% (Mg\(^{2+}\)-loaded) (Table 1). Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.

#### In vitro mutagenesis

A QuickChange II site-directed mutagenesis kit (Agilent Technologies) was used to obtain two mutants of pRAT4-PITRAP-A, i.e. R200S and a double mutant R141T/R181S (2XArg), in order to interfere with the stability of loop Cys\(^{205}\)-Cys\(^{212}\) and the proposed heparin-mediated cell binding respectively.
Confirmed constructs were used to express, refold and purify the corresponding recombinant proteins as described above. CD spectropolarimetry and one-dimensional NMR spectroscopy were used to analyse the effect of mutations on protein folding.

Cell attachment assays

An aliquot of PfTRAP-A was incubated with a 4-fold molar excess of biotinamidocaproate N-hydroxysuccinimide ester (Sigma) to obtain a low level of amine biotinylation. Free reagent was removed from quenched reaction mixture by gel filtration. Cell-attachment studies were carried out as described in [19] using Huh7 hepatoma cells fixed on to Maxisorp 96-well plates (Nunc). Instead of antisera, horseradish-peroxidase-coupled Neutravidin (Thermo Scientific) and o-phenylenediamine dihydrochloride in a stable peroxide substrate buffer (Thermo Scientific) were used to detect the amount of biotinylated PfTRAP-A bound to cells in the absence or presence of 6 kDa heparin (Sigma) and/or EDTA.

In another series of experiments, Maxisorp plates were coated with 25 μg/ml wild-type or mutant PfTRAP-A, and blocked with BSA. Huh7 cells were cultured to 60–80% confluence in EMEM (Eagle’s minimal essential medium) (Lonza) supplemented with 0.1 at 37° C. The amount of bound cells was quantified by a 30 min incubation with 25 μg/ml wild-type or mutant PfTRAP-A, and blocked with BSA, 1 mM MgCl2 and a 1/10 vol. of either PBS or a competing substance dissolved in PBS. Unbound cells were washed away twice with PBS supplemented with MgCl2, and the amount of bound cells was quantified by a 30 min incubation at 37° C in the presence of 3 mg/ml 4-nitrophenyl phosphate in 50 mM sodium acetate buffer (pH 5.0) and 1% (w/v) Triton X-100, stopping reaction with 0.33 M NaOH and measuring absorbance at 405 nm.

RESULTS

Refolding and purification of PfTRAP-A

A refolding protocol originally used for the complete PfTRAP extracellular domain [19] (Figure 1A) was modified for the production of PfTRAP-A. In the first chromatographic purification step, the refolded PfTRAP-A was bound to a heparin-affinity column and eluted with a linear salt gradient, yielding two purification steps, the refolded PfTRAP-A was bound to a heparin-affinity column and eluted with a linear salt gradient, yielding two affinity columns. The production of PfTRAP-A. In the first chromatographic purification step, the refolded PfTRAP-A was bound to a heparin-affinity column and eluted with a linear salt gradient, yielding two purification steps, the refolded PfTRAP-A was bound to a heparin-affinity column and eluted with a linear salt gradient, yielding two purification steps, the refolded PfTRAP-A was bound to a heparin-affinity column and eluted with a linear salt gradient, yielding two purification steps, the refolded PfTRAP-A was bound to a heparin-affinity column and eluted with a linear salt gradient, yielding two purification steps.

