Structural and functional dissection of the adhesive domains of *Plasmodium falciparum* thrombospondin-related anonymous protein (TRAP)

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TRAP (thrombospondin-related anonymous protein) is a sporozoite surface protein that plays a central role in hepatocyte invasion. We have developed procedures for recombinant production of the entire ECD (extracellular domain) and A domain of TRAP using bacterial- and baculovirus-expression systems respectively. The ECD and A domain were purified to homogeneity and migrated on gel-filtration columns as non-aggregated, monomeric proteins. These adhesive modules bound to HepG2 cells in a dose-dependent and bivalent cation-independent manner. The binding of ECD and the A domain to HepG2 cells was inhibited poorly by an excess of sulphatide analogues, suggesting the presence of as yet unidentified receptors for the A domain on hepatocytes. Using surface-plasmon-resonance-based sensor technology (Biacore), we demonstrate that TRAP ECD has higher affinity for heparin ($K_D = 40$ nM) compared with the A domain ($K_D = 79$ nM). We also present a three-dimensional structure of the A domain based on the crystal structure of the homologous von Willebrand factor A1 domain. The TRAP A domain shows two spatially distinct ligand-binding surfaces. One surface on the A domain contains the MIDAS (metal-ion-dependent adhesion site) motif, where point mutations of Thr^{131} and Asp^{162} correlate with impairment of cell infectivity by sporozoites. The other surface contains a putative heparin-binding site and consists of a basic residue cluster. Our studies suggest that TRAP interacts with multiple receptors during the hepatocyte invasion process. Our results also pave the way for inclusion of these high-quality recombinant TRAP domains in subunit-based vaccines against malaria.

Key words: A domain, heparin binding, hepatocyte invasion, malaria sporozoite, monomeric domain, surface-plasmon resonance, thrombospondin-related anonymous protein.

INTRODUCTION

Malaria accounts for more than two million deaths annually [1,2]. Sporozoites are the transmissible form of the malaria parasite from mosquito to the host, and are responsible for hepatocyte invasion. Two major surface proteins implicated in sporozoite infectivity are the CS (circumsporozoite) protein and TRAP (thrombospondin-related anonymous protein). Together, these two proteins are involved in the process of recognition and entry of sporozoites into hepatocytes [3–9]. Several studies have highlighted the roles played by CS protein in sporozoite invasion [3,10,11]. TRAP also assists the sporozoite in several pivotal functions, such as sporozoite gliding motility, hepatocyte invasion and establishment of infection in the vertebrate host [6,12,13].

The TRAP family of proteins are type I membrane proteins and possess multiple adhesive domains in their extracellular regions that bind to the host cells. TRAPs also contain intracellular domains that extend into the cytosol of the parasite and enable binding to the cytoplasmic motor system [14,15]. The ECD (extracellular domain) of *Plasmodium falciparum* TRAP consists of three adhesive regions, which include the A domain, the heparin-binding motif TRM (thrombospondin repeat motif) and an RGD sequence [4,16]. The significance of these adhesive modules was established by mutagenesis experiments, which showed that point mutations in the A domain and TRM severely impeded sporozoite infectivity in both insect and mammalian host cells [8].

TRAP A domain is homologous with the A1 domain of the vWF (von Willebrand factor) family of proteins, and bears close resemblance to the I domain of integrins [17]. This homology suggests that the TRAP A domain is probably involved in binding to cell-surface or cell-matrix molecules [18,19]. The A domain has the so-called MIDAS (metal-ion-dependent adhesion site), which contains the Asp-X-Ser-X-Ser (DXSXS) motif and which, by analogy to vWF and integrins, probably plays a role in bivalent cation binding and receptor recognition [20,21]. In support of this, a recent study indicated that a point mutation in the bivalent cation-binding site of the A domain affected sporozoite infectivity *in vivo* [8].

To dissect the structural and functional attributes of TRAP, we have established expression, refolding and purification procedures for the production of the A domain and ECD. A number of previous reports have suggested that recombinant A domain and ECD of TRAP exist as protein aggregates [5,7,9,22]. Our results show that both the recombinant proteins can be produced as soluble, monomeric domains. Our recombinant A domain and the ECD bound to HepG2 cells with specificity and in a dose-dependent manner. Inhibition of binding to HepG2 cells using heparin analogues suggested that no particular analogue is able to abrogate binding completely, indicating the presence of hitherto unknown receptor–ligand interactions between TRAP and hepatocytes. Homology modelling-based structural analysis of the A domain shows distinct molecular surfaces, which may be independently involved in the recognition of multiple receptors. Binding experiments between TRAP and heparin under flow conditions show that ECD binds to heparin with 2-fold higher affinity compared with the A domain. Taken together, our results

Abbreviations used: CS, circumsporozoite; ECD, extracellular domain; GdnHCl, guanidine hydrochloride; MIDAS, metal-ion-dependent adhesion site; Ni-NTA, Ni^{2+}-nitrilotriacetate; SPR, surface plasmon resonance; TRAP, thrombospondin-related anonymous protein; TRM, thrombospondin repeat motif; vWF, von Willebrand factor.

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pave the way for the identification of new cellular receptors for TRAP adhesive domains, and for the use of these adhesive domains in vaccines against malaria.

EXPERIMENTAL

Molecular cloning of *P. falciparum* TRAP A domain

A DNA fragment coding for TRAP A domain (residues 26–242) was amplified by PCR using a forward primer 5′-CTCggATCCAgAgATgTgCAAAACAAT-3′ and a reverse primer 5′-gCggCCgCAAgCTTTCagTgATggTgATggTgATgTTTgCCTCCTgCTgTTTTTTCTACTTCAACA-3′. The primers were designed to include a His_6 site at the C-terminus of the recombinant protein. TRAP A domain was subsequently subcloned in a baculovirus transfer vector pACGP67B at *Bam*HI and *Not*I sites. TRAP ECD (residues 26–500) was cloned and expressed in pQE30 (Qiagen) vector at *Bam*HI and *Hin*dIII sites with an N-terminal His tag. All clones were confirmed by DNA sequencing.

Transfection, expression and purification of TRAP A domain using the baculovirus system

SF21 insect cells were co-transfected with the TRAP A domain construct in the transfer vector and linearized baculovirus genomic DNA (BD PharMingen, Franklin Lakes, NJ, U.S.A.). The expression of TRAP A domain in High Five cells was verified by Western-blot analysis. The A domain was expressed in High Five cells using high-titre viruses at a cell density of 1 million/ml and at an MOI (multiplicity of infection) of 7. The baculovirus supernatant was harvested 70 h after infection and extensively dialysed against 20 mM Tris and 300 mM NaCl (pH 7.5) to remove chelators from the media. The dialysed supernatant was then passed through equilibrated Ni-NTA (Ni^{2+}-nitrilotriacetate) superflow beads (Qiagen). The A domain was eluted in 250 mM imidazole buffer (in 20 mM Tris and 300 mM NaCl, pH 7.4) and the purity of the eluted fractions was checked on a 12 % (w/v) SDS/polyacrylamide gel. The A domain was then concentrated in Centricon-10 and subjected to gel-filtration chromatography on a Superdex 200 column (Amersham Biosciences), which had been pre-equilibrated in 50 mM Tris (pH 7.5), 300 mM NaCl and 0.02 % sodium azide. To test for glycosylation of the A domain, Western-blot analysis was performed with concanavalin antibodies (Sigma).

Expression, purification and refolding of TRAP ECD from *Escherichia coli*

The TRAP ECD gene was cloned in pQE30 (Qiagen) vector with an N-terminal His_6 tag and expressed in M15 bacterial cells. These cells were grown to an absorbance $A_{600} \sim 0.6$ and induced with 1 mM IPTG (isopropyl β-D-thiogalactoside) for 4 h at 37 °C. *E. coli* cells were harvested and resuspended in chilled lysis buffer [50 mM Tris/HCl, pH 7.5/1 mM PMSF/10 mM dithiothreitol/10 mM EDTA/100 mM NaCl/100 μg/ml lysozyme] and sonicated. The overexpressed recombinant ECD forms inclusion bodies in *E. coli*. Therefore, a purification and refolding procedure was developed to produce soluble, recombinant protein. Briefly, the inclusion bodies were collected by centrifugation at 28 000 ***g*** at 4 °C and washed twice with chilled lysis buffer (without dithiothreitol and EDTA). The inclusion bodies were then solubilized in 6 M GdnHCl (guanidine hydrochloride), containing 10 mM Tris buffer (pH 8.0), 100 mM NaH_2PO_4 and 100 mM NaCl, for 10 h at room temperature (25 °C). The protein was subsequently loaded on to an Ni-NTA affinity column equilibrated in a buffer (10 mM Tris, pH 8.0/6 M GdnHCl/100 mM NaH_2PO_4/100 mM NaCl). Beads were washed with 20 column volumes of equilibration buffer at pH 6.3 and the bound protein was eluted in a buffer (10 mM Tris, pH 8.0/6 M GdnHCl/100 mM NaH_2PO_4/100 mM NaCl; pH 4.3). This affinity-purified ECD was then refolded by 40-fold rapid dilution in a buffer containing 50 mM Tris (pH 7.2), 2 mM GSH, 0.2 mM GSSG, 1 M urea and 0.5 M L-arginine at a final protein concentration of approx. 30 μg/ml. Refolding was allowed to proceed at 10 °C for 36 h. The refolding mixture was then dialysed for 48 h against 50 mM phosphate buffer (pH 7.0) containing 1 M urea to remove L-arginine before purification by ion-exchange chromatography. The refolded and dialysed protein was loaded on to a Q-Sepharose ion-exchange column equilibrated in buffer A (50 mM phosphate buffer, pH 7.0). The bound protein was eluted using a step gradient of 20 % buffer B (50 mM phosphate buffer, pH 7.0/1.5 M NaCl), which corresponds to 300 mM NaCl. Protein aggregates eluted at 50 % buffer B, which corresponds to 750 mM NaCl. Appropriate ion-exchange fractions were concentrated and loaded on to a gel-filtration column. The protein peak consistent with migration of monomers was collected and the purity of the proteins was verified on 12 % SDS/polyacrylamide gels under both reducing and non-reducing conditions.

Free cysteine assay to detect the number of disulphide bonds in the A domain

The TRAP ECD contains 11 cysteine residues: five in the A domain and six in the thrombospondin repeat region. Given the odd number of cysteine residues in the A domain, we attempted to detect the presence of free cysteine residue(s) in the baculovirus using Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid); Pierce] under native conditions. An aliquot (45 μl) of the A domain protein (approx. 5 mg/ml) was mixed with 446 μl of the assay buffer (0.5 M Na_2HPO_4/1 M NaH_2PO_4, pH 8.0), containing 9 μl of Ellman's reagent (stock solution, 4 mg/ml assay buffer). After 15 min incubation at room temperature, the absorbance was measured at 412 nm. A cysteine standard curve was prepared according to the manufacturer's instructions.

HepG2 cell-binding and competition assays

HepG2 cells were cultured in Dulbecco's modified Eagle's medium and 10 % (w/v) foetal bovine serum (Invitrogen) at 37 °C. Cells (10^5) were coated in a 96-well cell-culture plate and grown for 24 h [23]. HepG2 cells were fixed with 4 % (w/v) paraformaldehyde for 10 min at room temperature, followed by blocking with 1 % BSA for 2 h at room temperature. The A domain and ECD were incubated with HepG2 cells at increasing concentrations of 120, 250 and 500 nM. The bound protein was quantified using anti-TRAP antibodies and horseradish peroxide-conjugated secondary antibodies. Wells with only BSA and HepG2 cells served as controls and showed no binding.