Crystal structures of Mg2+ -free and Mg2+ -loaded forms of PfTRAP-A

The crystal structure of Mg2+ -free form of PfTRAP-A was solved at 1.9 Å resolution by SIRAS (single isomorphous replacement with anomalous scattering), and refined to a crystallographic R-factor of 18.8% (Table 1). In the structure, 200 residues (positions 41–240) were built into electron density. The folds of the two monomers of the asymmetric unit are very similar [RMSD (root mean square deviation) of 0.18 Å over 200 residues], with minor differences occurring mostly at crystal contacts. The structure shows the characteristic αβα sandwich, or αβ Rossmann fold, of vWA family members (Figure 2A). The structure is stabilized by the predicted disulfide bond between Cys24 and Cys254, tying the N- and C-termini together. Another disulfide link was found between Cys211 and Cys212, creating a hitherto unseen protruding surface loop of six residues, additionally stabilized by the side chain of conserved Arg200 which co-ordinates to three main-chain carbonyl groups of the loop (Figures 2B and 2C). The proposed MIDAS of the PfTRAP-A Mg2+ -free form is occupied by water only. Instead, strong globular electron density with peak height of ~7σ was observed at a distance of ~11 Å from the MIDAS. On the basis of apparent co-ordination geometry, the types of the ligands and the composition of the sample buffer used, this density was modelled as Cl−. The binding pocket is formed on one side by loop α2–α3 and the N-terminus of helix α3, and on the other side by loop βD–α4 and the N-terminus of helix α4 (Figure 2D). The average distance for the co-ordinating bonds in the two monomers of the asymmetric unit are 3.3 and 3.4 Å, ranging from 3.1 to 3.6 Å, suggesting relatively weak interaction.

The structure of the Mg2+ -loaded PfTRAP-A was solved using the Mg2+ -free model. The protein fold is nearly identical (RMSD 0.108 Å between the A monomers of the two models). In the presence of Mg2+, PfTRAP-A crystallized with a metal attached to its MIDAS with a typical binding geometry and expected co-ordinating residues (Figure 2E). The MIDAS is rather rigid, showing only a less than 0.2 Å increase in the radius of the binding pocket and minor changes in side-chain orientations upon Mg2+ binding. The average bond length is 2.08 Å, in good agreement with the values in literature [31]. Similar to the
Figure 2  Crystal structure of PITRAP-A shows a typical vWA fold with some previously unseen features

(A) Ribbon presentation for the crystal structural model of PITRAP-A in Mg\(^{2+}\)-loaded form. The model is coloured as a continuum from the N-terminus (blue) to the C-terminus (red), and helices are named. Side chains are shown only for the yellow-coloured cysteine residues involved in disulfide bonds. The Mg\(^{2+}\) bound to the MIDAS is shown as small green sphere, and Cl\(^{-}\) is shown as big green sphere. (B) PITRAP-A model shown in pink, with the attached ions coloured as above, superimposed on the closest structural homologue, the mouse vWF A1 domain (PDB code 1U0O) [33]. Whereas \(\alpha\)-helices and \(\beta\)-strands align nicely, some of the loops are clearly divergent. (C) A close-up of the arrangement of the Cys\(^{205}\)-Cys\(^{212}\) loop with the surrounding residues shows how the structure is stabilized by a disulfide bond, and by co-ordination of the Arg\(^{200}\) side chain as indicated. The conserved residues Tyr\(^{215}\) and Asn\(^{199}\) anchor the loop to the hydrophobic core of the domain. The Cys\(^{205}\)-Cys\(^{212}\) loop is shown as stick presentation, whereas the rest of the molecule is shown as ribbon with side chains, coloured as in (A). (D) Both the Mg\(^{2+}\)-loaded and Mg\(^{2+}\)-free forms of PITRAP-A contain Cl\(^{-}\) (green sphere) between \(\alpha\)4 and \(\alpha\)5, co-ordinated as shown. Two backbone hydrogen bonds are shown as blue lines. (E) At the MIDAS, an Mg\(^{2+}\) was observed (green sphere), co-ordinating directly to the side-chain oxygens of Ser\(^{56}\), Ser\(^{58}\) and Asp\(^{162}\). Water-mediated co-ordinates are donated by Asp\(^{54}\), Thr\(^{131}\) and a solvent molecule. Upon metal binding, the co-ordinated water moves 0.5 Å away from Ser\(^{56}\) and towards Asp\(^{54}\) and Thr\(^{131}\). (F) An apparent second Mg\(^{2+}\) (small green sphere) was also found to be present with a low occupancy, connected to Cl\(^{-}\) (big green sphere) via the shared co-ordinate of the Ser\(^{134}\) side chain and two water molecules bound to the Asn\(^{132}\) side chain. This Mg\(^{2+}\) receives direct protein ligands from Ser\(^{134}\) and from Asp\(^{170}\) in one alternative conformation, whereas in its other conformation Asp\(^{170}\) co-ordinates via a water molecule. The co-ordination is completed by a network of waters.