Effects of metal ions on A domain and ECD binding to HepG2 cells

To assess the effects of metal ions on the interaction of the A domain and ECD with HepG2 cells, the binding assays were repeated with samples containing either 5 mM EDTA or 10 mM each of $MgCl_2$, $CaCl_2$ and $MnCl_2$.

Homology modelling of the A domain and structural mapping of point mutations

Molecular modelling was performed using 3DPSSM (http://www.sbg.bio.ic.ac.uk/~3dpssm/). Modelling of the A domain was based on the sequence and X-ray crystal structure of vWF A1 domain (PDB code 1auq) [24]. Sequence alignment of plasmodial

A domains was performed using ClustalW (http://www.ebi.ac.uk/clustalw/).

SPR (surface plasmon resonance) analysis of TRAP–heparin interaction

SPR-based detection permits real-time interaction analysis of interacting molecular species under flow conditions [25,26]. Therefore we used a Biacore 2000 instrument to investigate the binding of ECD and the A domain to heparin. The Biacore 2000 biosensor chip SA (flat carboxymethylated dextran coupled with streptavidin) was purchased from Amersham Biosciences (Uppsala, Sweden). Kinetic rate constants for the interaction of heparin with ECD and the A domain were determined by SPR techniques [26]. Two sensor-chip flow cell surfaces were activated by three consecutive injections of 50 mM NaOH and 1 M NaCl for 1 min at a flow rate of 10 μl/min. Biotinylated BSA-heparin (Sigma) was injected through flow cell 2 at 100 nM until approx. 250 RU (resonance units) was captured by the streptavidin surface. For a negative control, biotinylated BSA (Sigma) was injected through flow cell 1 at 100 nM until saturation (reference surface). Kinetic binding analysis was performed in a buffer containing 10 mM Hepes (pH 7.4) and 150 mM NaCl, supplemented with 3 mM EDTA and 0.005 % surfactant P20 (Biacore, Amersham Biosciences, Uppsala, Sweden), at a constant flow rate of 30 μl/min, and the instrument was equilibrated at 25 °C. Kinetic data for ECD and the A domain were collected by injecting increasing concentrations of the protein at concentrations of 12.5, 25, 50, 100 nM and 5.6, 11.2, 22.4, 44.8, 89.6 and 179.2 nM respectively in independent experiments. The proteins were injected in series for 5 min at a flow rate of 30 μl/min at kinject mode and dissociation was monitored for 300 s at the same flow rate. After the observation of dissociation for 5 min, the experimental surface and the reference surface were regenerated by injecting 50 μl of regeneration solution (10 mM Hepes, 1.5 M NaCl, 3 mM EDTA and 0.005 % surfactant P20, pH 7.4) at a flow rate of 50 μl/min. To establish the level of experimental noise, each binding study was repeated three times, including blank injections of running buffer. To correct for refractive index change and instrument noise, the response data from the reference surface were subtracted from the response obtained from the reaction surface. To check whether the binding reactions were limited by mass transport, the flow rates were varied from 10 to 75 μl/min while keeping the concentration of the analyte constant. Evaluation of the association curves using BIAevaluation 3.2RC1 software indicated no mass-transfer-limited binding in our experiments.

Kinetic rate constants for the A domain were determined by fitting the corrected response data to a simple 1:1 (Langmuir) binding model with drifting baseline, whereas the kinetic rate constants for ECD were fitted to a two-state conformation change model. The rate equations were numerically integrated, and the results were simultaneously fitted to association and dissociation phase-response data using the non-linear least-squares data analysis program (BIAevaluation 3.2RC1). The K_d value for each kinetic interaction was calculated using k_d/k_a.

Competition of A domain and ECD binding to HepG2 cells with heparin and its analogues

Inhibition of the interaction of ECD and/or A domain with HepG2 cells using heparin was studied. Increasing amounts of molar excess of heparin were preincubated with purified proteins and, subsequently, these proteins were added to HepG2 cells.

For competition assays, 10^5 HepG2 cells/well were seeded in a 96-well cell-culture plate and were grown for 24 h, fixed with 4 % paraformaldehyde and blocked with 1 % BSA. Equimolar concentrations of the A domain and ECD were initially preincubated with a 50-fold molar excess each of chondroitin sulphates A and B, heparin, dextran sulphate, hyaluronic acid, sulphatides and fucoidan for 1 h at room temperature. Each of the complexes was then incubated with HepG2 cells for 1 h at room temperature. The cells were then washed with PBS, and the bound domains were detected using TRAP-specific antibodies.

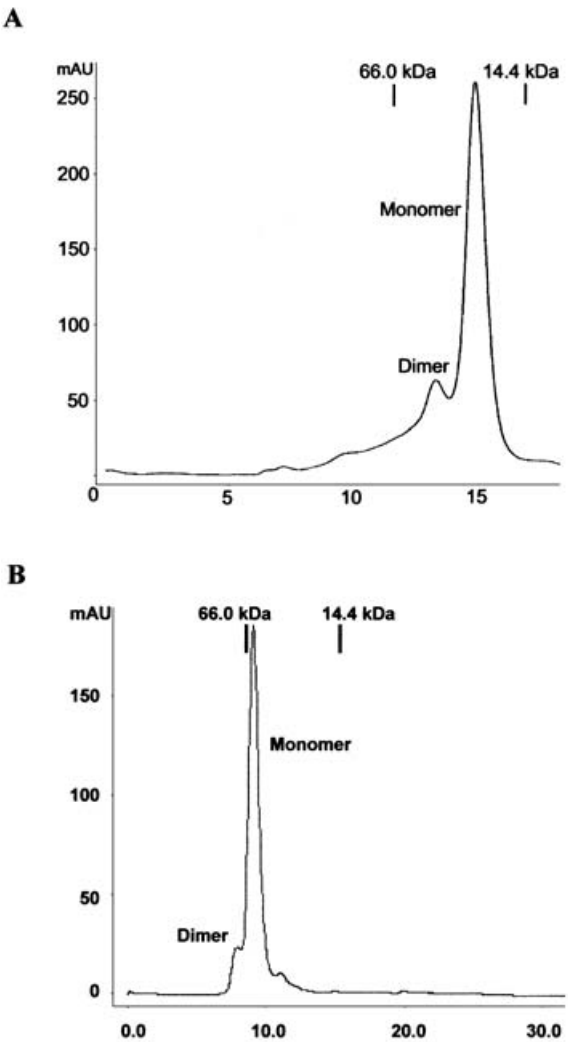

Figure 1 Gel-filtration analyses of recombinant TRAPs

(**A**) The Ni^{2+} affinity-purified recombinant A domain migrates on a gel-filtration column as a monomer with a very small fraction of dimer. (**B**) Bacterially expressed, purified and refolded ECD also migrates as a monomer on a gel-filtration column. Positions of the molecular-mass standards BSA (66 kDa) and lysozyme (14.4 kDa) are shown.

RESULTS

Recombinant production of TRAP A domain and ECD

Baculovirus-expressed A domain was purified using Ni-NTA and gel-filtration chromatographies. The migration of the A domain under reducing and non-reducing conditions in SDS/PAGE gel suggests a monomeric state, and is consistent with its elution profile on gel-filtration columns, where it migrates as an approx. 25 kDa protein (Figures 1A and 2A). A small fraction of the

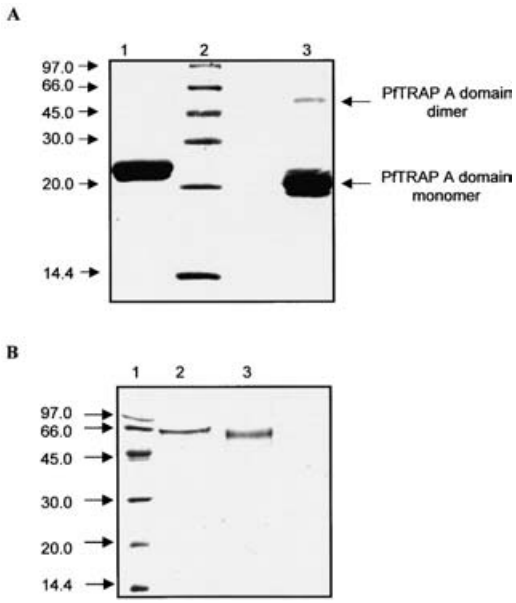

Figure 2 SDS/PAGE of TRAP domains under reducing and non-reducing conditions

(**A**) The baculovirus-expressed and purified A domain migrates at a molecular mass of approx. 25 kDa under reducing conditions (lane 1). Lane 2, molecular-mass standards (in kDa). Under non-reducing conditions, the A domain migrates as two bands (approx. 25 and 50 kDa), representing the monomeric and dimeric forms of the protein respectively (lane 3). (**B**) The bacterially expressed and refolded ECD migrates at a molecular mass of approx. 60 kDa. The protein shows the expected mobility shift when run under reducing (lane 2) or non-reducing conditions (lane 3). Lane 1, molecular-mass standards.

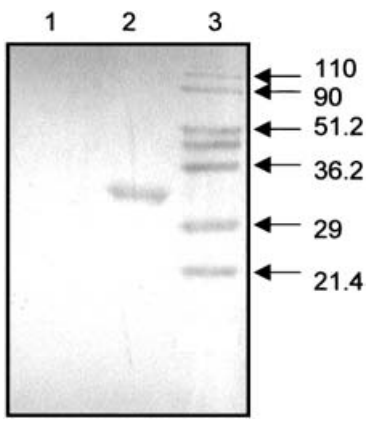

Figure 3 Glycosylation of the A domain

The baculovirus-expressed A domain was loaded on to a 12% SDS/polyacrylamide gel and glycosylation was verified by Western-blot analysis (lane 2). Specific antibodies were used to detect glycosylation. *E. coli*-expressed protein was used as a negative control (lane 1). Lane 3, molecular-mass standards (in kDa).

A domain exhibited dimerization under non-reducing conditions, but no higher-order oligomers were observed. This suggested the presence of one free cysteine residue in the A domain (out of five). The baculovirus-expressed A domain is glycosylated, as suggested by Western-blot analysis with a concanavalin A antibody (Figure 3). We also expressed and purified ECD in a functional form, as judged by its migration pattern on SDS/PAGE and gel-filtration columns (Figures 1B and 2B). Monomeric ECD

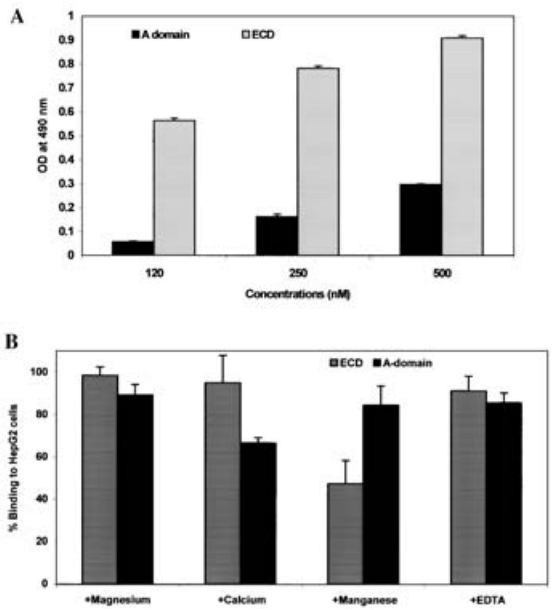

Figure 4 Binding of the purified A domain and ECD to HepG2 cells in a dose-dependent manner