Mg\(^{2+}\)-free structure, a Cl\(^{-}\) ion is found here also, with nearly identical bond distances. Surprisingly, globular electron density reminiscent of another Mg\(^{2+}\) was found only 4.9 Å away from the Cl\(^{-}\) pocket. The octahedral binding geometry, with an average bond distance of 2.08 Å, clearly supports the assignment of this electron density as Mg\(^{2+}\) (Figure 2F), but it has a low occupancy. This second Mg\(^{2+}\) shares the strictly conserved (see below) Ser\(^{134}\) side-chain ligand with the Cl\(^{-}\).

Comparison of the PITRAP-A structure with other members of the vWA family

A structural comparison using the DALI server [32] for the PITRAP-A Mg\(^{2+}\)-loaded form confirmed high homology with the existing vWA structures in the PDB (Supplementary Figure S1 at http://www.biochemj.org/bj/450/bj4500469add.htm). The highest Z-score of 24.3 with an RMSD of 2.0 for 185 residues was obtained for vWA1 (PDB code 1U0O) [33]. The only available apicomplexan model, the crystal structure of a fragment of TgMIC2 vWA/I domain (PDB code 2XGG) [34], aligned with a Z-score of 17.2 and an RMSD of 2.0 over 140 residues.

Alignment with a selection of the highest scoring unique proteins (Supplementary Figure S1) showed clear homology of the overall fold and the MIDAS, as well as partial conservation of the N- and C-terminal stabilizing cysteine residues, but demonstrated more variations at the loop regions. The most prominent structural feature of PITRAP-A is the clearly divergent structure of two loops revealed upon superimposition of PITRAP-A and vWA1 (Figure 2B). The first loop, formed by Ser\(^{123}\), Thr\(^{124}\) and Asn\(^{125}\) of the \(\alpha2\)-\(\alpha3\) loop, forms a minor protrusion on the surface, 15 Å from the MIDAS. Upon the superimposition of 275 DALI hits showing a significant Z-score of at least 10, only the mutually related complement fragments component C2A (PDB code 2I6Q) [35] and Factor B (PDB code 1Q0P) [36] show somewhat analogous surface loops, whereas DNA-repair protein KU70 (PDB code 1JEQ) has a ten-residue \(\alpha\)-helix at a corresponding location [37]. Inspection of a ClustalW sequence alignment for the available plasmodial TRAP sequences (Supplementary Figure S2 at http://www.biochemj.org/bj/450/bj4500469add.htm), as well as further comparison with three apicomplexan A domain homologues [34], suggests that this protrusion is likely to be conserved in the plasmodial TRAP A domains.
The second protrusion between Cys^{205} and Cys^{212}, already discussed above (Figure 2C), does not have a clear homologue in any of the 275 highest scoring DALI hits. The only potential exception is DNA-repair protein KU70, which has an acidic loop of similar size, but undefined in crystal structure [37]. Both of the two complement fragments discussed above show a threeresidue-smaller loop with different orientation in the crystal [35,36]. None of the loops in these three homologues are disulfide-bonded, however. On the basis of complete conservation of the two cysteine residues (Supplementary Figure S2), this surface determinant is likely to be found on all plasmodial TRAP A domains, as well as other apicomplexan homologues [34]. Also, the flanking residues Asn^{199} and Tyr^{215} that anchor the loop to the core of the fold (Figure 2C), are strictly conserved in plasmodial TRAPs. The latter is present on other apicomplexan sequences, whereas the former is replaced by a cysteine residue, probably pairing with another additional cysteine residue in TgMIC2 and NcTRAP (N. caninum TRAP). The accommodation of a basic side chain inside the loop appears to be a further conserved feature, since all apicomplexan sequences carry an arginine or lysine residue at the position of PITRAP-A Arg^{201}.