(**A**) HepG2 cells (approx. 10^5) were incubated with the A domain and ECD in equimolar amounts and at 120, 250 and 500 nM. The binding assay was performed in the absence of Ca^{2+}, Mg^{2+} or Mn^{2+} cations. Results are the means $\pm$ S.D. for $n = 3$ experiments, each performed in quadruplicate. (**B**) HepG2 cell binding was also tested in the presence of 10 mM each of the bivalent cations Ca^{2+}, Mg^{2+} and Mn^{2+} or in the presence of 5 mM EDTA. Results are the means $\pm$ S.D. for $n = 3$ experiments, each performed in quadruplicate.

showed the expected mobility difference under reducing and non-reducing conditions on SDS/PAGE (Figure 2B), which suggested correct formation of disulphide bonds in the recombinant protein.

A domain and ECD have different binding affinities for HepG2 cells

HepG2-cell-binding studies were performed using equimolar concentrations of the A domain and ECD. HepG2 cells were grown in 96-well cell-culture plates and incubated with increasing amounts of the recombinant proteins. As shown in Figure 4, both proteins bound to HepG2 cells in a dose-dependent manner. ECD showed stronger binding to HepG2 cells compared with the A domain (Figure 4).

Homology modelling-based structure of TRAP A domain

The A domain of TRAP is homologous with A1 domains of the vWF family of proteins and I domain of integrins. As X-ray crystallographic information is available for the latter modules, we identified the vWF A1 domain [24] as a close homologue of TRAP A domain. Using 3DPSSM, we built a homology modelling-based structure of the A domain. We then mapped the bivalent cation-binding motif, the two point mutations (Thr^{131} and Asp^{162}) [8] and a putative heparin-binding site on this homology model. In addition, we mapped the five cysteine residues contained in the A domain (Figures 5 and 6). Our modelling studies suggest that, similar to the A1 domains, the A domain of TRAP probably has a disulphide linkage between Cys^{43} and Cys^{235} (these correspond to residues Cys^{509} and Cys^{695} in the vWF A1 domain) [24]. The model also indicates that Cys^{55} is buried within the protein core (which is a conserved glycine in all other plasmodial A domains). The two extra residues, Cys^{212} and Cys^{205}, which are absent from

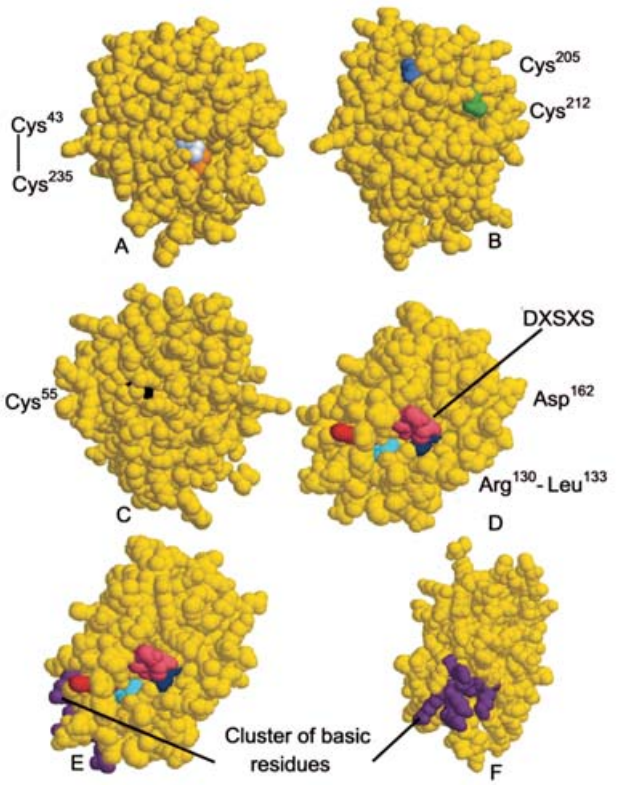

Figure 5 Homology modelling-based structure of the A domain

(**A**) A space-filling model in yellow with the disulphide bond between Cys[43] (white) and Cys[235] (orange). (**B**) A model showing the exposed Cys[205] (grey) and the partially buried Cys[212] residues (green). (**C**) The fifth cysteine Cys[55] (black) is completely buried in the protein core. (**D**) Location of Asp[162] (blue), which is adjacent to the DXSXS motif (magenta) and whose mutation impairs cell entry [8]. The point mutation of Thr[131] is also deleterious to sporozoite invasion. This model suggests that Thr[131] is probably exposed and flanked by Arg[130] (red) and Leu[133] (turquoise). (**E**) Juxtaposition of the putative heparin-binding region (purple), the DXSXS motif (magenta) and the two point mutations (as in **D**). (**F**) A cluster of basic residues (purple) that form the putative heparin-binding region. This site is immediately adjacent to, and partially overlaps, the heparin-binding residues in the homologous A1 domain of vWF [24].

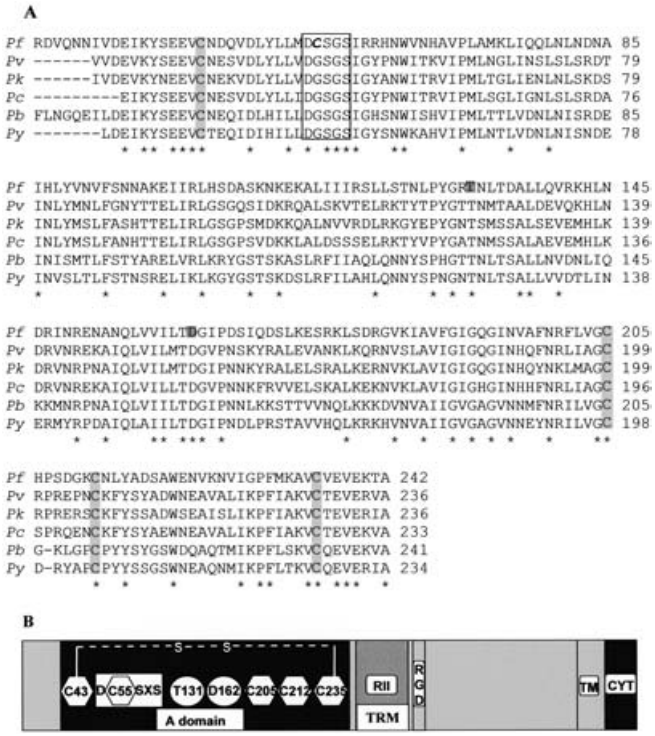

Figure 6 Sequence alignment of A domains and domain architecture of TRAP

(**A**) Multiple sequence alignment of TRAP A domains from various plasmodial species: *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. cynamolgi*, *P. berghei* and *P. yoelli*. Light grey boxes indicate the conserved cysteine residues. Notably, the odd fifth cysteine residue (in boldface and italicized; Cys[55]) in *P. falciparum* is a glycine residue in other TRAPs. The highly conserved DXSXS motif in the metal adhesion site is outlined as a box. Dark grey boxes indicate the conserved and functionally important Thr[131] and Asp[162] residues. (**B**) Overall domain architecture structure of *P. falciparum* TRAP, showing the proposed disulphide linkage between Cys[43] and Cys[235], the conserved DXSXS motif, the mutated residues (Thr[131] and Asp[162]) in the A domain, the TRM region, the yet unannotated RGD sequence, the transmembrane (TM) domain and the cytoplasmic tail (CYT).

the homologous vWF A1 domains but conserved in plasmodial A domains, are partially buried and accessible respectively (Figures 5 and 6). This analysis is consistent with the biochemical results, which suggested the presence of an unpaired cysteine residue in the free thiol assays (results not shown), as well as with the tendency of a minor fraction of the A domain to form dimers but not higher-order oligomers.

Mapping of the critical residues Thr[131] and Asp[162], which are involved in sporozoite infectivity [8], on the A domain structure shows that Thr[131] is in an exposed loop with the flanking residues Arg[130] and Leu[133], whereas Asp[162] is part of the MIDAS motif (Figure 5). In addition, the A domain has a distinct cluster of basic residues that come together to make a continuous patch of electropositive surface. This cluster is formed by Arg[130], Arg[141], Lys[142], Lys[173], Arg[176], Lys[177] and Arg[181] (Figure 5). Crystallographic and biochemical studies have shown previously that lysine and arginine residues often mediate the binding to heparin and other sulphatides. We propose that the cluster of basic residues identified on the A domain surface probably confers heparin-binding ability on the A domain. The homologous A1 domain from the vWF protein family has a similar heparin-binding surface [27]. A notable feature of the homology model of TRAP A domain presented here is the spatial separation of the putative heparin-binding site from the bivalent cation-binding region (Figure 5).

Multiple sequence alignment of the TRAP A domains from various plasmodial species

The extent of sequence conservation in the A domains of TRAPs from *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. berghei*, *P. yoelii* and *P. cynomolgi* was studied using ClustalW (Figure 6). Plasmodial A domains contain the prototypical bivalent cation-binding motif, which in other systems has been implicated in ligand binding and integrin activation [20]. Interestingly, the MIDAS motif in the A domain of *P. falciparum* TRAP contains an extra cysteine residue (Cys[55]), whereas in all other plasmodial A domains this residue is a glycine. Besides Cys[55], four other cysteine residues, namely Cys[43], Cys[205], Cys[212] and Cys[235], are conserved throughout plasmodial A domains.

SPR analysis of heparin binding to the A domain and ECD

Biacore-based analysis of the binding of ECD and the A domain to heparin demonstrates a tight and specific interaction of these domains with heparin. The A domain kinetic data fitted a simple univalent interaction with heparin, whereas the ECD data fitted a