**Surface charge analysis shows the presence of distinct local patches**

In an earlier study [19], homology modelling suggested that Arg^{130}, Arg^{141}, Lys^{142}, Lys^{173}, Arg^{176}, Lys^{177} and Arg^{181} would form a condensation of positive charge, possibly mediating the heparin interaction as suggested for a corresponding surface on TgMIC2 [34]. A simple coulombic surface charge analysis of PITRAP-A confirmed the participation of these residues in a positively charged patch, possibly mediating the heparin interaction as suggested for a corresponding surface on TgMIC2 [34]. A simple coulombic surface charge analysis of PITRAP-A confirmed the participation of these residues in patch formation (Figures 3A and 3B), with the exception of Arg^{130}. The potential role of this positive patch in heparin binding is indirectly supported by the apparent presence of an SO_{4}^{2−} molecule next to the Arg^{141}, Lys^{177} and Arg^{181} side chains in the crystal. A negatively charged patch was detected below the Cys^{205}–Cys^{212} loop (Figure 3B), formed by six acidic residues, of which Asp^{65}, Glu^{237} and Glu^{239} are strictly conserved among Plasmodia (Supplementary Figure S2).

**Cell binding assays support the suggested location of heparin binding site and indicate the importance of correctly folded loop Cys^{205}–Cys^{212}**

Solid-phase cell attachment assays were set up to investigate the binding of wild-type recombinant PITRAP-A and two mutant constructs (Figure 1A) to Huh7 hepatoma cells. Wild-type protein was able to bind to immobilized Huh7 cells (Figure 4A), and this interaction could be partially blocked both by EDTA and by heparin, as expected from earlier studies [18,19,23]. In a reverse set-up using immobilized PITRAP-A, and cells in suspension (Figure 4B), EDTA was found to have no or minimal inhibitory effect on the interaction. Instead, heparin could efficiently block binding of cells to PITRAP-A. The R141T/R181S double mutant, designed to remove two adjacent basic side chains from the centre of the presumed heparin-binding site, was unable to bind cells, suggesting that the PITRAP-A heparin-binding site was located correctly. Finally, the R200S mutation abolished cell binding, apparently because of partial misfolding of the domain, as observed upon CD and one-dimensional 1H-NMR analyses (Supplementary Figure S3 at http://www.biochemj.org/bj/450/bj4500469add.htm). The R141T/R181S mutant appears to fold in a similar manner as the wild-type protein (Supplementary Figure S3).

**DISCUSSION**

The multifaceted adhesion module vWA is present on dozens of mostly extracellular human proteins. The same unit is found in single or tandem copies in numerous proteins of the apicomplexan protists which parasitize a multitude of animals, including humans. The two PITRAP-A crystal structures reported in the present paper represent the first structural models for a complete vWA domain of a protein from this large protozoan phylum.

Although the conservation of the overall fold and the MIDAS on PITRAP-A clearly witness a close relationship to the mammalian vWA units, the structure also reveals interesting differences of potential functional significance, such as the presence of the extra loop with sequence CHPSDGKC and the apparently strictly conserved mechanism of stabilization associated with it (Figure 2C). In a search for an explanation for the observed dimerization tendency (Figure 1B), an interface analysis using the PDBe PISA server (http://www.ebi.ac.uk/msd-srv/prot_int/
EDTA (black bars). In a parallel experiment (white bars), the two arginine mutants, R141T/R181S, wild-type PfTRAP-A resulted in binding reaction which was inhibitable by heparin, but not with (n = 6) and are means ± S.D., but analogous results were obtained in parallel experiments with two forms differ in the co-ordination geometry of the metal and in the regulation of the ligand affinity in integrin \( \alpha \beta \) heterodimers. The two forms differ in the co-ordination geometry of the metal and in the position of the C-terminal \( \alpha \)-helix, and the ‘open’ form is believed to be the high-affinity form \([44,45]\). For the \( \text{Mg}^{2+} \)-loaded form of PfTRAP-A, the direct co-ordination of the remote Asp162 to the metal ion and the elevated position of the C-terminal helix \( \alpha \)-helix demonstrate that PfTRAP-A represents a typical ‘closed’ form. This conformation is maintained by the Cys41–Cys335 disulfide bond and by the hydrophobic packing of the side chains of Val221, Ile227, Phe230 and Val234. These residues are conserved in all plasmodial TRAP proteins, as well as in TgMIC2, NcTRAP and EtMIC1, suggesting that the integrin-type conformational affinity regulation will not take place in apicomplexan vWA domains, and that their MIDAS metals are permanently in the low-affinity less-electrophilic state.