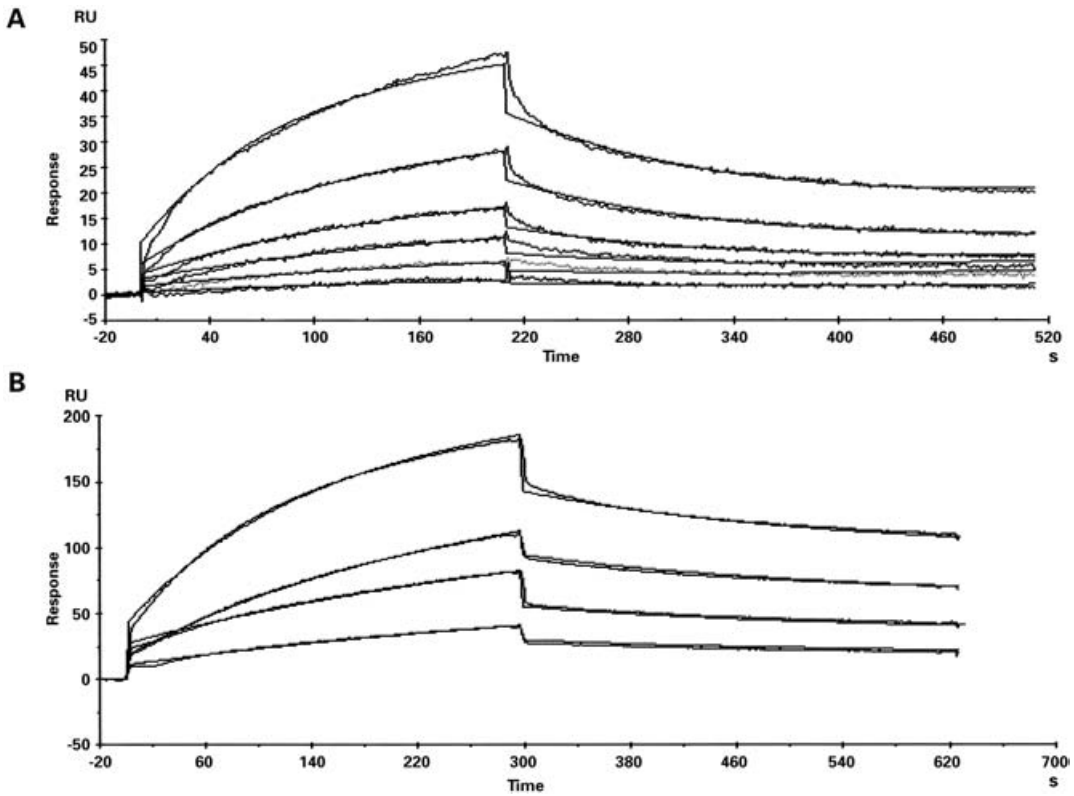

Figure 7 Kinetic studies of TRAP–heparin interaction

Global fitting of the sensograms obtained by injecting increasing concentrations of TRAP A domain (**A**) and ECD (**B**) on to a heparin-coated surface. Concentrations of the analytes injected are given in the Experimental section. Analytes were injected on to captured biotinylated BSA-heparin using biosensor chip SA (Biacore) for 5 min at 30 μl/min. Dissociations were monitored for 5 min in HBS-EP buffer (10 mM Hepes, pH 7.4/150 mM NaCl/0.005 % surfactant P20/3 mM EDTA; Biacore). In both cases, the response of the control surface (captured BSA) was subtracted from that of the BSA-heparin-immobilized surface. (RU, resonance units) The global fitting plots for the experimental results were calculated using the BIAevaluation 3.2RC1 program. (**A**) Curves for the A domain fitted a 1:1 (Langmuir) binding model ($K_D = 79$ nM); (**B**) a two-state reaction conformation change model was fitted for ECD ($K_D = 40$ nM).

two-state reaction conformational change model for heparin binding (Figure 7). The calculated binding constant for the A domain was $K_D = 79$ nM ($\chi^2 = 0.21$). The calculated binding constant for ECD was $K_D = 40$ nM, with $K_{a_1} = 5.23e^4$, $K_{d_1} = 2.12e^{-3}$, $K_{a_2} = 2.24e^{-3}$ and $K_{d_2} = 6.76e^{-6}$ ($\chi^2 = 1.81$). The presence of two contiguous heparin-binding modules in ECD (the A domain and the TRM; Figure 6B) is consistent with our Biacore-based kinetic analysis. The derived binding constant for ECD–heparin interaction of 40 nM is very similar to that obtained for the other TRM-containing sporozoite surface protein (CS) of 41 nM [11].

Heparin or its analogues are poor inhibitors of the binding of the A domain and ECD to HepG2 cells

Previous reports have shown that the A domain interacts with heparin on the surface of HepG2 cells [7,9,22]. However, when we used increasing concentrations of heparin to inhibit the binding of the A domain and ECD to HepG2 cells, we observed that even a 1000-fold excess of heparin could not abrogate the TRAP–heparin interaction (Figure 8A). These results suggest the presence of uncharacterized hepatocyte receptor(s) for the A domain and ECD on HepG2 cells. We also performed TRAP–HepG2-binding experiments in the presence of different heparin analogues (Figure 8B). The binding of either the A domain or ECD to HepG2 cells was decreased only partially in the presence of a 50-fold molar excess of various sulphated glycoconjugates. In these assays, hyaluronic acid, which is not a sulphated glycoconjugate, was used as a negative control.

DISCUSSION

TRAP is involved in sporozoite gliding and liver cell infectivity [6,8,10]. There has been considerable interest in TRAP for the development of sulphatide-based inhibitors that may disrupt the interaction of sporozoites with hepatocytes, and for use of TRAP as a vaccine [7,28,29]. The sporozoite surface protein CS, in conjunction with TRAP, interacts with heparan sulphate proteoglycans on hepatocytes and plays an important role in homing of sporozoites to liver [8,10,30–33]. Previous studies using bacterially produced recombinant TRAP and its domains have suggested that the A domain and ECD exist *in vitro* as oligomeric aggregates [5,7,9,22]. This is the first report to provide evidence that both the A domain and ECD of TRAP can be produced in heterologous expression systems and purified as soluble, functional and, more significantly, as monomeric proteins. The monomeric nature of recombinant TRAP is an important biophysical attribute in understanding the biological basis of TRAP action and for its potential in vaccine design. We conducted binding assays with HepG2 cells to confirm the functional integrity of the recombinant proteins. Our results show a dose-dependent and bivalent cation-independent binding of both the A domain and the ECD to HepG2 cells.

Our structural model of the A domain, and the striking conservation of cysteine residues among all plasmodial A domains, together suggest the presence of a disulphide bond between Cys^{43} and Cys^{235}. This covalent bond may tie the N-terminus of the A domain to its C-terminus, as in other vWF A1 domains

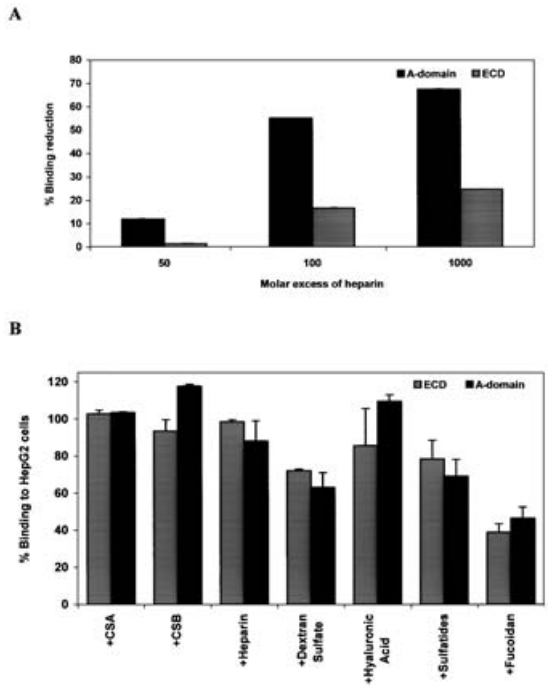

Figure 8 Competition of A domain and ECD binding to HepG2 cells with sulphated glycoconjugates

(**A**) Concentration-dependent inhibition of ECD and A domain binding to HepG2 cells using up to 1000-fold molar excess of heparin in solution. (**B**) Equimolar amounts (100 nM) of the A domain and ECD were preincubated with 50-fold molar excess each of chondroitin sulphate A (CSA), CSB, heparin, dextran sulphate, hyaluronic acid, sulphatides and fucoidan for 1 h at room temperature. HepG2 cell binding was then performed as detailed above.

[24]. Interestingly, TRAP A domains contain additional cysteine residues (Cys^{205} and Cys^{212}), which are highly conserved in plasmodial TRAPs. Our homology model of the A domain indicates that, whereas Cys^{205} is potentially exposed, Cys^{212} may be partially buried. The fifth cysteine residue, Cys^{55}, unique to the A domain of *P. falciparum* TRAP, is not a conserved residue among plasmodial A domains and is proximal to the DXSXS motif.

The homology-modelled structure of the A domain shows two spatially distinct surfaces for ligand binding. These surfaces contain the MIDAS motif, where point mutations of Thr^{131} and Asp^{162} correlate with impairment of cell infectivity by sporozoites [8] and a putative heparin-binding site respectively. Interestingly, the latter site shows a partial overlap with the known heparin-binding site on the A1 domain of vWF [24]. Indeed, many A1 and I domains are able to interact with heparin and other sulphatides, in conjunction with their ability to recognize independently and specifically other receptors.

A series of elegant experiments have shown previously that both the A domain and TRM integrally contribute to sporozoite infectivity [8]. Hence, these two adhesive modules of TRAP probably participate in hepatocyte invasion and may be crucial mediators in receptor–ligand interactions. Specifically, mutagenesis of Thr^{131} and Asp^{162} in the A domain and the resulting impairment of cell infectivity by sporozoites show that the MIDAS motif is important for sporozoite entry *in vivo* and *in vitro* [8]. The former residue lies in close proximity to the MIDAS motif, whereas the latter is in an exposed loop and is flanked by Arg^{130} and Leu^{133} (Figure 5). These results indicate that, in addition to the interaction of the A domain with heparin, there may exist a metal-ion-dependent mode of entry for sporozoites using the A domain of TRAP [8]. On the basis of the structural model presented above, it is feasible that TRAP A domain has two non-overlapping regions for interaction with heparin and an unidentified HepG2 cell receptor. This hypothesis is further supported by our competition assay data, which indicate that the binding of the A domain and ECD to HepG2 cells cannot be significantly inhibited by a large molar excess of heparin or its analogues such as sulphatide, fucoidan, chondroitin sulphate and dextran sulphate.

Our protein–cell and protein–heparin binding studies indicate that the A domain binds to HepG2 cells with lower affinity when compared with ECD. The latter contains multiple adhesive domains (the A domain, TRM and the RGD sequence); together, these domains probably increase the avidity of ECD for HepG2 cells. Prompted by these observations, we conducted a series of binding experiments between TRAP modules and heparin under flow conditions and using the SPR technology. Our results suggest that ECD has approx. 2-fold higher affinity for heparin (K_D = 40 nM) when compared with the A domain alone (K_D = 79 nM). The Biacore results also indicate that the ECD–heparin binding is probably a two-state reaction with a conformational change, consistent with the presence of two heparin-binding modules in the ECD.

Taken together, the current investigation highlights the following facts regarding TRAP: (i) the A domain and ECD do not exist as molecular aggregates *in vitro*, but are soluble, monomeric domains; (ii) the A domain and ECD are disulphide-containing modules; (iii) the A domain resembles A1 domains of vWF, which typically have several surfaces for interaction with multiple receptors; (iv) the structural model of the A domain suggests spatially distinct binding surfaces for bivalent cation binding (MIDAS motif) and for heparin binding; (v) the two point mutations that severely impair hepatocyte infectivity [8] map close to and within the MIDAS motif, but significantly away from the putative heparin-binding region; (vi) the A domain and ECD have different affinities for heparin, the latter binds heparin more tightly; and (vii) the interaction of TRAP with HepG2 cells is not completely inhibited even by high concentrations of heparin analogues.

It is feasible that the interaction of the A domain and ECD with heparin is responsible for homing of sporozoite to the liver, whereas the A domain MIDAS motif participates in cation-dependent recognition of unknown receptors on hepatocytes. The three adhesive modules of TRAP (the A domain, TRM and the RGD sequence) may participate in sequential and overlapping steps, leading to the attachment of sporozoites to hepatocytes and their subsequent entry into hepatocytes. Whereas the role of the TRM is clear, the reason for the conservation of the RGD sequence in *P. falciparum* TRAP remains undiscovered. Furthermore, the multiple biochemical facets of the A domain have made the identification of a potential protein ligand difficult. In this regard, we propose that expression of recombinant TRAP A domain/ECD in soluble and monomeric forms and our biochemical/structural analysis of these modules paves the way for the identification of additional cellular ligands which mediate sporozoite invasion into hepatocytes.

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