Several studies have suggested that binding of TRAP, in addition to circumsporozoite protein, to cell-surface HSPG receptors would contribute to the sporozoite invasion/infectivity, albeit with a minor role \([16–18]\). Using computational modelling, the binding site for heparin, an oversulfated analogue of heparan sulfate, on PfTRAP-A has been tentatively located to a region analogous to the binding site on vWF A1 domain \([19,46,47]\). We used in vitro mutagenesis to demonstrate the involvement of the proposed site in cell binding (Figure 4B). It should be noted that in this experiment, using Huh7 cells in suspension, chelation of \( \text{Mg}^{2+} \) did not result in inhibition of cell binding. This is obviously caused by the experimental set-up, perhaps by non-
random orientation of surface attachment of PfTRAP-A, because in the reverse set-up, both EDTA and heparin were effective inhibitors, as expected (Figure 4A). The apparent heparin-binding site of PfTRAP-A, spanning approximately 15 Å distance, is comparable in size with the heparin-binding epitope on the TSR unit of the same protein [22]. Apparently the higher amount of readily available basic side chains explains the reported higher heparin affinity of the A domain [19]. Unfortunately, our attempts to investigate the heparin affinity of PfTRAP-A using solution-state NMR failed due to increased aggregation of the protein in the presence of heparin fragments. In full-length TRAP, these two binding epitopes are likely to reside close to each other (Supplementary Figure S4C). This could explain the slight increase in heparin affinity in the presence of both domains, and the suggested occurrence of a conformational change upon heparin binding [19]. In the absence of structural data on a fragment spanning both the A and TSR units, this potential combined heparin binding surface, however, remains speculative.

The significance of the possible new binding sites for secondary Mg$^{2+}$ and Cl$^{-}$ on PfTRAP-A is currently unknown. Sequence comparison suggests only limited conservation for either the Mg$^{2+}$ or the Cl$^{-}$ sites across Plasmodium species and related organisms, and the partial occupancy of the secondary Mg$^{2+}$-site in crystal hints very loose binding. Co-ordinated additional ions have not been found at analogous positions on the closest mammalian vWA homologues. Although all available plasmodial vWA sequences, as well as that of T.gMIC2, show a conserved serine/threonine residue at the position corresponding to PfTRAP-A Ser 134, the presence of the auxiliary ions in the PfTRAP-A crystals may simply be a crystallographic artefact. Detailed biophysical and functional experimentation will be required to address the question of the possible physiological function of these ions.

In conclusion, the PfTRAP-A crystal structure reveals novel aspects of apicomplexan vWA domains, unpredictable using simple computational modelling with mammalian homologues [19,48]. It also places previous functional observations into a structural context, and corrects some false assumptions on the presence of heparin fragments. In full-length TRAP, these two binding epitopes are likely to reside close to each other (Supplementary Figure S4C). This could explain the slight increase in heparin affinity in the presence of both domains, and the suggested occurrence of a conformational change upon heparin binding [19]. In the absence of structural data on a fragment spanning both the A and TSR units, this potential combined heparin binding surface, however, remains speculative.

AUTHOR CONTRIBUTION
Tero Pihlajamaa, Tommi Kajander, Juho Knudt, Amit Sharma and Perttu Permi designed the experiments. Tero Pihlajamaa, Tommi Kajander, Juho Knudt, Kaisa Hurka and Perttu Permi performed the experiments and analysed data. All authors participated in writing the paper.

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SUPPLEMENTARY ONLINE DATA

Structure of Plasmodium falciparum TRAP (thrombospondin-related anonymous protein) A domain highlights distinct features in apicomplexan von Willebrand factor A homologues

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Figure S1  Structural alignment of PITRAP-A with ten of the closest structural homologues in PDB and with the apicomplexan homologue TgMIC2 (PDB code 2XGG) [1], obtained with DALL server [2]

All cysteine residues are shown in yellow and conserved residues are highlighted in colour (red, acidic; blue, basic; green, polar uncharged; grey, bulky aromatic or hydrophobic). For PITRAP-A, the nomenclature of secondary-structural elements and residue numbering are indicated. Conserved MIDAS residues are marked with an asterisk.

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The atomic co-ordinates and structure factors for Mg2⁺-loaded and Mg2⁺-free Plasmodium falciparum TRAP have been deposited in the PDB under codes 4F1J and 4F1K respectively.

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The two distinctive loop regions Ser\textsuperscript{123}–Asn\textsuperscript{125} and Cys\textsuperscript{205}–Cys\textsuperscript{212}, with its stabilizing residues Asn\textsuperscript{199}, Arg\textsuperscript{200} and Tyr\textsuperscript{215}, are shown in blue and yellow boxes respectively. For loop Cys\textsuperscript{205}–Cys\textsuperscript{212}, note the strict conservation of cysteine residues, and the difference in proline residue positioning in *P. berghei* and *P. yoelii*.
Figure S3  CD and NMR analyses of wild-type and mutated PITRAP-A

(A) Secondary-structure analysis was carried out by far-UV CD, as described in [3]. Results show highly similar spectra for the R141T/R181S (grey spheres) mutant protein and wild-type PITRAP-A (black spheres), suggesting nearly identical secondary structures. In contrast, the secondary structure of R200S mutant (grey triangles) appears to be clearly different. (B) Observation of ellipticity ($\theta$) at the wavelength of 220 nm as a function of increasing the temperature at a slope of 1°C/min demonstrates thermal stability of the wild-type PITRAP-A protein at temperatures below 40°C. (C) Comparison of one-dimensional ¹H–NMR spectra of wild-type (WT; thin black) and R141T/R181S (thin grey) PITRAP-A shows spreading of signals over 4 p.p.m. range, characteristic of a stable folded protein. In contrast, the R200S mutant (thick black) yields a poorly resolved spectrum with narrower signal distribution, suggesting partial misfolding. NMR experiments were carried out at 20°C on a Varian INOVA 500 or 600 MHz spectrometer, equipped with a $^{15}$N/$^{13}$C/$^1$H triple-resonance cold probe and a z-axis gradient system. The samples were in 50 mM NaCl, 20 mM Bis-Tris buffer (pH 6.6), 5 mM sodium phosphate, 95% $^1$H$_2$O, 5% $^2$H$_2$O, 50 μM MgCl$_2$ and 0.04% sodium azide.
Figure S4  Illustrations of possible complexes of PITRAP-A

(A) Putative dimerization interface of PITRAP-A, obtained with the PDBe PISA server [4], with a ΔG of −30 kJ/mol. The elements involved are the N-terminal half of loop Cys205–Cys212 (blue), and helices α4 (green) and α5 (orange). (B) Close-up of (A). Side chains are shown for the interface only. (C) A hypothetical complex of PITRAP-A (top) and TSR (bottom) domains with heparin. The complex was modelled by connecting the PITRAP-A Mg2⁺-loaded crystal structure to the NMR structure of the TSR domain (PDB code 2BBX) [5] and manual docking with heparin (PDB code 3IRJ) [6], followed by energy minimization and removal of steric clashes with the YASARA server (http://www.yasara.org/minimizationserver.htm).

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